Oxysterols: Modulators of Cholesterol Metabolism and Other Processes

GEORGE J. SCHROEPFER, JR.†

Departments of Biochemistry and Cell Biology and of Chemistry, Rice University, Houston, Texas

I. Introduction 362
II. Separation and Identification of Oxysterols 364
A. Chromatography of oxysterols 364  
B. Mass spectrometry of oxysterols 369  
C. NMR of oxysterols 369  
D. X-ray crystal structures of oxysterols 370  
III. Formation of Oxysterols 370
A. Formation of 26-, 25-, and 24-hydroxysterols 370  
B. Formation of 7-oxygenated sterols 380  
C. Formation of 5,6-epoxides of cholesterol and cholestane-3β,5α,6β-triol 391  
D. Formation of 5,6-chlorohydrins of cholesterol 393  
E. Formation of 24,25-epoxysterols 394  
F. Formation of 32-oxygenated sterols 395  
G. Formation of 4-hydroxysterols 396  
H. Formation of 19-hydroxysterols 396  
I. Formation of 15-oxygenated sterols 397  
J. Bioorganic syntheses of oxygenated sterols 397  
K. Formation of oxysterols in vivo 398  
L. Oxysterol formation in membrane preparations 401  
IV. Occurrence and Levels of Oxysterols 402
A. Oxysterols in plasma 402  
B. Possible physiological regulation of levels of oxysterols in plasma 412  
C. State of esterification of sterols in plasma 415  
D. Oxysterols in LDL 416  
E. Oxysterols in LDL modified by oxidation 419  
F. Oxysterols in other lipoprotein fractions 423  
G. Oxysterols in tissues 425  
H. Oxysterols in meconium 430  
I. Oxysterols in cerebrospinal fluid 430  
J. Oxysterols in food products 430  
V. Metabolism of Oxysterols 432
A. Metabolism of oxysterols in in vitro preparations 432  
B. Metabolism of oxysterols in cultured cells 436  
C. Metabolism of oxysterols in intact animals and human subjects 440  
D. Formation of fatty acid esters of oxysterols 448  
VI. Actions of Oxysterols 449
A. Effects of oxysterols in sterol synthesis in cultured cells 449  
B. Effects of oxysterols on HMG-CoA reductase in cultured cells 450  
C. Effects of direct addition of oxysterols to microsomes and cell-free preparations 458  
D. Effects of oxysterols on enzymes involved in cholesterol biosynthesis other than HMG-CoA reductase 459  
E. Oxysterol binding protein 461  
F. Sterol regulatory element binding proteins 462  
G. Oxysterols and orphan nuclear receptors 464  
H. Oxysterols as regulators of gene transcription of other species 466  
I. Oxysterols and antiestrogen binding activity 466
† Deceased December 11, 1998.
I. INTRODUCTION

Oxysterols represent a class of potent regulatory molecules with remarkably diverse, important biological actions. Early research on oxysterols concerned their generation from cholesterol (Chol) by autoxidation. Important studies by Bergström and Wintersteiner (64–66) and by others (681), reviewed by Bergström and Samuelsson (63), were extensively expanded by Smith and colleagues (1000, 1001). The ease of autoxidation of Chol...
noted in these important papers appears to have been ignored in the design and/or interpretation of many recent studies. Other investigations concentrated on selected oxysterols as a part of studies attempting to elucidate pathways and individual reactions involved in the formation of bile acids. These studies, spanning many decades, continue today. A major milestone was the demonstration by Kandutsch and Chen (466, 467) that certain oxygenated derivatives of Chol, but not highly purified Chol itself, caused an inhibition of sterol biosynthesis and a lowering of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity in cultured mammalian cells. These findings stimulated a tremendous amount of research on oxysterols, including their actions on a wide variety of other processes and the chemical preparation of a large number of natural and synthetic oxygenated sterols, leading to the demonstration that some 15-oxygenated sterols were not only extraordinarily potent in suppressing sterol synthesis in cultured cells but also showed significant hypocholesterolemic action upon administration to rodents and nonhuman primates. An increasing amount of evidence indicates that oxysterols represent major natural regulators of sterol synthesis and of HMG-CoA reductase. Seminal studies by Brown and Goldstein (139, 140, 142) defined the metabolic defect in familial hypercholesterolemia (FH) and identified the receptor for low-density lipoprotein (LDL). These discoveries resulted in an explosion of research on LDL, including its roles in the control of sterol biosynthesis and a large variety of other important cellular processes, and studies on the levels of oxysterols in LDL. A considerable amount of research has been focused on the possible involvement of oxysterols, as components of oxidatively modified LDL, in the pathogenesis of atherosclerosis. Recent research has shown that oxysterols can induce programmed cell death in a variety of cell types and can also affect the development of calcification in vascular cells. Brown and Goldstein and their associates have recently discovered extraordinarily complex mechanisms involved in the processing of transcription factors that affect genes for critical enzymes involved in Chol biosynthesis. In addition, the same transcription factors have also been shown to be involved in biosynthesis of other important lipids. Oxysterols have also been reported to affect other transcription factors including several nuclear orphan receptors, and specific oxysterols may represent their natural ligands. Despite continuing advances in studies of the chemistry of oxysterols, most investigators have relied on commercial materials that are unfortunately very limited with regard to structural types, available quantities, and reasonable costs. This situation has resulted in the acquisition of a large amount of information on the effects of one oxysterol, 25-OH-Chol, on a wide variety of parameters in cultured mammalian cells. Unfortunately, other oxysterols may be of considerably more physiological importance. Moreover, the results of studies with 25-OH-Chol (or the combination of 25-OH-Chol and Chol) have been frequently generalized to other oxysterols without experimentation. The limited availability of oxysterols is also a major factor responsible for the very restricted number of studies of their in vivo effects in animals.

Knowledge of the metabolism of individual oxysterols is very important in the interpretation of the effects of their addition to a given cell type in tissue culture studies or of their administration to animals. For example, administration of one oxysterol, 3β-hydroxy-5α-cholest-8(14)-en-15-one, to animals is associated with very rapid and substantial conversion to a complex array of polar metabolites of varying potencies in their biological actions. Moreover, construction of analogs in which the major in vivo metabolism of the 15-ketosterol is blocked gave compounds with improved potency with regard to hypocholesterolemic action and other effects. To understand the potential physiological or pathophysiological importance of oxysterols in biology and medicine, many investigations have focused on the determination of the levels of oxysterols in blood plasma, in plasma lipoproteins, various tissues, and food preparations. High levels of certain oxysterols have been reported in cataracts, in membranes of red blood cells from patients with sickle cell disease, and in plasma from patients with liver disorders. Major advances have been made in the technologies for the separation, identification, and quantitation of oxysterols. Unfortunately, a good deal of the information currently available on the levels (and even identity) of oxysterols is of dubious validity due to lack of appreciation and/or lack of attention to critical aspects in the application of existing methodologies. Oxygenated sterols have significant potential for applications in medicine because of their effects on Chol metabolism, cell growth (both of normal and transformed cells), and other processes.

I have attempted to provide a critical, fairly comprehensive review of important, very rapidly expanding, multiple areas of investigation on oxysterols. My coverage concentrates on mammalian systems and, with a few exceptions, does not include oxysterols in plants and lower forms. The review is offered in a spirit of attempting to assist young, and not so young, investigators seeking to initiate or expand research on oxysterols. Over 25 years of participation in investigations on oxysterols provides some measure of personal experience on many, but not all, of the topics covered. Restriction to a review of our own studies would have been a relatively simple task. Past personal experience with reviews of broad fields of research (313, 908, 909, 910) provided the recognition of the effort and masochism required. Nonetheless, the present review furnished a special challenge that I hope will be useful to others.
the separation and identification of oxysterols, a topic of considerable practical importance for research with these compounds. It is hoped that this coverage facilitates the selection and critical employment of the various methodologies. Sections on the formation, occurrence, and metabolism of the oxysterols are very important in understanding the actions and physiological significance of the various oxysterols. Critical coverage of some of these matters (with considerations of details of methodology) is provided not merely to correct errors but also to avoid perpetuation of the same in future research. Sections relating to the effects or activities of oxysterols include provision of their precise concentrations so as to allow the reader to appreciate the potencies of some of the oxysterols and to judge the potential physiological relevance of the reported observations.

As with many areas of research, some specialized nomenclature is involved. Presented in Figure 1 is the chemical structure and numbering system for cholestane. To conserve space, cholesterol is abbreviated as Chol, and hydroxy derivatives are presented as OH-Chol, e.g., 5α-OH-Chol for 25-hydroxycholesterol. 5α,6α-Epox½-Chol, 5β,6β-epoxy-Chol, and 5α,6β-diol OH-Chol have been employed for 5α,6α-epoxycholestan-3β-ol, 5β,6β-epoxycholestan-3β-ol, and cholestan-3β,5α,6β-triol, respectively. In many recent articles in the biochemical and molecular biology literature, the important oxysterol (25R)-cholestan-5α,6α-epoxycholesterol
diol [or (25R)-26-hydroxycholesterol] is referred to as 27-hydroxycholesterol. In this review, except in a few passages, I have used the more traditional nomenclature 26-hydroxycholesterol (26-OH-Chol), which is consistent with the systematic nomenclature utilized by Chemical Abstracts and the International Union of Pure and Applied Chemistry and does not imply a particular configuration at C-25 unless specified by the 25R- or 25S-designation. This nomenclature recognizes the fact that, in almost all biochemical studies, the configuration of C-25 in 26-hydroxy steroids has not been established and, in those cases in which this matter has been studied in 26-hydroxy steroids of biological origin, the concerned steroids are mixtures of the 25R- and 25S-isomers. 24,25-Epox½lanost-8-en-3β-ol is designated as 24,25-epox½lanosterol, a name derived from the alkene (as in 2,3-epoxysqualene) rather than the alkane moiety (as in 24,25-epoxycholesterol). Many specialized abbreviations have been used and are identified in the text at their first use.

A number of general or specialized reviews of various aspects of research on oxygenated sterols have been published (56, 63, 80, 83, 93, 105, 187, 254, 303, 330, 331, 343, 358, 417, 442, 443, 465, 470, 469, 591, 768, 790, 792, 846, 876, 909, 911, 1000–1006, 1028, 1099, 1113, 1125).

II. SEPARATION AND IDENTIFICATION OF OXYSTEROLS

The isolation, characterization, and quantitation of oxysterols, as well as definition of their biosynthesis and metabolism, are critically dependent on methods for their separation. Presented below is an updating of current chromatographic methods (and their limitations) for the separation of various oxysterols and their derivatives. Almost all of the studies attempting to define the chromatographic properties of oxysterols have been limited by a lack of a truly comprehensive collection of authentic compounds, a situation resulting from the lack of the commercial availability of many oxysterols and the considerable effort and/or skill required to prepare these compounds by chemical synthesis. Uncritical application of chromatographic methods, in the absence of knowledge of the behavior of a substantial number of standards of defined structure and purity, is unfortunately frequently encountered and does not provide a firm basis for the identification and/or quantitation of a given oxysterol. Also presented are brief sections on mass spectrometry, NMR, and X-ray crystal analyses of oxysterols. Even these rigorous methods of identification are usually dependent on obtaining purified samples by chromatography.

A. Chromatography of Oxysterols

1. Thin-layer chromatography

Thin-layer chromatography (TLC) provides a very simple, rapid separation method. This approach has been extensively used in various studies of oxysterols. Useful chromatographic data have been provided by a number of investigators (21, 22, 44, 133, 360, 609, 844, 1108). Thin-layer chromatography can be very valuable in separation of certain mixtures, especially those from synthetic reaction mixtures, composed of relatively few and often predictable components. However, studies such as those of Aringer et al. (21) and Aringer and Nordström (22), in which the TLC behavior of a significant number of oxygenated sterol standards was evaluated (21, 22), clearly demonstrate the very limited capability of TLC to provide useful separations of oxygenated sterols that could be
OXYSTEROLS: MODULATORS OF CHOLESTEROL METABOLISM 365

January 2000

anticipated in complex mixtures as encountered in samples such as blood or tissues.

Brooks et al. (133) presented TLC data for the following oxysterols and their TMS ether derivatives: (24S)-24-OH-Chol, 26-OH-Chol, 7β-OH-Chol, 7α-OH-Chol, 25-OH-Chol, and (20S)-20-OH-Chol. Rennert et al. (844) reported TLC data for a limited number of oxygenated derivatives on Whatman HP silica plates (in order of increasing Rf): 7α-OH-Chol, 4β-OH-Chol, 26-OH-Chol, 25-OH-Chol, (22S)-22-OH-Chol, (22R)-22-OH-Chol, and 20α-OH-Chol. Björkhem et al. (87) reported the TLC separation of the following oxysterols and their TMS ether derivatives: (24S)-24-OH-Chol, 7β-OH-Chol, 7α-OH-Chol. Teng and Smith (1108) presented TLC behavior of a number of oxygenated derivatives of Chol and their acetate derivatives on Whatman HP silica plates (in order of decreasing polarity): 7α,12α-dihydroxycholesterol, 5β-cholestane-3α,7α,12α-triol, 7α-OH-Chol, 7β-OH-Chol, 7-keto-Chol, 26-OH-Chol, and 7α-hydroxycholest-4-en-3-one. Malavasi et al. (609) reported the TLC behavior of Chol, 7α-hydroperoxycholest-5-en-3β-ol (Chol 7α-hydroperoxide), 7-keto-Chol, 7β-OH-Chol, and 7α-OH-Chol on silica gel F254 plates. Bachowski et al. (44) provided data for a large number of oxygenated derivatives of Chol and their acetate derivatives obtained after incubation of the individual oxysterols with Chol oxidase. Parent oxysterols included 7α-OH-Chol, 7β-OH-Chol, 19-OH-Chol, (20S)-20-OH-Chol, 7β-OH-Chol, and (25R)-26-OH-Chol. The authors noted that 7-keto-Chol was unaltered upon incubation with cholesterol oxidase. Malavasi et al. (609) reported the TLC behavior of Chol, 7α-hydroperoxycholest-5-en-3β-ol (Chol 7α-hydroperoxide), 7-keto-Chol, 7β-OH-Chol, and 7α-OH-Chol on silica gel F254 plates. Bachowski et al. (44) reported the TLC behavior on silica gel G-60 F254 plates for (in order of decreasing Rf) Chol, 5α-hydroperoxycholest-6-en-3β-ol, 7β-hydroperoxycholest-5-en-3β-ol, 7α-hydroperoxycholest-5-en-3β-ol, 7-keto-Chol, 5α-cholest-6-ene-3β,5α-diol, 7β-OH-Chol, and 7α-OH-Chol. The studies of Aringer et al. (21) and Aringer and Nordström (22) provided data for a large number of oxygenated sterols, including those with oxygen functions at carbon atoms 3, 6, 7, 12, 15, 24, 25, and 26, with Δ5 or Δ4 olefinic bonds or a saturated sterol nucleus (with 5α- and 5β-isomers), and 5,6- and 4,5-epoxysterols. Even this relatively large collection of C-27 oxysterols did not include physiologically relevant oxysterols with oxygen functions at carbon atoms 4, 20, and 22 and the 24,25-epoxysterols. Aringer et al. (21) also presented data for a number of oxygenated derivatives of plant sterols. Gray et al. (348) reported the separation of the TMS derivatives of 25-OH-Chol and 5α-cholestane-3β,5-diol on silica gel G-AgNO3 plates.

2. Liquid chromatography

Liquid chromatography (LC), medium-pressure LC (MPLC), and HPLC on various supports have been utilized extensively in attempts to separate various oxygenated sterols. Retention data for a limited number of oxysterols on columns of Sephadex LH-20 and hydroxyalkylated Sephadex LH-20 have been presented (20–22). MPLC on silica gel (521) provided separation of the acetate derivatives of a number of C-27 oxysterols. However, the 5α,6α- and 5β,6β-isomers of 3β-acetoxy-5,6-epoxycholestan-6-one were incompletely separated, and no separation of the diacetate derivatives of 7α-OH-Chol and 7β-OH-Chol was observed. MPLC on alumina-AgNO3 provided complete separations of the acetate derivatives of 7α-OH-Chol and 7β-OH-Chol and of 5α,6α-epoxy-Chol and 5β,6β-epoxy-Chol (521). These striking separations, coupled with the relatively high capacity of this form of chromatography, should be of value in semi-preparative scale work involving the chemical syntheses of the concerned compounds.

Saucier et al. (899) presented HPLC retention time data for a limited number of oxysterols on normal and reverse-phase columns. Oxysterols studied (in order of elution on normal phase HPLC) were (24S)-24-OH-Chol, 7-keto-Chol, 25-OH-Chol, (25R)-26-OH-Chol, 7β-OH-Chol, and 7α-OH-Chol. The order of elution on the reverse-phase HPLC column was 7α-OH-Chol, 7β-OH-Chol, 24-OH-Chol, 7-keto-Chol, 25-OH-Chol, and 26-OH-Chol. Kudo et al. (521) presented normal phase and reverse-phase retention time data for the acetate derivatives of a number of oxygenated sterols including the following: 7α-OH-Chol, 7β-OH-Chol, 19-OH-Chol, (20S)-20-OH-Chol, (20S)-21-OH-Chol, (22R)-22-OH-Chol, (24S)-24-OH-Chol, 25-OH-Chol, (25R)-26-OH-Chol, 5α,6β-diOH-Chol, 5α,6α-epoxy-Chol, 5β,6β-epoxy-Chol, (24RS)-24,25-epoxycholestan-7β-ol, and (24RS)-24,25-epoxylanosterol. The use of acetate derivatives provided the opportunity, with the use of 14C- or 3H-labeled acetic anhydride, to follow the elution of the various compounds and, with knowledge of the specific activity of the labeled acetic anhydride, to quantitate the various oxysterols. Kermasha et al. (482) presented retention data for a limited number of oxygenated derivatives of Chol on normal-phase HPLC. Oxysterols included were (in order of elution) 20α-OH-Chol, 25-OH-Chol, 5α,6α-epoxy-Chol, 5β,6β-epoxy-Chol, 7-keto-Chol, 7β-OH-Chol, 7α-OH-Chol, and 5α,6β-diOH-Chol. Caboni et al. (151) reported separations of a limited number of oxysterols by normal-phase HPLC. Oxysterols studied (in order of elution) were 5α,6α-epoxy-Chol, 5β,6β-epoxy-Chol (not separated from 4β-OH-Chol), 20α-OH-Chol, 7-keto-Chol, 25-OH-Chol, 19-OH-Chol, 7β-OH-Chol, 7α-OH-Chol, 7α,6α-epoxycholest-5-en-3β-ol, 7β-hydroperoxycholest-5-en-3β-ol, and 5α,6β-diOH-Chol. Brown et al. (135) reported retention data for a number of oxysterols on normal-phase HPLC on a silica column. Oxysterols studied included the following (in order of elution): 26-OH-Chol, 7β-hydroperoxycholest-5-en-3β-ol, 7-keto-Chol, 7α,6α-epoxycholest-5-en-3β-ol, 7β-hydroperoxycholest-5-en-3β-ol, 19-OH-Chol, 7β-OH-Chol, and 7α-OH-Chol. Lacritz and Jones (539) reported the use of normal-phase HPLC on an alumina-silica (16:84) column with gradient elution to separate a number of oxysterols. Included were (in order of elution) 20α-OH-Chol, 7-keto-Chol, 5β,6β-epoxy-Chol, 5α,6α-epoxy-Chol, 3β-hydroxy-5α-cholestan-6-one, 7β-OH-Chol, and 7α-OH-Chol.
Chol. This work employed an evaporative light-scattering detector.

Ansari and Smith (13) presented data on the HPLC behavior of several oxysterols on a μPorasil column using hexane-isopropyl alcohol (24:1) as solvent and on a μBondapak C_{18} column with acetonitrile-water (9:1). Data were presented on the following sterols: Chol, cholest-3,5-dien-7-one, cholesta-4,6-dien-3-one, cholest-5-en-3-one, cholestan-4-en-3-one, cholest-4-ene-3,6-dione, 25-OH-Chol, 7-keto-Chol, 5-hydroperoxy-5α-choleste-6-en-3β-ol, 7α-hydroperoxycholesterol-5-en-3β-ol, 7β-hydroperoxycholesterol-5-en-3β-ol, 5α-cholest-6-ene-3β,5-diol, 7α-OH-Chol, and 7β-OH-Chol. On the μPorasil column, little or no separation of cholest-5-en-3-one from cholesta-4,6-dien-3-one was observed. Little separation of numerous pairs of the oxysterols was observed on the μBondapak C_{18} column (including 7α-OH-Chol and 7β-OH-Chol). Teng and Smith (1108) reported normal-phase HPLC retention times for a number of oxysterols that included (in order of elution): 25-OH-Chol, 7-keto-Chol, 7α-OH-Chol, and 7α-OH-Chol. Also presented were data for the corresponding Δ^1-3-ketosterols formed upon incubation of the oxysterols with Chol oxidase. Sevaniyan and McLeod (952) presented data for normal-phase HPLC of the following sterols (in order of elution): 25-OH-Chol, 5α,6α-epoxy-Chol, 5β,6β-epoxy-Chol, 7-keto-Chol, 7α-hydroperoxycholesterol-5-en-3β-ol, and 7α-OH-Chol. Lund et al. (592) presented retention data for reverse-phase HPLC of the following oxysterols (in order of elution): 24-OH-Chol, 25-OH-Chol, and 26-OH-Chol.

The HPLC data on hydroperoxide derivatives of Chol have been presented (13, 43, 506). Korytowski et al. (506) reported partial separations, by reverse-phase HPLC on a C_{18} Ultrasphere column, of the following hydroperoxides (in order of elution): 7β-hydroperoxycholesterol-5-en-3β-ol, 7α-hydroperoxycholesterol-5-en-3β-ol, 5α-hydroperoxycholesterol-6-en-3β-ol, and 6β-hydroperoxycholesterol-5-en-3β-ol. Also reported was the normal-phase HPLC separation of the following hydroperoxides (in order of elution) on a silica column: 6β-hydroperoxycholesterol-4-en-3β-ol, 5α-hydroperoxycholesterol-6-en-3β-ol, and a mixture (not resolved) of the 7α- and 7β-hydroperoxides of Chol. Ba-chowski et al. (43) reported on the reverse-phase HPLC behavior of hydroperoxides of Chol on a C_{18} Ultrasphere column. Under the conditions studied, the 7α- and 7β-hydroperoxides of Chol eluted together, and they were followed by 5-hydroperoxy-5α-cholesterol-6-en-3β-ol and 6β-hydroperoxycholesterol-4-en-3β-ol.

Saucier et al. (898) presented normal- and reverse-phase HPLC data for lanost-8-ene-3β,32-diol and 3β-hydroxylanost-8-en-32-al and for two isolated sterols believed to be lanosta-8,24-diene-3β,32-diol and 3β-hydroxylanosta-8,24-dien-32-al. Shiao et al. (968) reported reverse-phase HPLC data on oxygenated triterpenoids [specifically oxygenated derivatives of Δ^7,9(11),24-lanostatrien-26-oic acid].

First applied in the sterol field for the separation of sterols differing in the number and location of olefinic double bonds (869, 870), Ag⁺-HPLC has been found to be valuable for the separation of the 3β-acetate derivatives of (20R,22R)- and (20S,22S)-isomers of 20,22-di-CH_{2}-Chol (872).

3. Gas chromatography

Brooks et al. (131) reported gas chromatography (GC) retention data for the TMS ether derivatives (in order of elution) 24-keto-Chol, 7-keto-Chol, 24-OH-Chol, and 25-OH-Chol on an SE-30 column. Gustafsson and Sjövall (368) presented GC data for the TMS ethers of (22R)-22-OH-Chol, (22S)-22-OH-Chol, 24-OH-Chol, and 26-OH-Chol on SE-30 and QF-1 columns. Under the conditions studied, little or no separation of the isomers of 22-OH-Chol was observed. Brooks et al. (133) provided retention data for the TMS ether derivatives of the following oxysterols on SE-30, OV-17, and QF-1 columns: 7α-OH-Chol, 7β-OH-Chol, (20S)-20-OH-Chol, (24S)-24-OH-Chol, 25-OH-Chol, and 26-OH-Chol. Burstein et al. (148) reported GC data for the TMS ether derivatives of (22R)-22-OH-Chol and (20R,22R)-dihydroxy-Chol. Also presented were data for the mono-TMS and bis-TMS ether derivatives of 5α-cholestan-3β,5-diol, the bis-TMS ether derivative of 25-OH-Chol, and the TMS ether derivative of 5α,6α-epoxy-Chol on OV-1 and OV-17 columns. Also were presented were data for the underivatized sterols. Aringer and Eneroth (20) reported GC retention data on the mono-, bis-, and tris-TMS ether derivatives of 5α, 6β-diOH-Chol on SE-30 and QF-1 columns. Also presented were data for the bis- and tris-TMS derivatives of 5α,6α-dihydroxy-Chol on an SE-30 column as well as data for 5α,6α-epoxy-Chol and 5β,6β-epoxy-Chol (and the corresponding epoxides of the 24-ethyl substituted sterols) as the free sterol and TMS and acetate derivatives. Aringer et al. (21) reported retention data on an SP-2100 column for the TMS derivatives of a number of oxygenated sterols, including (in order of elution) 7α-OH-Chol, 7β-OH-Chol, 5α,6α-epoxy-Chol (same retention time as 7β-OH-Chol), (22R)-22-OH-Chol, 20α-OH-Chol, 7-keto-Chol, 24-OH-Chol, 25-OH-Chol, and (25R)-26-OH-Chol. Also presented were data for a number of oxygenated derivatives of several plant sterols.

Aringer and Nordström (22) provided GC retention data for the TMS ether derivatives of a large number of dioxygenated C-27 sterols on an SP-2100 column (with additional data on a QF-1 column for some of the compounds). The various oxysterols included those with oxygen functions at carbon atoms 3, 6, 7, 12, 15, 24, 25, and 26, with Δ^3- or Δ^2-olefinic bonds or a saturated sterol.
nucleus (with 5α- and 5β-isomers), and 5,6- and 4,5-epoxy-sterols. Other potentially important oxysterols with oxygen functions at carbon atoms 4, 20, and 22 and 24,25-epoxy-sterols were not studied, nor were oxygenated derivatives of plant sterols. The data of Aringer and Nordström indicate that reliance on GC retention time data alone does not provide firm evidence for assignment of structure to an unknown oxysterol from a biological source in which considerable complexity can be anticipated. Brooks et al. (129) used selective enzymatic oxidation of the 3β-hydroxyl groups of oxysterols with Chol oxidase to generate 3-ketosteroids that were then analyzed by GC-MS of TMS derivatives. Kraaijpoel et al. (511) reported the separation of the TMS ether derivatives of (in order of elution) (22R)-22-OH-Chol, (20S)-20-OH-Chol, (20R,22S)-20,22-diOH-Chol, and (20R,22R)-20,22-diOH-Chol on a capillary column coated with SE-30. Gumulka et al. (360) reported the capillary GC separations of 5α,6α-epoxy-Chol and 5β,6β-epoxy-Chol as the free sterols and as their acetate derivatives on SE-30 and SE-54 columns. Also presented were data for the TMS derivatives on the SE-54 column. Brooks et al. (132) presented retention data for the TMS derivatives of 5α,6α-epoxy-Chol, 5β,6β-epoxy-Chol, 7-keto-Chol, and 26-OH-Chol on DB-1 and DB-5 capillary columns.

Park and Addis (772) presented capillary GC data (DB-5 column) for the TMS derivatives of the following sterols (in order of elution): 7α-OH-Chol, 7β-OH-Chol, 4β-OH-Chol, 5α,6α-epoxy-Chol, 3β-hydroxy-5α-cholestan-6-one, 7-keto-Chol, 25-OH-Chol, and 5α,6β-diOH-Chol. Under the conditions studied, the latter two sterols differed very little in retention time as was the case for the 4β-hydroxy and 5α,6α-epoxy sterols. On a DB-1 column, the TMS ethers showed the following order of elution: 7α-OH-Chol, 5α,6α-epoxy-Chol, 7β-OH-Chol, 4β-OH-Chol, 5α,6β-diOH-Chol, 7-keto-Chol, and 25-OH-Chol. Under the conditions studied, the retention times of the 7β-hydroxy and 4β-hydroxy sterols were very similar. Koopman et al. (505) presented capillary GC data for the TMS derivatives of a limited number of oxysterols on a fused silica capillary column coated with CP-Sil-19-CB. The sterols studied (in order of elution) were 7α-OH-Chol, Chol, 19-OH-Chol, 7β-OH-Chol, (22S)-22-OH-Chol, (22R)-22-OH-Chol, 20α-OH-Chol, 25-OH-Chol, and 26-OH-Chol. Schmarr et al. (906) reported the capillary GC behavior of the TMS derivatives of a number of oxysterols on a modified 50% phenyl-50% methyl polysiloxane deactivated fused silica column. The following order of elution of the sterols was reported: 7α-OH-Chol, 19-OH-Chol, Chol (eluted immediately after the 19-hydroxysterol and incompletely separated from it), 7β-OH-Chol, 5β,6β-epoxy-Chol, 5α,6α-epoxy-Chol, 5α,6β-diOH-Chol, 25-OH-Chol, 20α-OH-Chol, 7-keto-Chol, and 3β,5α-dihydroxy-5α-cholestan-6-one. Rennert et al. (844) presented data for a limited number of oxysterols on capillary GC on a DB-17 column (as free sterols). The oxysterols studied and their retention times (relative to cholesterol) were 20α-OH-Chol (1.35), 7α-OH-Chol (1.40), 25-OH-Chol (1.41), (22R)-22-OH-Chol (1.44), (22S)-22-OH-Chol (1.44), (24RS)-24-OH-Chol (1.46), and 26-OH-Chol (1.63). Pizzoferrato et al. (811) reported baseline separations of the TMS ether derivatives of the following sterols (in order of elution) on an HP-1 cross-linked methyl silicone capillary column: 19-OH-Chol, 7β-OH-Chol, 5α,6α-epoxy-Chol, 20α-OH-Chol, 5α,6β-diOH-Chol, 7-keto-Chol, and 25-OH-Chol. Breuer (121) reported the capillary GC (HP Ultra-1 fused silica column) behavior of the TMS derivatives of the following oxysterols (in order of elution): 7α-OH-Chol, 7β-OH-Chol, 4β-OH-Chol, 4α-OH-Chol, 24-OH-Chol, 25-OH-Chol, and 26-OH-Chol. Lai et al. (538) presented data on the capillary GC behavior of the TMS ether derivatives of the following oxysterols (in order of elution) on a DB-1 column: Chol, 7α-OH-Chol, 5β,6β-epoxy-Chol, 5α,6α-epoxy-Chol, 7β-OH-Chol, 20α-OH-Chol, 25-OH-Chol, 6-ketocholestanol, 7-keto-Chol, and 5α,6β-diOH-Chol.

Breuer and Björkhem (123) reported capillary GC (HP Ultra-1 fused silica column) data for the tert-butyldimethylsilyl ether derivatives of the following oxysterols (in order of elution): 5α,6α-epoxy-Chol, 7-keto-Chol, 7β-OH-Chol, 4β-OH-Chol, 24-OH-Chol, and 26-OH-Chol. Breuer et al. (124) reported that the TMS ether derivative of cholestane-3β,5α,6α-triol eluted shortly after the TMS ether derivative of the corresponding 3β,5α,6β-triol and shortly before that of 24-OH-Chol on an HP Ultra-1 fused silica capillary column. Breuer et al. (124) described a nice separation of the tert-butyldimethylsilyl ether derivatives of 4β-OH-Chol and 4α-OH-Chol. As noted above, Breuer (121) indicated that the TMS ethers of 4β-OH-Chol and 4α-OH-Chol are also separable on the same column. Mori et al. (673) presented capillary GC data on the TMS derivatives of the following oxysterols on an HP-1 cross-linked methyl silicone column: 7β-OH-Chol, 5α,6α-epoxy-Chol, 4β-OH-Chol, 5α,6β-diOH-Chol, 7-keto-Chol, and 25-OH-Chol. Pie et al. (801) and Pie and Seillan (802) reported essentially baseline separations of the TMS ether derivatives of the following compounds (in order of elution) on a 30-μm DB-5 capillary column: 7α-OH-Chol, Chol, 19-OH-Chol, cholesta-3,5-dien-7-one, 7β-OH-Chol, 5β,6β-epoxy-Chol, 5α,6α-epoxy-Chol, 20-OH-Chol, 5α,6β-diOH-Chol, 25-OH-Chol, 7-keto-Chol, and 26-OH-Chol. Garcia Regueiro and Maraschiello (322) reported baseline separations of the TMS derivatives of the following oxysterols (in order of elution) on a fused silica column coated with 5% phenylmethylsilicone: 7α-OH-Chol, Chol, 19-OH-Chol, 7β-OH-Chol, 5β,6β-epoxy-Chol, 5α,6α-epoxy-Chol, 20α-OH-Chol, 5α,6β-diOH-Chol, 25-OH-Chol, and 7-keto-Chol.

The thermal instability of the hydroperoxides of Chol precludes their analyses by GC. Lercker et al. (558) studied the thermal decomposition of the 7α- and 7β-hydroperox-
ides of Chol acetate. Each of the hydroperoxides gave the corresponding 7-keto and 7α- and 7β-hydroxy analogs as the major products of thermal decomposition. Considerably lower levels of other products were reported and included materials believed to be cholesta-3,5,7-triene, cholesta-3,5-dien-7-one, Chol acetate, 7-dehydrocholesterol, 3β-acetoxy-5β,6β-epoxycholestan-7α-ol, 3β-acetoxy-5α,6α-epoxycholestan-7α-ol, and 3β-acetoxy-5β,6β-epoxycholestan-7β-ol. The GC data on the TMS derivatives of the various decomposition products were presented.

Brown et al. (134) reported retention data for the TMS ether derivatives of a selected group of oxysterols on a 30-m fused silica column coated with DB-5 MS [5% phenyl)methylpolysiloxane]. The reported order of elution of the sterols was as follows: 7α-OH-Chol, Chol, cholest-4-ene-3β,6β-diol, 19-OH-Chol, 7β-OH-Chol, cholest-3,5-dien-7-one, cholestan-3β,5α,6α-diol, 5β,6β-epoxycholesterol, cholest-4-en-3-one (with no separation from the 5β,6β-epoxide), 6β-hydroxycholest-4-en-3-one, (22R)-22-OH-Chol, 5α,6α-epoxy-Chol, 20α-OH-Chol, 5α,6α-dioH-Chol, 25-OH-Chol, 3β-hydroxy-5α-cholestan-6-one, 7-ketochol, (25R)-26-OH-Chol, (25S)-26-OH-Chol (with no separation from its 25R-isomer). Axelsson and Larsson (33) presented capillary retention data for the TMS derivatives of a number of oxysterols and the TMS derivatives of the methyl esters of several cholestenic acid derivatives on a fused silica column (25 m × 0.32 mm) coated with a 0.17-μm layer of cross-linked methyl silicone with temperature programming. The sterols studied (in order of elution) were 7α-OH-Chol, 7α-hydroxycholest-4-en-3-one, 7β-OH-Chol, 7-keto-Chol, 24-OH-Chol, 7α,25-dihydroxycholesterol, 25-OH-Chol, 7α,25-dihydroxycholesteryl, 3β-hydroxycholest-5-en-26-oic acid methyl ester, 7α,26-dihydroxycholesterol, 26-OH-Chol, 7α,25-dihydroxycholest-4-en-3-one, 7α-hydroxy-3-oxo-cholesterol-4-en-26-oic acid methyl ester, 3β,7β-dihydroxycholest-5-en-26-oic acid methyl ester, 7α,26-dihydroxycholest-4-en-3-one, 7β,26-dihydroxycholesterol, 3β-hydroxy-7-oxo-cholesterol-5-en-26-oic acid methyl ester, and 26-hydroxy-7-oxo-cholesterol.

4. Chromatographic separations of oxysterol isomers

Gustafsson and Sjövall (368) reported the separation of the 22R- and 22S-isomers of 22-OH-Chol, as their TMS derivatives, by GC on an SE-30 column. No separation was observed on a QF-1 column. Burrows et al. (145) reported separation of the 22S- and 22R-isomers of 22-OH-Chol by column chromatography and TLC of their 3β-benzoate derivatives. Rennert et al. (844) reported no differences in the retention times of the 22R- and 22S-isomers of 22-OH-Chol upon capillary GC of the free sterols on a DB-17 column. The 22R- and 22S-isomers can be nicely separated by partition chromatography on columns of Celite 545 (149). The 23R- and 23S-isomers of 23-OH-Chol have been reported to have been cleanly separated by simple TLC of their dibenzoate derivatives (1159) and, as the free sterols, by silica gel chromatography or by TLC (1158, 1159) but not by GC as the free sterols or as their diacetate derivatives on SE-30 or QF-1 columns (1158). As noted previously, the 20R,22R- and 20S,22S-isomers of 20,22-dihydroxycholesterol have been separated, as their 3β-acetate derivatives, by Ag⁺-HPLC (872). 24R- and 24S-24-OH-Chol, as their TMS derivatives, can be resolved by capillary GC (592, 596). The 24R- and 24S-isomers of 24-OH-Chol can be separated with difficulty by TLC in the form of their 3β-benzoate derivatives (856, 1039, 1159) or more readily by HPLC of the 3β,24-dibenzoate derivatives (501). The 24R- and 24S-isomers of 24-OH-Chol can also be resolved, as their diacetate derivatives, by reverse-phase HPLC (521). The same sterols can also be nicely separated without derivatization by HPLC using a chiral column (899).

The 25R- and 25S-isomers of 26-OH-Chol or appropriate derivatives have been reported to be separable by HPLC (24, 839, 840, 1146). Redel and Capillon (840) reported the separation of the 3β,26-diacetate derivatives of 25R- and 25S-26-OH-Chol ("at a 25-mg level") using 2 μPorasil columns (30 cm × 7.9 mm) using 2.5% ethyl acetate in hexane with 10 recycles. Uomori et al. (1146) reported a partial separation of the 25S- and 25R-isomers of 26-OH-Chol by HPLC on a TSK gel ODS-120T column (250 × 4 mm I.D.) using 7% water in methanol.

The 5α,6α- and 5β,6β-isomers of 5,6-epoxy-Chol can be separated (at times considerably less than completely) by GC in the form of the free sterols (360), their TMS ether derivatives (20, 360, 538, 772), and, more cleanly, as their acetate derivatives (22, 360). Simple TLC on silica gel plates provides little separation of the two compounds (20, 360). However, notable separations have been achieved by TLC on silica gel-AgNO₃ plates in the form of their acetate derivatives (20) or by TLC of their TMS derivatives on silica gel G plates pretreated with hexamethyldisilazane (20). Although only very slight separation of the acetates of the 5α,6α- and 5β,6β-epoxides was achieved on silica gel MPLC (521), remarkable separation of the two compounds has been effected by MPLC on an alumina-AgNO₃ column (521). Excellent separations of the acetate or benzoate derivatives of the two epoxides can be achieved by both normal-phase and reverse-phase HPLC (13, 521). Sevanian and McLeod (952) also reported separation of the 5,6-epoxide isomers as the free sterols by normal-phase HPLC. Cholestan-3β,5α,6β-triol and cholestan-3β,5α,6α-triol can be separated by TLC, LC, or GC (as either di-TMS or tri-TMS derivatives) (20).

The 24R- and 24S-isomers of 24,25-epoxy-Chol and of 24,25-epoxylanosterol are resistant to separation by capillary GC as their acetate derivatives (280) or by reverse-phase HPLC on routine C₁₈ columns (280, 758, 759) either as the free sterols or their acetate derivatives. However,
these isomers are separable on a special Vydac C18 column (280, 759). The benzoates of the 24R- and 24S-isomers of 24,25-epoxy-Chol have been separated by normal-phase HPLC (280, 946), by MPLC on silica gel 60 (897), and by preparative TLC (701). The separation of the 24R- and 24S-isomers of 24,25-epoxycholesteryl by TLC of the acetate or benzoate derivatives has also been described (1102, 1103). The free sterols have been reported to show slightly different retention times on normal-phase HPLC (1102), with retention times of 16.6 and 16.9 min for the 24S- and 24R-isomers, respectively.

5. Chromatographic separations of fatty acid esters of oxysterols

Oxygenated sterols are often found as their fatty acid esters. Reverse-phase HPLC procedures for the separation of fatty acid esters of 3β-hydroxy-5α-cholest-8(14)-en-15-one have been described (207). With the use of chemically synthesized (825) fatty acid esters of the 15-ketosterol, rather clean separations of the linolenate, arachidonate, linoleate, elaidate, oleate, and stearate esters were achieved using a C18 Microsorb column. With the use of this system, separation of the oleate and palmitate esters was not achieved. However, the use of a C6 Spherisorb column permitted the separation of the linoleate, arachidonate, linoleate, elaidate, oleate, and stearate esters were achieved using a C18 Microsorb column. With the use of this system, separation of the oleate and palmitate esters was not achieved. However, the use of a C6 Spherisorb column permitted the separation of the linoleate, arachidonate, linoleate, palmitate, oleate, stearate, and arachidate esters of the 15-ketosterol. Lin and Morel (571) reported TLC and reverse-phase HPLC data for the mono-and diol esters of 25-OH-Chol. Brown et al. (134) described reverse-phase HPLC separations of the palmitate, stearate, oleate, linoleate, arachidonate, and docosahexaenoate esters of 7-keto-Chol. The individual esters were prepared by chemical synthesis, but they were not isolated in pure form (except for the oleate ester) and were not characterized. Szedlacsek et al. (1082) presented retention time data for a number of diesters and 3β-acyl monoesters of (25R)-26-OH-Chol on an Adsorbosphere C18 column using mixtures of acetonitrile and isopropyl alcohol as the eluting solvent. Also presented were data for the 3β-oleyl esters of 7α-OH-Chol, 7β-OH-Chol, 7-keto-Chol, 5α,6α-epoxy-Chol, 5β,6β-epoxy-Chol, 5α,6β-diOH-Chol, and 25-OH-Chol.

B. Mass Spectrometry of Oxysterols

Full or partial MS data for a number of oxygenated derivatives of Chol and/or their TMS derivatives have been presented. These include 26-OH-Chol (21, 32), 25-OH-Chol (131, 155, 772, 811), 24-OH-Chol (131, 368), 22-OH-Chol (148, 368, 1063), 20α-OH-Chol (811), 19-OH-Chol (562), 7α-OH-Chol (562, 772), 7β-OH-Chol (155, 562, 772, 811), 4α-OH-Chol (124), 4β-OH-Chol (124, 562, 772), 5α,6β-diOH-Chol (124, 562, 772, 811), 5α,6α-diOH-Chol (124), 5α,6α-epoxy-Chol (74, 348, 562, 772, 811, 155), 5β,6β-epoxy-Chol (20, 562), 7-keto-Chol (131, 155, 562, 772, 811), 24-keto-Chol (131), cholestan-3β,5α-diol (155), 7α,26-dioH-Chol (1222), 7α,26-dihydroxycholest-4-en-3-one (562), 7β,26-dihydroxycholest-4-en-3-one (562), (20R,22R)-20,22-diOH-Chol (148, 511, 872, 1063), (20S,22S)-20,22-diOH-Chol (872), and cholesta-3,5-dien-7-one (74). In addition, Aringer and Nordström (22) presented fragmentation data for the TMS derivatives of a large number of oxygenated sterols. These included the mono-TMS ether derivatives of a number of 3-hydroxy-sterols with ketone functions at carbon atoms 6, 7, 12, 15, and 24, the TMS ether derivatives of 3-hydroxyxysterols with either 5,6- or 4,5-epoxide functions, and bis-TMS ether derivatives of 3-hydroxysterols with an additional hydroxy function at carbon atoms 6, 7, 12, 15, 24, 25, or 26. Partial mass spectral data for a number of oxygenated derivatives of β-sitosterol have been presented by Daly et al. (232). MS of the TMS ether derivatives of a number of oxygenated derivatives of campestero sterol and sitosterol were presented by Dutta and Appelqvist (270). Dutta (269) presented mass spectra for several oxygenated derivatives of stigmasterol. However, the oxidized derivatives of these plant sterols were not isolated and fully characterized prior to GC-MS analyses.

Considerable effort has been expended toward an understanding of the electron impact-induced fragmentations of 3β-hydroxy-5α-cholest-8(14)-en-15-one. Studies of the 15-ketosterol and its derivatives (including its TMS dienol ethers) and analogs (including deuterium-labeled analogs) along with analyses of high-resolution MS data on individual fragment ions provided important information regarding its fragmentation on electron impact (825, 827, 828) which have facilitated studies of the structures of metabolites (485, 826, 827, 916) and new analogs (382–384, 486, 978–983, 1058, 1071, 1072, 1074–1076, 1201) of the parent 15-ketosterol. MS data have been presented for a number of chemically synthesized fatty acid esters of 3β-hydroxy-5α-cholest-8(14)-en-15-one (825). Included were data for the palmitate, palmitoleate, stearate, oleate, linoleate, linolenate, arachidate, and arachidonate esters.

C. NMR of Oxysterols

With continued improvements in instrumentation and techniques, NMR spectroscopy provides an increasingly powerful approach for the identification of oxygenated sterols. Selected aspects of the NMR analyses of oxygenated sterols have been reviewed elsewhere (1198). In efforts led by Dr. W. K. Wilson, the 1H- and 13C-NMR spectral properties of 3β-hydroxy-5α-cholest-8(14)-en-15-one and its analogs have been extremely studied and provided important evidence for the structures of metabolites and analogs of the 15-ketosterol (382, 384, 485, 486, 826, 827, 978–983, 1072–1074, 1076, 1129, 1130,
In addition, they have provided critical information leading to the first assignments of the resonances for each of the protons of the side chain of sterols with a saturated C_8H_17 side chain (as in Chol) or of sterols with a Δ^24 double bond in the side chain (980, 1199).

Carr et al. (163) applied ^1H- and ^13C-NMR to the study of 6a-chloro-5b-cholestan-3β,5-diol, the major product obtained upon treatment of Chol-lecithin liposomes with hypochlorous acid. ^13C-NMR assignments were provided for this halohydrin and for two other halohydrins of Chol, i.e., 6β-chloro-5α-cholestan-3β,5-diol and 5-chloro-5α-cholestan-3β,6β-diol. Partial ^1H-NMR assignments were provided for the 6β-chloro-3β,5β-diol. Emmons et al. (279) presented detailed analyses of the ^13C- and ^1H-NMR spectra of a number of oxygenated derivatives of lanosterol and 24,25-dihydrolanosterol. Emmons et al. (280) also presented full ^13C-NMR assignments for the acetate derivatives of (24R)-24,25-epoxylanosterol, (24S)-24,25-epoxylanosterol, (24R)-24,25-epoxy-Chol, and (24S)-24,25-epoxy-Chol. The 24R- and 24S-isomers of the acetates of 24,25-epoxylanosterol and of 24,25-epoxy-Chol could be differentiated by ^13C-NMR. In contrast, the ^1H-NMR spectra of the 24R- and 24S-isomers of the epoxylanosterol and epoxycholesterol were essentially indistinguishable, as had been noted previously for the acetates of 24,25-epoxylanosterol (100). The 24R- and 24S-isomers of 24-hydroxysterols can be differentiated by ^13C-NMR (500). The 25R- and 25S-isomers of 26-hydroxysterols can be differentiated by ^13C-NMR (54, 826, 949, 1146) and by ^1H-NMR of their (+) or (-)-methoxy(trifluoromethyl) phenylacetate esters (711, 1146) or acetate esters (826).

A nice application of ^1H- and ^13C-NMR to the elucidation of structure of a derivative of an oxysterol is found in studies of the structure of squalamine (674, 1192), a novel compound isolated from the shark that has notable antimicrobial activities. Another case is that of boophilin, a 3-sulfate derivative of 3β-hydroxycholest-5-en-26-oic acid in which the acid is conjugated in an amide linkage to l-leucine (819). This novel compound, isolated from a cattle tick, was reported to have antifungal and antibacterial activity (819). Kobayashi (498) analyzed the ^13C-NMR spectra of a number of polyhydroxy 5α,14α-steroids and determined additivity relationships of hydroxyl substituent effects. Fontana and co-workers (307, 308) applied ^1H-NMR in studies of the levels of certain oxygenated derivatives of Chol in egg powders. Their studies involved examination of the resonances corresponding to C-6 and C-18 protons of free oxysterol fractions obtained after chromatography. The detection limit under the conditions employed was ~0.3 ppm or 5 mg from a 16-g sample of egg powder. The oxygenated derivatives of Chol studied (307, 308) were the following: 5α,6α-epoxy-Chol, 5β,6β-epoxy-Chol, 7α-OH-Chol, 7β-OH-Chol, 7-keto-Chol, 5α,6β-diOH-Chol, 25-OH-Chol, and 20α-OH-Chol. Although the spectra were recorded at 500 MHz, only low-precision data were presented, and the number of oxysterols studied was limited.

D. X-ray Crystal Structures of Oxysterols

X-ray crystallographic analysis has been employed to establish unequivocally the structures of oxygenated sterols (or their derivatives) shown to be potent regulators of sterol synthesis (112, 337, 485, 665, 707, 767, 800, 922, 927, 1030, 1031) or of key intermediates or by-products in the chemical syntheses of 15-oxygenated sterols of potential importance in biology or medicine (214, 1195, 1196). X-ray crystallographic analyses of the 25R-isomer of 26-OH-Chol (493, 949) and its 25S-isomer (1146) have been presented. Duchamp et al. (262) reported X-ray data on the p-bromobenzoate ester of (25S)-26-hydroxycholest-4-en-3-one. McCourt et al. (629) provided X-ray crystal structure analyses for 25-OH-Chol and 7-keto-Chol. Ishida et al. (427) presented an X-ray crystal analysis of scymnol [(24R)-5β-cholestan-3α,7α,12α,24,26,27-hexol], a compound with reported medical utility from shark bile. Shimura et al. (974) reported the determination of the structure of aragusterol C by X-ray crystal analysis. Aragusterol C, a novel halogenated (chloro)oxysterol from an Okinawan sponge, was reported to inhibit the growth of KB cells (IC_{50} 0.04 μg/ml) and had antitumor activity in vivo.

III. FORMATION OF OXSTEROLS

A. Formation of 26-, 25-, and 24-Hydroxysterols

In 1956, Fredrickson and Ono (314) reported the formation of labeled 26-OH-Chol and 25-OH-Chol upon incubation of [4-14C]Chol with mouse liver mitochondria. The labeled products were characterized by their mobilities on paper chromatography and, in the case of the 26-hydroxysterol, by cocrystallization with the authentic, unlabeled sterol. In 1961, Danielsson (234) also characterized 26-OH-Chol as a product of the mitochondrial metabolism of [4-14C]Chol. The labeled 26-hydroxysterol was characterized by chromatography and cocrystallization experiments. In contrast to the study of Fredrickson and Ono, little or no enzymatic formation of labeled 25-OH-Chol could be detected. Upon incubation of the labeled 26-OH-Chol with mouse liver homogenates, the formation of cholest-5-ene-3β,7α,26-triol was shown by chromatography and cocrystallization experiments. In a separate study, Danielsson (235) reported the formation of cholest-5-ene-3β,7α,26-triol after incubation of mouse liver homogenates with labeled 7α-OH-Chol. Mitropoulos and Myant (659) reported the conversion of [4-14C]Chol to 26-OH-Chol, 3β-hydroxycholest-5-en-26-oic acid, and 3β-
hydroxycholesterol-5-en-24-oic acid (as well as lithocholic acid, Chenodeoxycholic acid, and α- and β-muricholic acids) when incubated with rat liver mitochondria in the presence of the soluble fraction of a rat liver homogenate. For the most part, characterization of the labeled products was limited to TLC. The C-24 acids were reported to be present in the form of taurine conjugates. Mitropoulos et al. (660) reported the conversion of [4-14C]Chol to 26-OH-Chol upon incubation with rat liver mitochondria. The labeled 26-OH-Chol was characterized by TLC behavior of the free sterol and its diacetate derivative and by the results of cocrystallization studies with the diacetate derivative.

Björkhem and Gustafsson (90) reported the formation of 26-OH-Chol from Chol with rat liver mitochondria and the requirement for NADPH and oxygen. Incubation under nitrogen or incubation with buffer alone or boiled mitochondria was reported to result in no formation of 26-OH-Chol. The product was characterized by chromatography (TLC and radio-GC). The formation of the 26-hydroxysterol was shown to occur with the incorporation of molecular oxygen. Björkhem and Gustafsson (90) also observed the formation of 25-OH-Chol from Chol with rat liver mitochondria in the presence of NADPH and oxygen. The formation of the 25-hydroxysterol was very much less than that of 26-OH-Chol under the same conditions. Identification was based on TLC and GC. No formation of labeled 25-OH-Chol was detected upon incubation with buffer alone or with boiled mitochondria. Evidence was presented indicating the origin of the 25-hydroxyl function from molecular oxygen. Aringer et al. (21) reported the enzymatic formation of 26-OH-Chol, 25-OH-Chol, and 24-OH-Chol upon incubation of [4-13C]Chol with rat liver mitochondria in the presence of NADPH or an NADPH-generating system. The relative amounts of the 26-hydroxy-, 25-hydroxy-, and 24-hydroxysterols were ~1.0, 0.3–0.5, and 0.1, respectively. The formation of these sterols from autoxidation of Chol was excluded on the basis of results with boiled mitochondrial controls. Characterization of the oxysterols was based on TLC and LC and on GC-MS studies of their TMS derivatives.

Pedersen and Saarem (785) reported the solubilization of a cytochrome P-450 from rat liver mitochondria that catalyzed the conversion of Chol into 26-OH-Chol and 25-OH-Chol (in a ratio of 9:1) in the presence of adrenodoxin, NADPH-ferredoxin reductase, and NADPH. The oxysterols were characterized by GC-MS as their TMS derivatives. Pedersen et al. (786) studied the substrate specificity of the soluble liver mitochondrial P-450 system. The most efficient substrates were 7α-OH-Chol and 7α-hydroxycholesterol-5-en-3-one followed by 5β-cholestane-3α,7α,12α-triol and 5β-cholestane-3α,7α,α-trihydroxy-β-cholestan-26-oic acid as a minor product. Gustafsson (363) demonstrated the 26-hydroxylation of exogenous and endogenous Chol by rat liver mitochondria. The 26-hydroxysterol was characterized by GC-MF of its bis-TMS derivative. Kok and Javitt (502) reported the 26-hydroxylation of endogenous Chol of hamster liver mitochondria using GC-MS methodology involving a deuterated internal standard of 26-OH-Chol with analyses of ion abundances in the region corresponding to M-CH$_3$COOH in the MS of the diacetate derivative. The description of the characterization of the labeled internal standard was limited.

Lund et al. (593) reported the conversion of Chol to 26-OH-Chol and 24-OH-Chol upon incubation with mouse liver mitochondria in the presence of NADPH and isocitrate. No formation of 25-OH-Chol was detected under the conditions employed. The amounts of the 26-OH-Chol and the 24-OH-Chol formed were not presented (although the former compound was reported to be more abundant). On the basis of 1-h incubations with mouse liver mitochondria, the presence of an isotope effect in the conversion of d$_6$-Chol (deuterium at C-26 and C-27) to 26-OH-Chol and in the conversion of d$_4$-Chol (deuterium at C-24) to 24-OH-Chol was reported. Lund et al. (592) demonstrated the conversion of Chol to 26-OH-Chol, 25-OH-Chol, and 24-OH-Chol (1.0:0.2:0.4, respectively) upon incubation with pig liver mitochondria in the presence of added isocitrate in Tris-HCl buffer containing 20% glycerol. The products were identified by GC-MS of TMS derivatives. Incubation of a highly purified preparation of the 26-hydroxylase from pig liver mitochondria with [6,7,7-D$_3$]Chol in the presence of adrenodoxin, adrenodoxin reductase, and NADPH gave not only 26-OH-Chol but also the 25-hydroxy and 24-hydroxy derivatives of Chol. The ratio of the 26-hydroxy-, 25-hydroxy-, and 24-hydroxy derivatives of Chol was reported to be approximately the same as that observed with the intact pig liver mitochondria. The 24-OH-Chol formed with the purified enzyme was reported to be one isomer that was tentatively assigned as the 24S configuration. The authors suggested that a single enzyme was responsible for the formation of the three hydroxy-sterols. The presence of an isotope effect was reported for 24-hydroxylation with the purified enzyme (reported as “$K_{II}/K_P > 10$”) but little or none for 25-hydroxylation or 26-hydroxylation. The absence of an isotope effect for 26-hydroxylation with the purified pig liver enzyme differed from the finding of a significant isotope for the same reaction catalyzed by mouse liver mitochondria (593). The magnitude of isotope effects was estimated from the results of a single time point experiment (30 min), and no information regarding the amounts of oxygenated sterol products formed was provided. The deuterated substrates used contained deuterium not only at the carbon atom of interest but also at adjacent carbons. For the 26-, 25-,
and 24-hydroxylations the substrates used were \([25,26,26,26,27,27,27,27,2H_2]\text{Chol}, [25,26,26,26,27,27,27,27,2H_2]\text{Chol}, \text{and } [23,23,24,24,25,2H_2]\text{Chol}, \text{respectively. The preparation of the 24-OH substrate was described in another paper by the same investigators (593). The product was obtained in low yield through a multistep synthesis. Little or no characterization of the product, or of the multiple intermediates in its synthesis, was provided. The final product was reported to contain 77.4% d_5 species accompanied by lower percentages of d_4, d_3, d_2, d_1, and d_0 species. In the critical experiment concerning the 24-hydroxylation, essentially no 24-hydroxylated species was formed from the \([23,23,24,24,25,2H_5]\text{Chol}.\)

In this experiment, the Chol substrate lacking deuterium at C-24 was a \([25,26,26,26,27,27,27,2H_2]\text{Chol} \text{preparation.}\)

Petrack and Lantario (798) described an assay method for 26-hydroxylase activity that involved normal-phase HPLC of the \(\Delta^1\)-3-ketones resulting from treatment of mitochondrial incubation mixtures with Chol oxidase. Products of the action of Chol oxidase on authentic oxy-
steroles (7α-OH-Chol, 7β-OH-Chol, 26-OH-Chol, and 25-OH-Chol) were studied by normal-phase HPLC. Only an authentic sample of 7α-hydroxycholest-4-en-3-one was described as being available. 24-OH-Chol was not studied. Products of the action of Chol oxidase on the authentic standards and on mitochondrial incubation mixtures were studied only by HPLC (absorbance at 240 nm). Using this assay system, the authors reported that rat liver mitochondria catalyzed the formation of 26-OH-Chol from endogenous Chol. No 26-OH-Chol was formed with boiled mitochondrial preparations or upon incubation in the presence of cyclosporin (20 μM) or carbon monoxide. The enzymatic formation of lesser amounts of 25-OH-Chol was noted. 2-Hydroxypropyl-β-cyclodextrin (0.9%) and exogenous Chol in α-hydroxypropyl-β-cyclodextrin (0.9%) stimulated 26-OH-Chol formation. It was suggested that stimulation by cyclodextrin itself was due to facilitation of access of the endogenous Chol substrate to the en-
zeyme. Fasting markedly lowered 7α-OH-Chol formation, but not 26-OH-Chol formation, in homogenates of rat liver.

Since early studies by Berseus (69) and Mitropoulos and Myant (658), the stereospecificity involved in the formation of 26-hydroxy derivatives of Chol and of other sterols (27, 55, 367, 374, 963, 1144, 1145) has been an area of interest. The experiments of Berseus (69) and of Mitropoulos and Myant (658), both involving the use of \([{}^{14}\text{C}]\text{Chol} \text{formed biosynthetically from } [2-{}^{14}\text{C}]\text{mevalonate, demonstrated that the formation of 26-OH-Chol by mouse liver mitochondria involved a high degree of stereospecificity. The in vivo results of Hanson et al. (374), based on studies of the 3α,7α,12α-trihydroxy-5β-chole-
stan-26-oic acid formed from [2-{}^{14}\text{C}]\text{mevalonate, were similar and, in addition, indicated that the 26-oic acid had predominantly the 25R-configuration. Batta et al. (55) reported that the 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid of human bile had the 25R-configuration. However, Une et al. (1145) reported that the 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid found in the unconjugated frac-
tion of urine from an infant with Zellweger’s syndrome was, as judged by HPLC analysis of its \(p\)-bromophenacyl derivative, a 7:3 mixture of the 25R- and 25S-isomers. It should be noted that the efficient in vivo conversion of both the 25R- and 25S-isomers of 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid to cholic acid has been observed in the rat (126, 365) and human (1078). Very recently, Van Veldhoven et al. (1161) reported that purified trihydroxy-
coprostanoyl-CoA oxidase of rat liver, catalyzing the intro-
duction of a \(\Delta^2\)-double bond, acted on only the 25S-
isomer of the CoA derivative of 3α,7α,12α-trihydroxy-5β-
cholestan-26-oic acid. However, isolated rat liver peroxisomes catalyzed the desaturation of both the 25S- and 25R-isomers, leading to the suggestion (1161) of the presence of a racemase in the peroxisomes.

The stereospecificity of the \(\omega\)-hydroxylation of sterols by mitochondria and by microsomes of liver has been reported to differ. In one study (963) with liver mitochondria and microsomes of the rat, guinea pig, and rabbit, incubation of 5β-cholestan-3α,7α-diol was reported to yield both the 25R- and 25S-isomers of cholestan-3α,7α,12α-triol. However, the 25R-isomer was the predominant product (as judged by GC of the TMS derivative) in the mitochondrial incubations. With liver microsomes of the rat and guinea pig, the 25S-isomer was the predominant product, whereas with rabbit, the 25R-isomer was reported to be the predominant product. The results with rat liver microsomes were in accord with an earlier report by Gustafsson and Sjöstedt (367) that indicated that the 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid formed upon incubation of biosynthetically labeled (from \([2-{}^{14}\text{C}]\text{mevalonate}) \text{Chol with rat liver mitochondria had the 25S-configuration. Une et al. (1144) reported that, upon incubation of 5β-cholestan-3α,7α,12α-triol with liver mitochondria of a number of different animals (rat, rabbit, ham-
ter, chicken, turtle, carp, and frog), both the 25R- and 25S-isomers of 5β-cholestan-3α,7α,12α-tetrol were formed. However, the 25R-isomer was the predominant product in each case (as judged by HPLC analysis of a 26-anthroyl derivative). Incubations of the triol with liver
micromones of hamster, chicken, frog, and carp were also reported to give both the 25R- and 25S-isomers of the tetrol. In accord with the reports of Shefer et al. (963) and Gustafsson and Sjöstedt (367), the 25S-isomer was the predominant product with rat liver micromones. However, the 25R-isomer was the major product with hamster and carp liver micromones. The conversion of 5β-cholestan-3α,7α,12α-triol to 5β-cholestan-3α,7α,12α,26-tetrol by a partially purified cytochrome P-450 from rat liver mitochondria was reported to give only the 25R-isomer of the tetrol (as judged by TLC) (27). However, the published chromatogram does not appear to exclude the possible formation of some of the 25S-isomer. As noted previously, the ω-hydroxylation of 3β-hydroxy-5α-cholesta-5(14)-en-15-one by rat liver mitochondria has been shown to yield both the 25R- and 25S-isomers of 3β,26-dihydroxy-5α-cholesta-8(14)-en-15-one. NMR studies indicated that the 25R- and 25S-isomers were present in a 4:1 ratio. A similar direct analysis of the stereochemistry of the in vitro enzymatic ω-hydroxylation of Chol has not been made. Whereas Chol serves as a substrate for the ω-hydroxylating system of mitochondria, it is not clear whether Chol is a substrate for the microsomal system (367). However, it is noteworthy that a sample of 26-OH-Chol isolated from human aorta has been reported (839) to be comprised of its 25R- and 25S-isomers in a 9:1 ratio (as judged by HPLC analysis of its diacetate derivative).

As noted previously, 24-hydroxysterols and 25-hydroxyysterols can arise from mitochondrial hydroxylation of sterols as shown in the cases of Chol (21, 592) or 3β-hydroxy-5α-cholesta-8(14)-en-15-one (826, 827). The formation of 24-OH-Chol has also been reported (251) after aerobic incubation of labeled Chol with a microsomal preparation of bovine liver in the presence of an NADPH-generating system or the combination of NADPH plus an NADPH-generating system. The characterization of the labeled product was based on TLC and cocrystallization experiments with the dibenzoate derivative. The results of the latter experiments were interpreted as indicating the 24S-configuration of the 24-hydroxysterol. The extent of conversion of Chol to 24-OH-Chol was very low (0.32–0.38%). No conversion was detected in an incubation carried out in the absence of oxygen. Similar results were obtained with rat brain microsomes (575), with conversions of Chol to 24-OH-Chol of ~0.15–0.18%. Björkhem et al. (94) reported that incubations of rat brain microsomes with [4,14C]Chol and NADPH gave negligible conversion to 24-OH-Chol, stated to be considerably <0.2%. The authors noted that “the small extent of conversion of the labeled cholesterol varied considerably in different sets of experiments, regardless of the time of incubation and the mode of addition of the cholesterol (acetone, cyclodextrin, or Tween 80).” Despite the difficulties in demonstration of the enzymatic formation of 24-OH-Chol using radiotracer methodology, the authors indicated the in vitro formation of the 24-hydroxysterol by two types of experiments. The first involved GC-MS measurement of the levels of 24-OH-Chol in rat brain microsomes before and after incubation for 2 or 20 h. The levels of the 24-hydroxysterol after 0, 2, and 20 h of incubation in a typical experiment were reported as 0.72, 0.82, and 0.86 μg/ml brain microsomal preparation. However, this approach apparently gave variable results, and it was stated that “in some experiments there was no linear increase in the amount of 24(S)-hydroxycholesterol.” The second approach involved the measurement, by GC-MS, of the incorporation of labeled oxygen of 18O2 into the 24-hydroxysterol formed upon incubation of rat brain microsomes in the presence of NADPH. The rate of formation of the 24-OH-Chol was estimated to be ~30 ng h−1 ml microsomal preparation−1 or ~0.015% of Chol in the preparation per hour. The mitochondrial fraction of rat brain also catalyzed the incorporation of labeled oxygen of 18O2 into 24-OH-Chol. The level of incorporation (~0.003% of endogenous Chol per hour) was lower than that observed with the microsomal fraction.

24-Hydroxysterols can also result from another process, i.e., the reduction of a 24,25-epoxysterol. Steckbeck et al. (1039) reported the formation of labeled 24R)-24-hydroxylanost-8-en-3β-ol and (24R)-24-OH-Chol upon incubation of [2-3H]-24R,25-epoxylanosterol with a rat liver homogenate preparation. However, it should be noted that the 24R-isomer of 24-OH-Chol is not considered to be the naturally occurring isomer present in mammalian blood and tissues. In subsequent studies (1029, 1102), no conversion of 3H-labeled (24S)-24,25-epoxy-Chol to 24-OH-Chol or 25-OH-Chol could be detected with mouse L fibroblasts. Similarly, with Chinese hamster lung cells, no conversion of (24S)-24,25-epoxy-Chol to 24-OH-Chol or 25-OH-Chol was detected. However, with these cells, conversion of the (24R)-24,25-epoxy-Chol to (24R)-24-OH-Chol was observed.

Saucier et al. (897) reported the formation of labeled 25-OH-Chol (identified by chromatographic and cocrystallization experiments) upon incubation of [3H]mevalonate with Chinese hamster lung cells. The mode of formation of the 25-OH-Chol was not established. Alsena et al. (7) reported experiments suggesting 25-hydroxylation of (20S)-20-OH-Chol by bovine adrenal cortex mitochondria. Formation of the 25-hydroxylated sterol was observed in incubations carried out at pH 7.80 (but not at pH 7.40). Assignment of structure was based on GC-MS studies of the TMS ether derivative (in the absence of an authentic standard).

Pedersen et al. (787) reported that a solubilized cytochrome P-450 from bovine brain mitochondria catalyzed the 26-hydroxylation of a number of C27 sterols (5β-cholestan-3α,7α,12α-triol, 7α-hydroxycholesterol-4-en-3-one, 7α,12α-dihydroxycholesterol-4-en-3-one, 5β-cholestan-3α,7α-diol, and 5β-cholestan-3α,7α,12α-triol). The activity of the brain preparation was reported to be
considerably lower than comparable preparations from rat and human liver. No formation of 26-OH-Chol from Chol was detected under the conditions studied. The products were characterized by GC-MS, although data on this matter were not presented. No mention was made regarding hydroxylation at C-24 or C-25 by the bovine brain enzyme preparation. Javitt et al. (149) reported the formation of labeled 26-OH-Chol (as well as Chol, 3β-hydroxychol-5-en-26-oic acid, chenodeoxycholic acid, and cholic acid) after the intravenous administration of [5-13C]mevalonate to animals. The title of this paper indicates that the studies were made in the Syrian hamster, whereas the text indicates that the studies involved Sprague-Dawley rats.

Rennert et al. (844) demonstrated the expression of the 26-hydroxylase gene in human granulosa cells. They also reported the NADPH-dependent formation of labeled 26-OH-Chol from [3H]Chol (location of label not specified) upon incubation with mitochondria from ovarian mitochondria from superovulated rats. All incubations were carried out in the presence of aminogluthimide (100 μg/ml) to inhibit side-chain cleavage activity that was reported to be much higher than that of 26-hydroxylase activity. Identification of the product was based on chromatographic and cocrystallization studies as well as GC-MS. However, it should be noted that at least one significant ion was present in the spectrum of the isolated sterol that was not present in that of authentic 26-hydroxysterol and that at least one major ion present in the MS of the authentic sample was absent in the MS of the isolated sterol. Ovarian mitochondrial 26-hydroxylase activity was markedly increased by calcium (200 μM), pregnenolone (IC50 <10 μM), or progesterone (IC50 ~10 μM). It was suggested that the levels of progestins that inhibit the 26-hydroxylase may be in the physiological range for their occurrence in ovaries. The authors also noted that physiological stimulation of steroidogenesis in ovary might, by virtue of the actions of progestins on the 26-hydroxylase, suppress the formation of 26-OH-Chol. Furthermore, such a reduction in 26-OH-Chol formation could result in increased Chol synthesis and increased LDL receptor activity, with a resulting continual supply of Chol for cell function and steroidogenesis.

Su et al. (1057) characterized a cDNA for a mitochondrial P-450 of rat liver and expressed it in COS cells. Mitochondrial preparations from the COS cells were reported to catalyze, in reconstituted systems, the 25-hydroxylation of vitamin D3 and the 26-hydroxylation of Chol. Characterization of the products was limited to HPLC analysis. Usui et al. (1147) presented evidence indicating that the vitamin D 25-hydroxylase of rat liver mitochondria also catalyzed the 26-hydroxylation of 5β-cholestan-3α,7α,12α-triol. The cDNA for the vitamin D 25-hydroxylase was transfected into COS cells, and the enzyme activities of a solubilized extract of mitochondria from the COS cells were studied. A reconstituted system containing a solubilized mitochondrial extract of the COS cells showed substantial activity not only for 25-hydroxylation of 1α-hydroxy vitamin D but also for 26-hydroxylation of 5β-cholestan-3α,7α,12α-triol.

Lund et al. (595) recently reported cloning of both human and mouse cDNA encoding an enzyme with cholesterol 25-hydroxylase activity. Cells overexpressing the 25-hydroxylase produced 25-OH-Chol from Chol and 24,25-diOH-Chol from 24-OH-Chol. Unlike cytochrome P-450 that catalyze many sterol hydroxylations, the 25-hydroxylase contains a diiron cofactor (Fe-O-Fe or Fe-O-H) bound to histidine clusters. Although it remains to be demonstrated that the primary function of this enzyme is 25-hydroxylation, results reported by Lund et al. (595) with transfected cells overexpressing the 25-hydroxylase appeared to be compatible with the suggested role of 25-OH-Chol in the regulation of cholesterol homeostasis. However, 25-OH-Chol levels in blood and tissues are extremely low (see section IV).

Cali and Russell (153) isolated the human 26-hydroxylase cDNA and determined its sequence. The human 26-hydroxylase was predicted to have an amino acid sequence that showed 81% amino acid homology with the 26-hydroxylase of rabbit liver mitochondria. The combination of Chol (31 μM) and 25-OH-Chol (5 μM) had no effect on the level of mRNA for the 26-hydroxylase in simian virus 40-transformed human fibroblasts. The human 26-hydroxylase cDNA and an expressible cDNA for bovine adrenodoxin were introduced into simian COS cells. Incubation of [7β-3H]-5β-cholestan-3α,7α,12α-triol with these cells led to the formation of the corresponding C-26 acid as the major product and small amounts of the C-26 alcohol (as judged by TLC). Incubation of [7β-3H]-5β-cholestan-3α,7α,12α,26-tetrol with the cells led to the formation of the corresponding acid (as judged by TLC). Cali et al. (152) localized the gene for the 26-hydroxylase to chromosome 2 in humans and to chromosome 1 in the mouse. Axen et al. (36) presented evidence indicating that the human cyp26 also catalyzes the 1α-hydroxylation and 25-hydroxylation of vitamin D3. They reported that recombinant expressed human cyp26 in Escherichia coli catalyzed not only the 26-hydroxylation of 5β-cholestan-3α,7α,12α-triol and the oxidation of 5β-cholestan-3α,7α,12α,26-tetrol to the corresponding acid but also the 25-hydroxylation of vitamin D3 and of 1α-hydroxy vitamin D3, and the 26- and 1α-hydroxylation of 25-hydroxy vitamin D3. The same workers also reported that COS-1 cells transfected with human cyp26 cDNA catalyzed not only the 26-hydroxylation of 5β-cholestan-3α,7α,12α-triol but also (to a lower extent) the 1α-hydroxylation and 26-hydroxylation of 25-hydroxy vitamin D3.

Pikulkeva et al. (803) also reported the expression of human cyp26 in E. coli. The purified recombinant enzyme
catalyzed the 26-hydroxylation of Chol and, with a much higher efficiency, 5β-cholestan-3α,7α,12α-triol. The enzyme was also found to catalyze the conversion of 25-hydroxyvitamin D₃ to material with the HPLC behavior of its 1α-hydroxy derivative. The catalytic activity for this reaction was found to be very considerably less than that for the 26-hydroxylation of Chol or of 5β-cholestan-3α,7α,12α-triol. It is of interest that studies with recombinant liver cyp26 expressed in E. coli (36, 803, 804) have not noted the formation of either 24- or 25-hydroxylated products after incubation with Chol or 5β-cholestan-3α,7α,12α-triol (as seen in incubations with liver mitochondria). No studies have been presented to establish the stereochemistry at C-25 in the 26-hydroxysterol formed with the recombinant enzyme.

P-450 cyp26 from liver mitochondria has been purified to homogeneity in the cases of rabbit (1194), rat (729), and pig (61). The enzyme from pig liver catalyzed the 26-hydroxylation of 5β-cholestan-3α,7α-diol, Chol, and 25-hydroxyvitamin D₃. Structural assignments for the products were based on HPLC. The rat cyp26 has been expressed in yeast (887), and the human cyp26 has been expressed in E. coli (36, 803, 804). The enzyme expressed in yeast catalyzed the 26-hydroxylation of 5β-cholestan-3α,7α,12α-triol and the 25-hydroxylation of 1α-hydroxyvitamin D₃ (887). The products were characterized by HPLC.

Reiss et al. (842) reported the formation of 26-OH-Chol by bovine aortic endothelial cells. 26-OH-Chol and the corresponding C₂₇ carboxylic acid were identified on the basis of GC-MS studies of metabolites recovered in the media. The levels of 26-OH-Chol and the carboxylic acid in the culture medium were markedly increased by incubation in the presence of added Chol (20 μM). Incubation of the cells with 5β-cholestan-3α,7α,12α-triol led to decreased levels of 26-OH-Chol and the C₂₇ carboxylic acid in the medium. Under similar cell culture conditions, the formation of 26-OH-Chol (along with the corresponding C₂₇ and C₂₄ carboxylic acids) was also detected in media from incubations of Hep G2 cells. 26-OH-Chol was not detected in the media after incubation of Chinese hamster ovary (CHO) cells. Björkhem et al. (84) reported a time-dependent increase in the levels of 26-OH-Chol and of 3β-hydroxycholesterol-5-en-26-oic acid in the medium after incubation of human alveolar macrophages for different periods of time in MEM supplemented with 10% heat-inactivated fetal calf serum (FCS). The levels of the 26-OH-Chol and the C₂₇ acid in the cells were reported to be “in general less than 20% and 5%, respectively, of the content of those compounds in the medium.” Cyclosporin suppressed the levels of 26-OH-Chol and the C₂₇ carboxylic acid in the medium. The effect on the levels of the cholestenoic acid was more marked than that of the levels of the 26-OH-Chol. With the use of trideuterated Chol (apparently labeled at C-6, C-7α, and C-7β; although this reviewer could not locate a description of the synthesis of this d₉-Chol), the formation of d₉-26-OH-Chol and d₉-3β-hydroxycholesterol-5-en-26-oic acid was also reported. The presence of the 26-hydroxylase was also shown by Western blot experiments. Björkhem et al. (84) reported that cultured human endothelial cells also formed 26-OH-Chol and 3β-hydroxycholesterol-5-en-26-oic acid, but in amounts very considerably less than they observed with pulmonary macrophages. It was proposed that “conversion of cholesterol into 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid represents a general defense mechanism for macrophages and possibly for other peripheral cells exposed to cholesterol. Absence of this defense mechanism may contribute to the premature atherosclerosis known to occur in patients with sterol 27-hydroxylase deficiency (cerebrotendinous xanthomatosis).” Lund et al. (590) extended these studies and reported that, with human alveolar macrophages preloaded with [¹⁴C]Chol, incubation of the cells with media containing FCS resulted in a significant recovery of [¹⁴C] in 26-oxygenated products (26-OH-Chol plus 3β-hydroxycholesterol-5-en-26-oic acid) in the culture medium. However, the recovery of [¹⁴C] in 26-oxygenated products was only ~10% that of [¹⁴C]Chol in the medium. In other experiments, the macrophages from different individuals were incubated with media containing 10% FCS. With cells from eight subjects, most (~76%) of the Chol was in the free state. Of the 26-oxygenated metabolites, most were found in the medium (82 ± 13% for 26-OH-Chol and 99 ± 1% for 3β-hydroxycholesterol-5-en-26-oic acid). It was stated that all of the 26-oxygenated products “recovered from the cell medium were always unesterified.” In another set of experiments, macrophages from four patients were incubated with media containing FCS and cyclosporin (20 μM) for 24 h. Cyclosporin decreased the amounts of 26-OH-Chol plus 3β-hydroxycholesterol-3-en-26-oic acid in the medium from 6.1 ± 1.6 to 0.4 ± 0.1 fmol·cell⁻¹·24 h⁻¹. Under these conditions, cyclosporin increased the Chol levels in the cell from 10.5 ± 1.5 to 17.8 ± 3.2 fmol·cell. The amount of esterified Chol in the cells was low (<8% of total). Lund et al. (590) also presented evidence (based on blood samples of hepatic vein, portal vein, and a peripheral artery) that indicated a significant arterial-hepatic venous difference for total 26-oxygenated metabolites and suggested uptake by liver of the 26-oxygenated compounds. The authors suggested “a protective role of sterol 27-hydroxylase in the development of atherosclerosis” on the basis of the above findings and the known predisposition of subjects with cerebrotendinous xanthomatosis (CTX) to develop premature atherosclerosis.

Babiker et al. (40) extended this research to a study of the capacity of various cells to secrete 26-oxygenated products, i.e., 26-OH-Chol and cholest-5-en-26-oic acid, into the culture medium as measured by GC-MS analyses. Human macrophages were reported to show a higher
capacity to secrete 26-oxygenated products than human endothelial cells. Human lung alveolar macrophages, human monocyte-derived macrophages, and human endothelial cells \((n = 3\) in each case) showed mean values of 1400, 300, and 38 ng·10^6 cells^{-1·24 h^{-1}}, respectively. One culture of human fibroblasts showed 20 ng·10^6 cells^{-1·24 h^{-1}}. As expected, cells (monocyte-derived macrophages or fibroblasts) from patients with CTX showed little or no secretion of 26-oxygenated products (<2 ng·10^6 cells^{-1·24 h^{-1}}). In the case of the lung macrophages, the great majority of the 26-oxygenated products present in the culture medium (and, to a lesser extent, in the cells) was found as the 26-carboxylic acid. Addition of FCS, cyclodextrin, cyclodextrin plus Chol, albumin, LDL, high-density lipoprotein (HDL), or apolipoprotein (apo) A-I increased the secretion of 26-oxygenated products from the cells. Incubation of the lung macrophages with 25-OH-Chol \((12.4 \mu M)\) decreased the secretion of 26-oxygenated products. Westman et al. \((1193)\) conducted additional investigations of the role of 26-OH-Chol (and its corresponding acid) in the net efflux of Chol from monocyte-derived macrophages. Cells loaded with Chol (from acetylated LDL) showed significant levels of 26-OH-Chol, and the efflux of the 26-hydroxysterol was proportional to its intracellular level. The export of Chol and 26-OH-Chol (and the corresponding acid) was markedly affected by the presence and the nature of acceptor species in the culture medium. Incubation with reconstituted HDL or native HDL resulted in a decreased efflux of 26-OH-Chol and cholestenoic acid from the cells and increased the efflux of free Chol into the medium. Because HDL is commonly considered to be an important physiological acceptor of sterols after their efflux from cells, these experiments raise a question as to the importance of the conversion of Chol to 26-OH-Chol and the efflux of 26-OH-Chol as an important protective defense mechanism in the net removal of Chol from cells.

The immunosuppressant cyclosporin A has been reported \((823)\) to show high potency in the inhibition of the 26-hydroxylation of Chol in rat liver mitochondria, with an \(IC_{50}\) of 4 \(\mu M\). In contrast, direct addition of cyclosporin A \((50 \mu M)\) had no effect on cytochrome P-450 activity in vitro \((823)\). Additional studies with rat hepatocytes indicated that cyclosporin A caused a concentration-dependent inhibition of the incorporation of \(^{14}C\)Chol into total bile acids, with an \(IC_{50}\) value of \(-10 \mu M\). The formation of labeled \(\beta\)-muricholic acid and of mono- and dihydroxylated bile acids was inhibited considerably more than the synthesis of cholic acid and other polar acids considered to be derived largely from cholic acid. Cyclosporin A \((10 \mu M)\) also decreased the levels of total bile acids in rat hepatocytes plus media. The decrease in the levels of total bile acids was found to largely reflect decreases in the amounts of chenodeoxycholic acid and \(\beta\)-muricholic acid since the levels of cholic acid did not change in the treated cells. The changes in bile acid formation and the major effect on chenodeoxycholic acid and \(\beta\)-muricholic acid formation were interpreted as being in accord with postulations of an alternative pathway for the biosynthesis of bile acids apart from the pathway in which \(7\alpha\)-hydroxylation of Chol is the initial reaction. It is important to note that cyclosporin A had other effects relative to Chol metabolism in the rat hepatocytes. Whereas cyclosporin A \((10 \mu M)\) had no effect on the levels of ATP in rat hepatocytes, the antibiotic caused a 14\% decrease in the concentration of cellular ATP at 20 \(\mu M\) cyclosporin A. The antibiotic \((10 \mu M)\) also caused a 46\% increase in the amount of cell-associated \(^{14}C\) after incubation of the rat hepatocytes with \(^{14}C\)Chol and a 30\% decrease in the incorporation of \([2\text{-}^{14}C\text{acetate into Chol (although methodology on the latter point was not presented).}

Dahlbäck-Sjöberg et al. \((231)\) reported that cyclosporin A inhibited the formation of 26-OH-Chol from Chol with either rat liver mitochondria or with a purified 26-hydroxylase from rabbit liver mitochondria. The antibiotic caused a 50\% inhibition of 26-OH-Chol formation at \(-4 \mu M\). Whereas cyclosporin A was highly active in the inhibition of 26-hydroxylation of Chol and \(7\alpha\)-OH-Chol with the rat liver mitochondria, it had little or no effect on the 26-hydroxylation of 5\(\beta\)-cholestan-3\(\alpha\),7\(\alpha\)-diol and 5\(\beta\)-cholestan-3\(\alpha\),7\(\alpha\),12\(\alpha\)-triol. With the purified 26-hydroxylase, the antibiotic also had no significant effect on the 26-hydroxylation of 5\(\beta\)-cholestan-3\(\alpha\),7\(\alpha\),12\(\alpha\)-triol. Kinetic studies with the purified cytochrome P-450 showed that the inhibition of 26-hydroxylation of Chol by cyclosporin A was noncompetitive in nature. Vinegar et al. \((1202)\) reported that cyclosporin inhibited 26-OH-Chol formation by Hep G2 cells \((IC_{50} = 1 \mu M)\) and by mitochondria from Hep G2 cells (inhibition constant = 0.25 \(\mu M\)). Identification of the product was based on the incorporation of \(^{14}C\)Chol (complexed with \(\beta\)-cyclodextrin) into 26-OH-Chol as judged by HPLC. The chromatograms presented did not include information of the mobilities of other oxygenated sterols such as 25-OH-Chol or 24-OH-Chol (which are expected to be formed in mitochondrial incubations) or other oxysterols that might arise from autoxidation of \(^{14}C\)Chol during incubations and/or sample processing. The cyclosporin used was apparently a commercial preparation for intravenous administration (containing substantial amounts of a polyoxyethylated castor oil) that was then added in dimethyl sulfoxide. The authors reported that, with freshly isolated mitochondria from Hep G2 cells, the inhibition caused by cyclosporin of 26-OH-Chol formation was competitive. It should be noted that cyclosporin itself undergoes metabolism by cytochrome P-450 systems similar to those involved in the initial steps of the side-chain oxidation of sterols, i.e., hydroxylation and oxidation to aldehydes and carboxylic acids \((205)\). Vinegar et al. \((1202)\) also reported that cyclosporin had other actions upon incubation with Hep G2.
cells. Cyclosporin, at 2.5 and 10 μM, inhibited the incorporation of [14C]Chol into Chol esters of cells (−19 and −32%, respectively); this was associated with increased levels of free [14C]Chol in the cells (+26% at 2.5 μM and +75% at 10 μM). Cyclosporin, at 0.25 and 1.0 μM, had no significant effect on the esterification of Chol. Winegar et al. (1202) also reported that cyclosporin, at 10 μM (but not at 1 μM), caused an inhibition of LDL uptake (as measured by cell-associated 125I-LDL). In these studies, possible effects of the castor oil derivative in the commercial sample of cyclosporin were not evaluated. Holmberg-Betzoltz and Wikvall (394) reported that purified rabbit liver cyp26 catalyzed the conversion of Chol to 26-OH-Chol, but the activity of the enzyme with this substrate was considerably less than that with 5β-cholestane-3α,7α-diol or 5β-cholestane-3α,7α,12α-triol. Incubation in the presence of carbon monoxide (CO:O₂, 98:2; vs. N₂:O₂, 98:2) inhibited the 26-hydroxylation of 5β-cholestane-3α,7α,12α-triol. Ketoconazole (an inhibitor of cytochrome P-450), but not disulfiram (an inhibitor of aldehyde dehydrogenase), inhibited the 26-hydroxylation of 5β-cholestane-3α,7α,12α-triol with the purified rabbit liver cyp26.

Cyclosporin administration has been reported to increase plasma Chol levels (9). Whether or not this effect is due to the inhibitory action of cyclosporin on the conversion of Chol to 26-OH-Chol is not known. 26-OH-Chol is a potent downregulator of HMG-CoA reductase activity and an inhibitor of sterol synthesis. In addition, as noted previously, the conversion of Chol to 26-OH-Chol has also been suggested to be important in providing a mechanism for the elimination of excess Chol from cells (84, 590).

Preincubation of mitochondria with several peripheral benzodiazepine receptor ligands or the induction of hepatic porphyria resulted in decreases in the conversion of labeled Chol to 26-OH-Chol (as judged by radio-TLC analysis) by rat liver mitochondria (1128). The effects of these treatments on the formation of 26-OH-Chol were ascribed to modulation of the intracellular transport and availability of the Chol substrate.

The presence of 26-hydroxylase has been demonstrated in frozen and thawed preparations of human skin fibroblasts (992). The following sterols were reported to be substrates for the 26-hydroxylase: 5β-cholestane-3α,7α,12α-triol, 5β-cholestane-3α,7α,12α-diol, 7α-OH-Chol, and 7α-hydroxycholest-4-en-3-one. Chol was not tested as a substrate.

Axelson and Larsson (32) reported the formation of 26-OH-Chol after incubation of FCS with normal human fibroblasts. Identification of the 26-OH-Chol was based on GC-MS of its TMS derivative. All GC-MS experiments involved the addition of an internal standard of a sample of 26-OH-[3H]Chol containing predominantly 7 atoms of deuterium. Almost all of the 26-OH-Chol reported to be formed was found in the medium. Increases in the amounts of 26-OH-Chol found in the medium at 24 h were ~3.5 times that found in cells incubated for only 15 min with FCS. Under the conditions studied, the levels of 25-OH-Chol and of 24-OH-Chol in the medium (or in cells) at 24 h did not appear to be different from those found after 15 min of incubation with FCS. Substantial levels of 7-keto-Chol were found in medium (and in cells) after either 15 min or 24 h of incubation of the cells with FCS. For example, the levels of 7-keto-Chol in medium at 15 and 24 h were ~12 and ~3 times, respectively, that of the levels of 26-OH-Chol in the same experiment. Significant levels of 7α-OH-Chol and 7β-OH-Chol were also detected in medium (and in cells) after incubation of the cells with FCS for 15 min or 24 h. Cyclosporin A appeared to abolish the increase in 26-OH-Chol found in the medium. Also, little or no increase in 26-OH-Chol in medium was observed upon incubation of cells from a homozygous FH subject. No analyses of the oxysterol levels in the FCS used were presented. Experiments with [3H]Chol and/or [3H]Chol olate incorporated into FCS, and analysis by HPLC also showed formation of 26-OH-[3H]Chol which was almost exclusively in medium. The formation of 26-OH-[3H]Chol under these conditions appeared to be substantially suppressed by cyclosporin A. It should be noted that only a small fraction of the labeled Chol was converted to 26-OH-Chol (~0.04% of the internalized LDL Chol). A study of the time course of the effects of the addition of FCS to fibroblasts on the levels of newly formed 26-OH-Chol (in the medium) and the levels of HMG-CoA reductase suggested that the changes might be related. However, the major decreases in reductase activity occurred before any substantial increase in 26-OH-Chol in the medium. Axelson and Larsson (33) described other experiments indicating the formation of 26-OH-Chol and, in lesser amounts, 25-OH-Chol from Chol of FCS (with ~70% of the Chol in LDL) in normal human fibroblasts (based on GC-MS analyses of compounds in the culture media). Also formed were significant amounts of cholest-5-ene-3β,7α,26-triol, 7α,26-dihydroxycholest-4-en-3-one, 3β-hydroxy-cholest-5-en-26-oic acid, 3β,7α-dihydroxycholest-5-en-26-oic acid, 3oxo-7α-hydroxycholest-4-en-26-oic acid, 3β,7α-dihydroxycholest-5-en-26-oic acid, and 3β-hydroxy-7-oxo-cholest-5-en-26-oic acid. The major 26-oxygenated sterols detected were 26-OH-Chol, 7α,26-dihydroxycholest-4-en-3-one, 7α,26-dihydroxycholest-5-en-7-one, and 3oxo-7α-hydroxycholest-4-en-26-oic acid. Quite different results were observed with a virus-transformed line of human fibroblasts. In this case, very little formation of the 26-oxygenated sterol or its metabolites was observed. In the same experiments, substantial amounts (in order of abundance) of 7-keto-Chol, 7β-OH-Chol, and 7α-OH-Chol were observed in the culture media from 15 min and 48 h incubations of both the normal fibroblasts and the virus-transformed fibroblasts with the FCS. The authors noted that “most of these sterols were probably formed by autoxidation of cholesterol during...
the purification of the samples, since their amounts did not differ significantly from those of the controls (15-min incubations)." This may be the case. In addition, the presence of these oxysterols in the FCS used in these experiments was not excluded since analyses of the FCS were not made. In the normal human fibroblasts cyclosporin A inhibited the formation of 26-OH-Chol, 7α,26-dihydroxycholest-4-en-3-one, 7α-hydroxycholest-4-en-26-oic acid (as judged by HPLC) from labeled Chol or labeled Chol oleate (preincubated with FCS before cellular incubations). It is important to note that the results of the experiments of Axelson and Larsson (32, 33) were interpreted as indicating the formation of 26-OH-Chol from Chol (or its esters) present in LDL. Whereas this is probably the case, the actual experiments did not involve incubation of the cells with preparations of isolated LDL; they involved incubations with FCS.

Reiss et al. (843) reported on the expression of sterol 26-hydroxylase in human endothelial cells from pulmonary artery and aorta as judged by measurement of levels of mRNA. mRNA for the 26-hydroxylase was also detected in Hep G2 cells but not in CHO-K1 cells. 26-OH-Chol and 3β-hydroxycholest-5-en-26-oic acid were observed in the culture media in the cases of endothelial cells (from both pulmonary artery and aorta) incubated for 72 h in media containing delipidated FCS. Increased levels of the two compounds in the culture medium were observed after incubations of the endothelial cells in the presence of added Chol. The formation of 26-OH-Chol and 3β-hydroxycholest-5-en-26-oic acid was also observed after incubation of Hep G2 cells for 72 h with exogenous Chol in media containing delipidated FCS. The level of 26-OH-Chol in the medium of the Hep G2 cells was comparable to that observed for endothelial cells from aorta (grown in the same medium containing added Chol). Under the conditions studied, the level of 3β-hydroxycholest-5-en-26-oic acid in the medium for the Hep G2 cells was ~10 times higher than that observed for the aortic endothelial cells. The authors proposed that "27-hydroxylase activity in arterial endothelium provides a local mechanism of defense against Chol accumulation within the arterial wall by serving as a pathway for elimination of intracellular Chol by conversion to more polar metabolites," a suggestion bearing remarkable similarity to the suggestion made by Björkhem et al. (84) for results obtained from human pulmonary alveolar macrophage cells. Reiss et al. (843) did not detect formation of 26-OH-Chol in the medium after incubation of CHO cells for 72 h in medium containing delipidated FCS and added Chol. Whereas the 26-OH-Chol was not detected, low levels of 3β-hydroxycholest-5-en-26-oic acid were reported.

Taurocholic acid (10, 30, and 50 μM) caused a significant reduction in the level of cyp26 activity in rat hepatocytes (1137). The extents of inhibition were 31 and 81% at concentrations of 10 and 50 μM taurocholate, respectively. Taurocholate (50 μM) also lowered the levels of cyp26 mRNA, and there was an even more striking decrease in the level of mRNA for cyp7a. No effect of taurocholate on the level of mRNA for lithocholic acid 6β-hydroxylase was observed. The lowering of mRNA for the 26-hydroxylase by taurocholate was dose dependent with significant reduction at 5–50 μM. Maximal lowering of cyp26 mRNA was observed at 30 and 50 μM. The concentrations of the taurocholate used were stated to be in the physiological range reported in portal vein blood of rats. Taurocholate, cholic acid, and deoxycholic acid, each at 50 μM, had essentially identical effects in lowering the levels of cyp26 mRNA in cultured rat hepatocytes. Chenodeoxycholic acid (50 μM) was less potent, and β-muricholic acid (50 μM) had no significant effect. Taurocholate (50 μM) suppressed transcriptional activity for both cyp26 and for cyp7a. In another paper, the same group (1139) extended these studies. At 50 μM, either deoxycholic acid or cholic acid resulted in marked lowering of cyp26 activity, mRNA levels, and transcriptional activity in rat hepatocytes. Similar findings were made with cyp7a. In contrast to these effects of deoxycholate and cholate, little or no effects were observed with β-muricholic acid and ursodiol bile acids. In contrast to these studies with cultured cells, Shefer et al. (966) reported that feeding deoxycholic acid to rats had no effect on the levels of hepatic cyp26 activity or on the levels of mRNA for this enzyme. In contrast, marked lowering of the levels of cyp7a activity was observed that was associated with a striking increase in the levels of mRNA for this enzyme. Xu et al. (1208) reported that the levels of hepatic mitochondrial cyp26 activity of New Zealand White (NZW) rabbits increased by +60% upon Chol feeding (2% Chol in a chow diet) for 10 days. Total biliary drainage for 7 days had no effect on the levels of the cyp26 activity in chow-fed or in the Chol-fed animals. The assay used was based on the incorporation of [4-14C]Chol into material with the TLC mobility of 26-OH-Chol and recrystallization to constant specific activity (data not presented). Xu et al. (1209) recently reported increased cyp26 activity in NZW and heterozygous (but not homozygous) WHHL rabbits on Chol feeding (3 g/day for 10 days).

Post et al. (815) observed that cafestol, a diterpene responsible for the hypercholesterolemic effect of boiled coffee, caused a dose-dependent decrease in cyp26 activity, bile acid synthesis, and cyp7 activity in cultured rat hepatocytes. The decrease in 26-hydroxylase activity was associated with decreases in its mRNA levels and in cyp26 gene transcriptional activity. Clear effects on each of the above were observed at 32 μM cafestol. Modest decreases (~20%) in mRNA levels for LDL receptor and HMG-CoA reductase were observed at 63 μM cafestol.

Twisk et al. (1138) reported that insulin, at physiological concentrations, inhibited the formation of bile ac-
ids and lowered cyp26 activity. The decrease in enzyme activity caused by insulin was associated with a decrease in the level of mRNA for the enzyme and decreased transcriptional activity. Very similar results were obtained with respect to the effects of insulin on cyp7a.

Sterol 26-hydroxylase activity is preferentially localized in the pericentral hepatocytes in rat liver (1140). The enzyme activity, steady-state mRNA level, and gene transcription for cyp26 were reported to be 2.9, 2.5, and 1.7 times higher, respectively, in pericentral hepatocytes than in perportal hepatocytes. A similar preferential localization in pericentral hepatocytes has also been reported for cyp7 (see sect. iiB).

Hasan and Kushwaha (376) reported the formation of 26-OH-Chol in homogenates of baboon liver in the presence of added NADPH-generating system. Identification of the 26-OH-Chol was limited to its behavior on a reverse-phase HPLC system for which only 26-OH-Chol, 7-keto-Chol, and Chol were used as authentic standards. The nature of the homogenate preparations was not specified except to note that they were prepared from frozen (-70°C) samples of liver and that “homogenates were prepared in phosphate-buffered saline by polytron homogenizer on ice.” The methodology for quantitation of the 26-OH-Chol involved saponification of the incubation mixture followed by reverse-phase HPLC. The base-labile 7-keto-Chol was added to the strongly alkaline saponified incubation mixture before extraction of the nonsaponifiable lipids (NSL). The levels of cyp26 activity were measured as the difference between results obtained at 4 and 37°C. The substrate involved in these assays was apparently Chol present in the homogenate preparation, since no addition of exogenous sterol was mentioned. The magnitude of “background” cyp26 activity at 4°C was not presented. Using this methodology, Hasan and Kushwaha (376) reported that the mean value of cyp26 activity of baboon liver homogenates was higher in samples from “low responders” to a high-fat, high-Chol diet [318 ± 95 (SE) μg 26-OH-Chol·g liver-1·h-1; n = 3] than in those from “high responders” [67 ± 13 (SE) μg 26-OH-Chol·g liver-1·h-1; n = 3]. The animals were fed the high-fat, high-Chol diet for an unspecified period of time. Kushwaha et al. (526) reported on the levels of mRNA for the cyp26 in livers of baboons on a chow diet and at various times after the administration of a high-fat (40% of calories), high-Chol (1.7 mg/kcal) diet. Three groups of animals were employed that differed in their levels of serum Chol upon administration of the high-Chol diet. The levels of cyp26 mRNA in the livers of low responders on the chow diet were higher than those observed in the medium responders and high responders. Upon changing from the chow diet to the high-Chol, high-fat diet, little or no change in 26-hydroxylase mRNA was observed in the high responders. In contrast, significant increases were observed in the low responders and medium responders.

The cyp26 mRNA levels in the medium and low responders on the high-fat, high-Chol diet were higher than those of the high responders at each of the time points studied. Except for the value at 3 wk after initiation of the high-fat, high-Chol diet, the levels of 26-hydroxylase mRNA in the low responders were higher than those of the medium responders. In the same study the low responders and the high responders did not differ with respect to the levels of mRNA in liver for cyp7a on the chow diet or on the high-fat, high-Chol diet (weeks 3, 10, and 78). Furthermore, no differences were observed between the levels of mRNA for cyp7a upon changing from a chow diet to the high-Chol diet (in either the low responders or the high responders). Chen et al. (190) reported increases in the levels of cyp26 activity and protein in low responder baboons on a high-Chol, high-fat diet after 6 and 10 wk (relative to values obtained at 3 wk). In the high responders, no significant increases were observed at 6 and 10 wk. Unfortunately, no data were provided on animals before the administration of the high-Chol, high-fat diet. Another study (527) from the same laboratory concerned studies in ovariectomized baboons on the high-Chol, high-fat diet. Hepatic and adrenal levels of mRNA for the cyp26 were higher in animals treated with estrogen (estradiol cypionate) or with the combination of estrogen plus progesterone. Wang et al. (1181) reported that hepatic mitochondrial cyp26 activity in piglets was decreased by fasting and increased by feeding (nursing).

Sugawara et al. (1066) observed that expression of steroidalogenic acute regulatory protein in COS-1 cells cotransfected with cyp26 and adrenodoxin caused a marked (6-fold) increase in the formation of 3β-hydroxycholest-5-en-26-oic acid. The steroidalogenic acute protein stimulates steroid formation in adrenal and gonadal cells and appears to be involved in the transport of Chol within mitochondria. The results of Sugawara et al. (1066) indicate that this protein can stimulate not only steroid hormone formation from Chol, but it can also stimulate the conversion of Chol to 26-OH-Chol and the cholestenoic acid. Other recent investigations of the regulation of the 26-hydroxylase of rat liver have indicated sequence complementarity between the 5′-terminal regions of the mRNA for the rat liver mitochondria 26-hydroxylase and serine protease inhibitors (960, 961), the levels of the latter previously shown (1216) to be regulated by growth hormone. The sequence overlap was noted to “probably represent the first observation of 5′-end overlap between two functional mRNA” (960). The mRNA levels of the 26-hydroxylase in liver were shown to be regulated by growth hormone (961). Further studies indicated that the mRNA for the serine protease inhibitors can regulate the expression of the cyp26 gene (960).

Bile duct ligation in male Sprague-Dawley rats has been reported (364) to result in increased in vitro 26-hydroxylation of Chol by liver mitochondria.
The importance of the 26-hydroxylase is clearly demonstrated in the case of CTX, an autosomal recessive sterol storage disease characterized by tendon xanthomas, neurological dysfunction with dementia and cerebellar ataxia, and premature atherosclerosis and cataracts (85). Homozygous subjects show very low levels or the absence of 26-hydroxylase activity in fibroblasts obtained by skin biopsy (152, 489, 992). A number of different mutations in the cyp26 gene have been reported (6, 152, 191–193, 323, 489, 553, 694, 1167, 1168). Very recently, Rosen et al. (862) described studies of mice with a disrupted sterol 26-hydroxylase gene. The level of bile acids in feces and bile was very much lower in a cyp26−/− mouse than in a control (cyp26 +/+ ) mouse. Furthermore, the conversion of [7β-3H]7α-OH-Chol to acidic material in feces was much lower in a cyp26−/− mouse than in a control (cyp26 +/+ ) mouse after its intraperitoneal injection. The mean level of 7α-OH-Chol in serum was reported to be elevated in the cyp26−/− mouse (n = 6) when compared with that for control (cyp26 +/+ mice; n = 5), with mean values of 5.0 ± 2.5 and 1.2 ± 0.5 (SE) μM, respectively. However, from the data presented, these mean levels did not differ significantly (P > 0.05). The mean level of 26-OH-Chol in serum from the cyp26−/− mice was reported as <0.0025 μM as compared with 0.20 ± 0.03 μM for the control mice. The levels of 7α-OH-Chol in liver, brain, and kidney were reported to be higher in cyp26−/− mice than in control mice, although it was not specified as to whether the results presented corresponded to analyses of pool samples or from individual animals from each group. The mean level of serum lathosterol, taken as an indirect measure of hepatic HMG-CoA reductase activity, was reported to be significantly elevated in the knockout mice, and the hepatic level of HMG-CoA reductase mRNA was stated to be two- to threefold higher in the knockout mice. Interestingly, indications of neurological or vascular abnormalities or marked elevation of tissue Chol, as seen in human CTX, were not detected.

B. Formation of 7-Oxygenated Sterols

The results of an important early study in 1958 by Bergström et al. (62) demonstrated that the 7α-hydroxylation involved in the overall conversion of Chol to cholic acid occurs with stereospecific loss of the 7α-hydrogen. In 1965, Mendelsohn et al. (642) reported the conversion of [26,14C]Chol to labeled 7α-OH-Chol and 7β-OH-Chol upon incubation with rat liver preparations. Identification of the labeled products rested largely on the results of cocrystallization studies. The authors ascribed the formation of both of the 7-hydroxysterols to enzymatic action and not to autoxidation of Chol. However, it should be noted that the reported incorporations of the labeled Chol into 7α-OH-Chol (i.e., ~1.1%) and 7β-OH-Chol (i.e., ~0.0%) were quite low and were comparable to that of the incorporation into 7α-OH-Chol with one heat-inactivated control incubation. The possibility that the 7-oxygenated derivatives of Chol arose from autoxidation of Chol under the incubation conditions employed is a very important matter in view of prior work of Danielsson (233). In another early study, Björkhem et al. (87) reported, upon incubation of labeled Chol with the 20,000-g supernatant fraction of a rat liver homogenate, the formation of the following oxygenated sterols: 7α-OH-Chol, 7-keto-Chol, 7α-hydroxycholest-4-en-3-one, 7α,12α-dihydroxycholest-4-en-3-one, and 5β-cholestane-3β,7α,12α-triol. The labeled products were characterized on the basis of TLC and cocrystallization experiments. Although the incubations were relatively short, no rigorous controls to assess the possible artificial formation of 7α-OH-Chol and 7-keto-Chol from autoxidation of Chol were employed. The formation of 7α-OH-Chol, 7β-OH-Chol, and 7-keto-Chol from Chol has also been reported (454) with rat liver microsomes in the presence of NADPH under conditions permitting peroxidation of lipids (i.e., in the absence of EDTA).

7α-OH-Chol, 7β-OH-Chol, and 7-keto-Chol, as well as the 7-hydroperoxides, are products of the autoxidation of Chol. An abundance of evidence has demonstrated that 7α-OH-Chol is also an enzymatic product. Whether or not 7β-OH-Chol is also the product of direct enzymatic action is less clear. Although Mendelsohn et al. (642) claimed the indication of the presence of Chol 7β-hydroxylase activity in rat liver preparations, the evidence presented on this point was very limited and inconclusive. Shoda et al. (976) reported that rat liver mitochondria have the capacity to epimerize certain 7α-hydroxysterols or their cholest-5-en-26-ol analog to give the corresponding 7β-hydroxy compounds. The epimerization was proposed to occur via 7-keto intermediates. The action of rat liver mitochondria on 7α-OH-Chol itself was apparently not studied. The enzymatic nature of the formation of the 7β-hydroxy compounds was indicated by the dependence of the reaction on the presence of added isocitrate and the amount of mitochondrial protein. Breuer and Björkhem (123) reported substantial enrichment of 18O in both oxygens of 7β-OH-Chol of liver and plasma after exposure of rats to 18O2. In the same experiments, very substantial enrichment of 5α-cholest-7-en-3β-ol with 18O was observed; however, the 18O content of Chol was minimal. The results (from 2 rats) were interpreted as demonstrating the formation of 7β-OH-Chol from Chol in vivo and suggested the possibility that the 3β,7β-dihydroxy-Δ5-sterol “is predominantly formed from newly synthesized cholesterol, or a cholesterol precursor, by enzymatic reactions in vivo.”

Song et al. (1015) reported the enzymatic conversion of 7α-OH-Chol to 7-keto-Chol with hamster liver micro-
some in an NADP-dependent reaction. This enzyme activity was also present in liver microsomes of human and bovine origin but was absent in liver microsomes of rat, mouse, and rabbit. 7β-OH-Chol was not tested as a substrate. Thus 7-keto-Chol can arise not only from decomposition of the 7α- and 7β-hydroperoxides of Chol but also by enzymatic dehydrogenation of 7α-OH-Chol (at least with hamster, human, and bovine liver microsomes). Song et al. (1014) purified this enzyme from hamster liver microsomes. The purified enzyme catalyzed the C-7 dehydrogenation of both 7α-OH-Chol and 7β-OH-Chol to form 7-keto-Chol. It also efficiently catalyzed the C-11 dehydrogenation of hydrocortisone and corticosterone (11β-hydroxysteroids). The purified enzyme was also reported to catalyze the C-3 dehydrogenation of Chol. The hamster 7α-OH-Chol dehydrogenase showed high sequence similarity to human 11β-hydroxysteroid dehydrogenase. Although low or absent in rat liver microsomes, immunochemical studies indicated the presence of 7α-OH-Chol dehydrogenase in human liver microsomes. This was the first description of an enzyme with 7α-hydroxysteroid dehydrogenase activity and provides an origin of 7-keto-Chol other than by autoxidation of Chol or peroxidative metabolism.

The formation of 7-oxygenated sterols from Chol has also been reported upon incubation of Chol and ethyl linoleate in the presence of horseradish peroxidase. Teng and Smith (1106) reported that the 7α- and 7β-hydroperoxides were the initial and chief products. Formation of 7-oxygenated sterols from Chol has also been reported upon incubation of Chol with soybean lipoxygenase in the presence of linoleic acid or ethyl linoleate (454, 594, 1106). Teng and Smith (1106) reported the formation of the 7α- and 7β-hydroperoxides (1:3 to 2:3 ratio of 7α- to 7β-products) of Chol upon incubation of [3H]Chol and ethyl linoleate with soybean lipoxygenase. The 7α- and 7β-hydroperoxides are thermolabile, and each undergoes thermal decomposition to 7-keto-Chol and to 7α- and 7β-OH-Chol (and to a number of other lesser products) (558). Johansson (454) reported the formation of 7α-OH-Chol, 7β-OH-Chol, and 7-keto-Chol (as judged by radio-TLC) after incubation of labeled Chol with soybean lipoxygenase and linoleic acid. The relative amounts of the three sterols formed were ~1.0:1.1:2.9, respectively. Lund et al. (594) reported the formation of 7α-OH-Chol, 7β-OH-Chol, and 7-keto-Chol upon incubation of Chol with lipoxygenase in the presence of linoleic acid. The 7α- and 7β-hydroxysterols were reported to be formed in approximately the same amounts. The 5α,6α- and 5β,6β-epoxides of Chol and 5α,6β-diOH-Chol were also formed under these conditions. Identification of products was based on behavior on HPLC and GC-MS studies (spectral data not presented). The formation of 7α-OH-Chol, 7β-OH-Chol, 7-keto-Chol, and the 5α,6α- and 5β,6β-epoxides of Chol has also been reported upon incubation of Chol with 13-hydroperoxyoctadeca-9,11-dienoic acid (594).

Chol 7α-hydroxylase (cyp7a) from different species has been purified (197, 449, 724), cloned (198, 210, 221, 449, 564, 718, 1131), and sequenced (198, 210, 221, 449, 564, 718, 719, 1131, 1178), and the cyp7a from rats and humans have been expressed in E. coli and purified (477, 565). A number of discrepancies in reported nucleotide sequences for the human cyp7a gene have been noted (1178). Several reviews of factors controlling cyp7a activity have been presented (93, 876, 1172). Chiang and Stroup (199) reported the identification of a putative bile acid-responsive element in the cyp7a gene promoter of rat liver. The cyp7a activity is localized primarily in the pericentral hepatocytes of rats (67, 1140, 1143). Enzyme activity, steady-state mRNA, and gene transcription for the 7α-hydroxylase were 7.9, 9.9, and 4.4 times higher in the pericentral hepatocytes than in perportal hepatocytes (1140). Very recently, Massimi et al. (622) reported that the localization of cyp7a mRNA in rat liver shows marked changes during development, with a homogeneous distribution at birth followed by changes to the adult pattern of pericentral localization at 28 days. A striking diurnal variation in the levels of enzyme activity, enzyme protein, and mRNA has been presented for the 7α-hydroxylase in liver of normal rats (462, 719) and for enzyme activity and mRNA in rabbit liver (462), and it has been suggested that pretranslational regulation is responsible for the circadian rhythm of the 7α-hydroxylase (719). The basal (10 AM) rat hepatic mRNA expression and gene transcription appears to be localized to only four or five hepatocytes of the liver cell plate located near the hepatic venules (rather than portal venules) (67). At the time of highest mRNA expression and gene transcription of the cyp7a gene (~10 PM), about one-half to two-thirds of the hepatocytes containing detectable mRNA for the 7α-hydroxylase were located near the hepatic venule. A diurnal variation of the levels of cyp7a (120) and of cyp7a mRNA in liver of cholestyramine-fed rats has also been presented (564). Starvation reduced the level of cyp7a activity of rat liver microsomes in male rats (719). Chol feeding and cholestyramine administration increased the levels of mRNA in liver for the 7α-hydroxylase (120, 449, 564, 702, 753, 874). Chol feeding (2% in diet) of rats for 14 days resulted in increases in microsomal cyp7a activity (+288%), mRNA levels in liver (+291%), and liver nuclear transcriptional activity (+220%) for the 7α-hydroxylase (449). Feeding of either cholic acid or chenodeoxycholic acid, at a level of 1% in diet, decreased the levels of hepatic mRNA for the 7α-hydroxylase. In contrast to the case of the cyp26 (11), cyp7a mRNA was detected only in liver (449) and was not detected in testes, adrenal, kidney, heart, lung, or brain. However, in another study (197), cyp7a activity was reported not only in liver but also in kidney, heart, and lung, albeit at lower levels than in liver.
Chol feeding results in increased levels of hepatic cyp7a activity in rats (82, 88 and references cited therein) and pigs (298). Björkhem et al. (88) reported that Chol feeding (2% in diet) increased cyp7a activity in livers of rats. At a lower level (1% in diet), Chol had no significant effect on enzyme activity. Interestingly, Chol feeding (2% in diet) increased the levels of cyp7a activity in liver in thoracic duct-cannulated rats. It was suggested that the Chol-induced increase in enzyme activity was due to factors "unrelated to the flux of Chol from the intestine to the liver." It was suggested that high levels of dietary Chol resulted in "increased binding of bile acids in the intestine and increased loss of bile acids in feces." The loss of bile acids was proposed to "lead to a reduced suppression of the cholesterol 7α-hydroxylase by the bile acids." Osada et al. (735) reported that dietary administration of Chol (0.5%) for 21 days to young or adult male rats was associated with a higher level of cyp7a activity in liver microsomes (relative to animals on a Chol-free diet).

It is important to note that Chol feeding does not always induce increases in the levels of cyp7a and/or cyp7a mRNA in liver (184, 512, 814, 873, 993, 1141). Administration of a high-Chol (1.7 mg/kcal), high-fat (40% of calories) diet to baboons had no effect (at weeks 3, 10, and 78) on the levels of cyp7a mRNA in liver (relative to values on a chow diet) (526). Furthermore, no differences were observed between high and low responders to dietary Chol (while on the chow diet or the high-fat, high-Chol diet) (993). Krause et al. (512) presented results indicating that NZW rabbits fed a chow diet supplemented with Chol (0.5%), peanut oil (3%), and coconut oil (3%) for 8 wk, sufficient to induce marked hypercholesterolemia, did not show an increase in hepatic cyp7a mRNA. In fact, an apparent decrease in the level of mRNA was observed that was of borderline statistical significance. Poorman et al. (814) reported that feeding Chol (0.25% in diet) had no effect in rabbits on the levels of hepatic cyp7a activity or on the levels of mRNA for this enzyme. Rudel et al. (873) reported substantially lower values of hepatic cyp7a activity and mRNA upon feeding of a high-Chol (0.8 mg Chol/kcal) diet relative to control animals receiving a monkey chow diet (low Chol, low fat). Xu et al. (1207) reported that feeding Chol, either 0.2% or 2% in a chow diet for 10 days, decreased the levels of hepatic microsomal cyp7a activity by 41 and −53%, respectively. Chol feeding (2%) lowered the levels of mRNA (−79%). In animals on a chow diet, the mean level of cyp7a activity and mRNA were lower in Watanabe heritable hyperlipidemic (WHHL) rabbits than in NZW rabbits (1207). Xu et al. (1208) reported that the levels of hepatic microsomal cyp7a activity decreased (−68%) in NZW rabbits upon Chol feeding (2% Chol in a chow diet) for 10 days. Total biliary drainage for 7 days resulted in decreases in cyp7a activity in both chow-fed control rabbits and the Chol-fed rabbits. The decreased levels of cyp7a activity in the Chol-fed rabbits were attributed to an increased bile acid pool resulting from an associated increase in hepatic cyp26 activity. Xu et al. (1209) recently reported decreased cyp7a activity in NZW and heterozygous WHHL rabbits after Chol feeding (3 g/day for 10 days).

Horton et al. (401) reported marked differences in the level of cyp7a activity in livers of rats and hamsters fed the same Chol-free diet. The levels in rats were ~16-fold higher than those in hamsters, a finding that was associated with a 20-fold higher synthesis of Chol in the rat, as measured by the incorporation of labeled hydrogen into digitonin precipitable sterols (DPS) in liver at 1 h after the intravenous injection of 3H-labeled water. Chol feeding resulted in a decrease in Chol synthesis in both species. However, the induction by Chol feeding of increased levels of cyp7a expression in the rat was not observed in the hamster.

Cheema et al. (184) presented important results that might explain at least a portion of the variable responses of cyp7a gene expression and enzyme activity after Chol feeding. Their studies, conducted in female mice (C57BL/6J), demonstrated that the responses to Chol administration (1% in diet) varied markedly in animals fed diets containing different fats [none or diets with a high level (20% by weight) of fats] containing either polyunsaturated fatty acids (safflower oil), monounsaturated fatty acids (olive oil), or saturated fatty acids (beef tallow). Mice fed Chol (1%) in a chow diet for 3 wk showed a very large increase in cyp7 mRNA in liver. However, administration of Chol (1%) in the three high-fat diets resulted in different responses. Animals on the diet high in fat containing polyunsaturated fatty acids showed significant increases in cyp7a mRNA and enzyme activity (relative to controls on the same diet). In contrast, animals on either the diet with fats high in monounsaturated fatty acids or saturated fatty acids showed significant decreases in cyp7a mRNA and enzyme activity relative to control animals on the same diets. Cheema et al. (184) noted that administration of the high-fat diets alone (without added Chol) resulted in increases in cyp7a mRNA and enzyme activity. Thus the results noted above reflect the findings that the addition of Chol to the various diets resulted in further increases in cyp7a mRNA and enzyme activity with the polyunsaturated fatty acid diet but resulted in a lowering of these values in the animals fed the monounsaturated or saturated fatty acid-rich diets.

Björkhem et al. (96) reported that liver microsomes from human subjects catalyzed the conversion of 26-OH-Chol to cholest-5-ene-3β,7α,26-triol. Evidence for the assignment of structure was obtained by TLC followed by GC-MS analysis of the TMS derivative. A partial MS was presented and compared with that of a synthetic sample. Whereas cholestyramine treatment increases the levels of cyp7a activity in liver microsomes, subjects treated with the resin did not appear to show an increased level of 7α-hydroxylase.
activity in liver microsomes for 26-OH-Chol. No conversion (limit of detection, 0.01 nmol/min⁻¹·mg protein⁻¹) of 26-OH-Chol to the Δ⁵,3β,7α,26-triol could be detected using human liver mitochondria. This finding differs from that made with pig liver mitochondria (35).

Axelson et al. (35) presented results indicating the first demonstration of the presence of 7α-hydroxylase activity for 26-oxygenated sterols in liver mitochondria. Mitochondria from pig liver catalyzed the formation of 7α-hydroxylated products from 26-OH-Chol and 3β-hydroxycholest-5-ene-26-oic acid. Products were characterized by GC-MS (TMS derivative of sterol and TMS derivative of the methyl ester of the acid). The combined results of this study indicated that the early reactions leading to the formation of bile acids can occur exclusively in mitochondria (i.e., not involving microsomal 7α-hydroxylase). Toll et al. (1118) reported that pig liver microsomes catalyze the 7α-hydroxylation not only of Chol but also of 26-OH-Chol, 3β-hydroxycholest-5-ene-26-oic acid, and 3β-hydroxycholest-5-ene acid. Two cytochrome P-450 species were isolated, one of which catalyzed the 7α-hydroxylation of 26-OH-Chol, 3β-hydroxycholest-5-ene-26-oic acid, and 3β-hydroxycholest-5-ene acid but not of Chol. The other cytochrome P-450 species catalyzed the 7α-hydroxylation of Chol and the other three substrates. The results were interpreted to “strongly indicate the presence of multiple microsomal sterol 7α-hydroxylases.” The same workers (976) also reported that human liver microsomes catalyzed the 7α-hydroxylation of 26-OH-Chol and 3β-hydroxycholest-5-ene-26-oic acid. However, only slight activity was observed when Chol was used as the substrate. A P-450 fraction prepared from the microsomes efficiently catalyzed the 7α-hydroxylation of 26-OH-Chol and 3β-hydroxycholest-5-ene-26-oic acid but had no detectable action on Chol. The 7α-hydroxylation activity in microsomes for 26-OH-Chol was much higher in humans and pigs than in rats. The mitochondrial fraction of human liver also catalyzed the 7α-hydroxylation of 3β-hydroxycholest-5-ene-26-oic acid. However, little or no 7α-hydroxylation of 26-OH-Chol was observed. The 7α-hydroxylation activity in mitochondria for 26-OH-Chol was much higher in pigs than in one human, rats, or rabbits. Human and pig mitochondria were also reported to catalyze the formation of 7β-hydroxy products that were proposed to originate from epimerization of the corresponding 7α-hydroxy compounds (through 7-keto intermediates). In a subsequent study, Toll et al. (1119) reported that liver microsomes from the rats, pigs, and humans catalyzed the 7α-hydroxylation of 25-OH-Chol. Whereas treatment of rats with cholestryamine caused a marked increase in cyp7a activity (+281%) in liver microsomes, no significant (P > 0.05) effect of cholestryamine administration on the 7α-hydroxylation of 25-OH-Chol was observed. Purification of Chol 7α-hydroxylation from pig liver showed two 7α-hydroxylase species, one for Chol and one for 25-OH-Chol (and 26-OH-Chol). The results indicated that at least 99% of the 7α-hydroxylase activity toward 25-OH-Chol of pig liver microsomes was separated from the species catalyzing the 7α-hydroxylation of Chol. Furthermore, transfection of the human liver cyp7a cDNA in COS cells showed significant cyp7a activity, whereas no significant 7α-hydroxylation of 25-OH-Chol was observed.

Martin et al. (619) reported the 7α-hydroxylation of 26-OH-Chol by microsomes of hamster liver, Hep G2 cells, and human liver. Little information was provided on the identification of the product. Under the specific assay conditions used in this study, the 7α-hydroxylase activity for 26-OH-Chol in hamster liver microsomes was very much higher than that of Chol 7α-hydroxylase, a finding also made with microsomes from human liver. In contrast, the 7α-hydroxylase activity for 26-OH-Chol in microsomes from Hep G2 cells was less than that for 7α-hydroxylation of Chol. Cholestryamine treatment of hamsters was reported to increase microsomal 7α-hydroxylase activity for 26-OH-Chol (+56%) and cyp7a activity (+75%). The results of several types of experiments suggested that the microsomal enzyme responsible for 7α-hydroxylation of 26-OH-Chol is different from cyp7a. Addition of cholesterol to hamster microsomes lowered cyp7a activity (~50%) but had little (~11%) effect on 7α-hydroxylation of 26-OH-Chol. In contrast, 26-hydroxycholesterol lowered 7α-hydroxylation of 26-OH-Chol (~68%) but had little or no (~20%; not significant) effect on cyp7a activity. Addition of a detergent, Emulgen 913, to hamster microsomes resulted in a differential lowering of activity; treatment with 0.2% detergent resulted in an ~70% lowering of 7α-hydroxylase activity for 26-OH-Chol but only a ~30% lowering of cyp7a activity. Martin et al. (619) noted that they could detect no cyp7a activity or 7α-hydroxylase activity for 26-OH-Chol in mitochondria preparations from hamster liver or Hep G2 cells. However, no data were presented on these points.

On the basis of studies of the metabolism of 26-OH-Chol and 7α-OH-Chol to bile acids in Hep G2 cells (444), it has been suggested that 7α-hydroxylation of 26-OH-Chol is not “well-expressed” in these cells. CHO-K1 cells have been reported neither to show cyp7a activity nor to contain detectable levels of mRNA for the 7α-hydroxylase (263). Østlund Farrants et al. (743) reported that the level of cyp7a activity in Hep G2 cells is ~17% that observed in rat hepatocytes and very much less than that of rat liver microsomes.

Whereas 7α-hydroxylation of Chol is commonly considered to occur exclusively in liver, the 7α-hydroxylation of 26-hydroxylated and 25-hydroxylated sterols in other cell types has been reported. Zhang et al. (1225) reported the formation of 7α-hydroxy derivatives upon incubation of 26-OH-Chol and 25-OH-Chol with human fibroblasts, indicating the potential extrahepatic formation of 7α-hydroxysterols.
Payne et al. (781) reported sterol 7α-hydroxylase activity for 25-OH-Chol (but not Chol or testosterone) in isolated granulosa cells from rat ovary and in whole ovarian preparations which appears to be distinct from Chol 7α-hydroxylase of liver. After incubation of 3H-labeled 25-OH-Chol with rat granulosa cells, two 7α-hydroxylated metabolites were isolated and identified as cholest-5-ene-3β,7α,25-trioli and 7α,25-dihydroxycholest-4-ene-3-one on the basis of MS and 1H-NMR data. The formation of these metabolites was markedly stimulated by interleukin-1β. Incubations of [3H]Chol or [3H]testosterone with rat ovarian dispersates (in the presence or absence of interleukin-1β) were unable to detect any mRNA transcripts for cyp7b. Portnow et al. (620) presented evidence indicating that cyp7b also catalyzes the 7α-hydroxylation of testosterone, androstenedione, and dihydrotestosterone. Martin et al. (861) investigated the presence of a 7α-hydroxylase of the 2,3-dihydroxycholest-4-ene-3-one on the basis of MS and 1H-NMR analyses of the sterols recovered from the incubation medium. Little or no 7α-hydroxylation was reported for the 2,3-dihydroxycholest-4-ene-3-one (at concentrations from 1.2 to 12 μM).

Schwarz et al. (943) reported transfection of human embryonic kidney 293 cells with an expression plasmid containing mouse cyp7b1 cDNA. Incubation of the transfected cells with [3H]25-OH-Chol led to the formation of labeled material with the TLC behavior of cholest-5-ene-3β,7α,25-trioli and 7α,25-dihydroxycholesterol-4-ene-3-one. In addition, the formation of a third metabolite was detected, for which the structure cholest-5-ene-2,3β,7α,25-tetrol was suggested on the basis of the results of GC-MS of its TMS ether derivative and the reported formation of an acetonide derivative (data not provided). The authors concluded that the cyp7b1 cDNA was both an oxysterol 7α-hydroxylase and a 2-hydroxylase. It is noteworthy that, in the same paper, studies of the metabolism of [3H]25-OH-Chol by liver microsomes (in the presence of NADPH) from a number of species (including human) appear to show little or no radioactive metabolite with the mobility of the 2,3β,7α,25-trioli. Chol feeding (2% in diet for 10 days) to male mice was reported to have no effect on the level of cyp7b mRNA in liver and little or no effect on cyp7b enzyme activity in liver microsomes (assayed by TLC with [3H]25-OH-Chol as substrate) or on hepatic oxysterol 7α-hydroxylase activity. Administration of colestipol (2% in diet) had little or no effect on hepatic cyp7b mRNA, enzyme activity, or enzyme protein. Feeding cholic acid (0.5% in diet) lowered cyp7b mRNA in liver (approximately −70%); however, this reduction was less than that observed for cyp7a mRNAs (i.e., almost complete absence). Cholic acid administration was associated with −40% lowering of cyp7b enzyme activity and enzyme protein. A limitation of these studies was the use of pooled samples of liver from individual mice (n = 4 or 5), thus precluding any statistical evaluation of the experimentation.

Norlin and Wikvall (717) recently reported that pig liver microsomes catalyzed the 7α-hydroxylation of 26-OH-Chol and 25-OH-Chol as well as dehydropiandrostosterone and pregnenolone. Chol and testosterone were also 7α-hydroxylated but to a very low extent. These authors reported on extensive efforts to determine if the cytochrome P-450 in the pig microsomes for 7α-hydroxylation of 26-OH-Chol was the same as that catalyzing the 7α-hydroxylation of dehydropiandrostosterone. An extensively purified enzyme catalyzed the 7α-hydroxylation of 26-OH-Chol, 25-OH-Chol, dehydropiandrostosterone, and pregnenolone. With the purified enzyme, no 7α-hydroxylation of Chol or testosterone was observed. No separation of activities for the 7α-hydroxylation of 26-OH-Chol and dehydropiandrostosterone was observed during the purification. However, notably large variations were observed in the ratio of 7α-hydroxylation of the two substrates in different experiments. Studies with five inhibitors indicated no selective effect on the 7α-hydroxyl-
ation of the two substrates. With the purified enzyme, addition of 26-OH-Chol had no inhibitory effect on the 7a-hydroxylation of dehydroepiandrosterone. However, dehydroepiandrosterone inhibited the 7a-hydroxylation of 26-OH-Chol. Interestingly, the inhibition was noncompetitive, suggesting, if one enzyme is involved, the possibility of the presence of more than one active site. In summarizing their studies the authors favored the idea that two enzymes are involved in the 7a-hydroxylation of the two substrates.

Post et al. (815) reported that cafestol, a diterpene responsible for the hypercholesterolemic effect of boiled coffee, caused a dose-dependent decrease in cyp7a activity, bile acid synthesis, and cyp26 activity in cultured rat hepatocytes. The decrease in 7a-hydroxylation activity was associated with decreases in its mRNA levels and in cyp7a gene transcriptional activity, which were observed at concentrations as low as 16 and 32 μM, respectively.

Stravitz et al. (965) reported that taurine conjugates of cholic acid and deoxycholic acid caused a concentration-dependent lowering of the levels of mRNA for cyp7a in primary cultures of rat hepatocytes. In contrast, the taurine conjugates of ursodeoxycholic acid, hyodeoxycholic acid, and ursolic acid had no effect under the same study conditions. Twisk et al. (1139) found that either deoxycholic acid or cholic acid (at 50 μM) caused a marked lowering of cyp7a activity, mRNA levels, and transcriptional activity in rat hepatocytes. Similar findings were made with cyp26. Little or no effects were observed with two hydrophilic bile acids, β-muricholic acid and ursolic acid. The results of studies of the effects of 27 different bile acids (at 50 μM) were presented. A moderate correlation between the hydrophobicity of the bile acids and cyp7a mRNA levels was observed. Pandak et al. (754) reported that, in Hep G2 cells, the hydrophobic bile salts, glycochenodeoxycholate and glycodeoxycholate, decreased bile acid synthesis and cyp7a mRNA levels in a time-dependent and concentration-dependent fashion. The hydrophilic bile salts, glycoursodeoxycholate and glycohyodeoxycholate, had no effect on cyp7a mRNA levels nor did glycocholate (intermediate hydrophobicity).

Whereas certain hydrophobic bile acids have striking effects in downregulating cyp7a in cultured hepatocytes, the regulation of cyp7a by bile acids in animals appears to be much more complicated. For example, Pandak et al. (752) observed that intraduodenal administration of taurocholate to bile fistula rats resulted in a clear decrease in hepatic cyp7a activity, mRNA levels, and transcriptional activity. However, intravenous administration of taurocholate had no effect on cyp7a. An intestinal factor, as yet unidentified, was proposed as a possible important regulatory species of cyp7a to account for this discrepancy.

Stravitz et al. (1049) reported findings indicating that protein kinase C is involved in the lowering of cyp7a gene transcription in primary cultures of rat hepatocytes caused by hydrophobic bile acids. Activation of protein kinase C with phorbol 12-myristate 13-acetate (0.1 μM) decreased mRNA for cyp7a (~71%) and transcriptional activity (~60%). Taurocholate (25 μM) decreased mRNA for the 7a-hydroxylase. However, this effect of taurocholate could be blocked by preincubation of the cells with inhibitors of protein kinase C. Further studies showed that hydrophobic bile acids caused an increase in the amount of protein kinase C that was associated with membranes. Stravitz et al. (1050) extended these studies. Nonselective activation of protein kinase C isoforms with phorbol 12-myristate 13-acetate resulted in a 75% decrease in cyp7a mRNA levels in rat hepatocytes. However, thymeleatoxin, a phorbol compound reported to selectively activate calcium-independent protein kinase C isoforms, had little effect on cyp7a mRNA levels in rat hepatocytes. These and other results led to the suggestion that calcium-independent protein kinase C isoforms are involved in the repression of cyp7a gene transcription by taurocholate.

Crestani et al. (222) reported that phorbol 12-myristate 13-acetate, an activator of protein kinase C, caused an inhibition of cyp7a promoter activity in Hep G2 cells transfected with rat cyp7a promoter/luciferase chimeric genes. The location of the negative phorbol ester response sequences were mapped. In contrast, the levels of 7-oxygenated sterols (7α-OH-Chol, 7β-OH-Chol, and 7-keto-Chol) as well as those for 5α,6α-epoxy-Chol, 5β,6β-epoxy-Chol, and 20α-OH-Chol in U 937 cells (a monocyte-like cell line) were reported to increase upon incubation with phorbol 12-myristate 13-acetate (100 ng/ml) for 60 min. The reported formation of the 20α-OH-Chol is noteworthy. However, it is important to note that characterization of the oxysterols was limited to chromatographic behavior on capillary GC of their TMS derivatives.

Taniguchi et al. (1094) studied the expression of the cyp7a gene in Hep G2 cells. Incubation of the cells in complete medium (containing 10% FCS) for 2, 4, 8, and 24 h was reported to show a gradual increase in the levels of mRNA for cyp7a with time with a maximum twofold increase at 24 h. However, considerable experimental variation was observed, and significant (P < 0.05) elevation of mRNA was observed only at 2 h. The claimed increase in mRNA levels under these conditions appeared to be blocked or suppressed by the addition of very-low-density lipoprotein (VLDL, 40 μg/ml) or 25-OH-Chol (12.4 μM). Similar incubations of the Hep G2 cells in serum-free medium was reported to result in substantial and significant (P < 0.05) increases in the levels of mRNA for cyp7a at all time points studied (i.e., 2, 4, 8, and 24 h) with a maximum increase (~5-fold) at 8 h. These substantial increases in mRNA levels in cells cultured in serum-free medium were blocked or suppressed by the addition of VLDL (40 μg/ml) or 25-OH-Chol (12.4 μM). The results of studies of the transcriptional activity for the cyp7a gene
with nuclei from Hep G2 cells (grown in serum-free or complete media for 8 h) indicated that the increased levels of mRNA appeared to be the result of transcriptional regulation.

Taniguchi et al. (1004) also reported that dexamethasone (1 μM) caused an increase in the levels of mRNA for cyp7a in Hep G2 cells grown in serum-free medium. Progesterone (32 μM) was reported to decrease the levels of mRNA for the 7α-hydroxylase. The same authors reported that chenodeoxycholic acid caused a dose-dependent decrease in the levels of cyp7a mRNA in Hep G2 cells grown in serum-free medium for 8 h. Chenodeoxycholate (100 μM) caused significant lowering as early as 2 h. Nuclei from cells incubated with this bile acid (100 μM) for 8 h were reported to show a lowering of cyp7a gene transcription. Ramirez et al. (835) also reported results indicating transcriptional control of cyp7a in a transformed hepatocyte cell line and in transgenic mice. Hylemon et al. (420) studied the hormonal control of the cyp7a mRNA levels and of transcriptional activity of the cyp7a gene in cultures of adult rat hepatocytes. The combination of dexamethasone (0.1 μM) and thyroxine (1.0 μM) resulted in a marked increase in the levels of cyp7a mRNA, whereas the individual hormones, at the same concentrations, had relatively little effect. The individual hormones increased the levels of transcriptional activity for cyp7a, and the combination of the two hormones resulted in a marked increase. Further hormonal control of the 7α-hydroxylase was indicated by the lowering of mRNA levels of either glucagon (0.2 μM) or dibutylryl cAMP (50 μM). Taurocholate lowered the levels of cyp7a mRNA in rat hepatocytes incubated in the presence of added thyroxine and dexamethasone, with an IC₅₀ of ~25 μM (420). Trawick et al. (1122) reported that dexamethasone (100 μM) increased cyp7a mRNA levels in rat hepatoma (L35) cells and that the increase caused by dexamethasone was augmented by the addition of dithiothreitol (DTT) or reduced glutathione. In the absence of the steroid, DTT or reduced glutathione had no effect on cyp7a mRNA levels. Dexamethasone plus DTT increased cyp7a transcriptional activity, and DTT reversed the repression of cyp7a transcription caused by insulin but not that caused by phorbol 12-myristate 13-acetate. Combined results were interpreted as indicating that the level of reduced glutathione has an important influence on the transcription and expression of the cyp7a gene.

Story et al. (1047) reported that administration of either β-thyroxine or L-thyroxine to rats elevated the levels of hepatic microsomal cyp7a activity, whereas propylthiouracil depressed the level of hepatic microsomal activity. However, when the results were expressed on the basis of enzyme activity per liver, no significant effects of the administration of β-thyroxine, L-thyroxine, or propylthiouracil were observed. Ness et al. (704) recently reported that intraperitoneal injection of L-triiodothyronine (100 μg/100 g body wt) to hypophysectomized male rats resulted in a very substantial increase (8-fold) in the levels of cyp7a mRNA at 6 h. Timed studies carried out after administration of L-triiodothyronine (100 μg/100 g body wt) indicated a maximal stimulation at 1 h. Hepatic microsomal cyp7a activity was also reported to increase and to reach a maximum at 90 min; however, no data were presented on this point. Increased levels of cyp7a mRNA in livers of thyroid hormone-deficient rats have been observed after administration of thyroid hormone (704, 705). The same authors (703) have reported that administration of triiodothyronine to thyroidectomized rats increases transcription of the cyp7a gene by liver nuclei. However, in humans with hypothyroidism, administration of thyroid hormone (either triiodothyronine or thyroxine) had no effect on the levels of 7α-hydroxycholesterol-4-en-3-one (an indirect measure of hepatic cyp7a activity) in serum despite the observation of significant decreases in LDL Chol and total Chol in serum (902). Medical treatment of hyperthyroidism resulted in significant increases in LDL Chol and total Chol in serum; however, no significant change in the levels of 7α-hydroxycholesterol-4-en-3-one was observed. Thus changes in thyroid hormone status, sufficient to modify Chol levels in serum, had no effect on hepatic cyp7a activity (at least as studied by effects on the levels of the 7α-hydroxy-3-ketosterol in serum).

Daily treatment of rats for 4 days with dexamethasone (100 mg/kg) or pregnenolone-16α-carbonitrile (50 mg/kg) by intraperitoneal injection in corn oil resulted in a very marked lowering of the level of cyp7a activity in liver microsomes (197). No effect of phenobarbital (100 mg/kg) administration was observed. Glucocorticoids (dexamethasone, cortisol, and corticosterone), but not other steroid hormones (17α-ethinylestradiol, progesterone, testosterone, androsterone, pregnenolone, and aldosterone), have been reported to increase the conversion of [14C]Chol to bile acids in cultured rat hepatocytes (822). Dexamethasone (1 μM) had no effect on cyp7a activity at 24 h; however, very large increases were observed at 48 and 72 h. In contrast, treatment of rats with dexamethasone (100 mg/kg) or with pregnenolone-16α-carbonitrile was reported to markedly reduce the levels of cyp7a mRNA in liver (564) and to lower the levels of enzyme activity and protein. Data on the latter two points were not presented. However, it should be noted that earlier studies reported a doubling of cyp7a activity in rat liver at 3 h after the intraperitoneal administration of synthetic or natural corticosteroids (1151). The diurnal rhythm for the 7α-hydroxylase in rats was also obliterated by hypophysectomy or adrenalectomy (335). Administration of either conjugated equine estrogen or medroxyprogesterone acetate has been reported to increase the levels of hepatic cyp7a mRNA in ovariectomized adult female cynomolgus monkeys (212).

In studies of the hormonal regulation of cyp7a, Cres-
tani et al. (222) reported that insulin inhibited cyp7a promoter activity in Hep G2 cells transfected with rat cyp7a promoter/luciferase chimeric genes. In the same study, a glucocorticoid (dexamethasone) and all *trans*-retinoic acid activated cyp7a promoter activity. In the same system, thyroxine had no effect on cyp7a promoter activity. Crestani et al. (223) reported the identification of a hormone response unit for cyp7a promoter activity that mediates the opposing effects of retinoic acid and phorbol 12-myristate 13-acetate. The activation of promoter activity was observed with both all *trans*-retinoic acid and 9-*cis*-retinoic acid. Sadeghpour et al. (880) reported identification of the portion of the sequence responsible for the effects of retinoic acid and phorbol 12-myristate 13-acetate (as well as insulin) and that mutations in this region resulted in the elimination of the effects of retinoic acid, phorbol 12-myristate 13-acetate, and insulin and a decrease in the effect of dexamethasone. Two bile acid response elements in the promoter region of the rat cyp7a gene have recently been identified (199, 1056). Foti and Chiang (311) reported findings indicating that a transcriptional factor [basic transcription element-binding protein (BTEB)] binds to a region corresponding to a bile acid response element of the rat cyp7a promoter and that overexpression of BTEB resulted in a repression of transcriptional activity of cyp7a.

Twisk et al. (1138) reported that insulin, at physiological concentrations, inhibited the formation of bile acids and lowered cyp7a activity. The decrease in enzyme activity caused by insulin was associated with a decrease in the level of mRNA for the enzyme and decreased transcriptional activity. As noted previously, very similar results were observed with regard to the effects of insulin on cyp26.

Rudling et al. (875) reported findings indicating the importance of growth hormone on hepatic cyp7a. Hypophysectomized rats showed lowered levels of enzyme activity (~64%). Interestingly, the levels of hepatic cyp7a mRNA were increased (+81%). It was proposed that a deficiency of growth hormone as a consequence of hypophysectomy results in decreased hepatic cyp7a activity, which in turn leads to a decreased synthesis and fecal excretion of bile acids and that the resulting decreased levels of bile acids in liver caused a derepression of the transcription of the cyp7a gene with resulting increased levels of cyp7a mRNA. Rudling et al. (875) also reported that infusion of growth hormone to hypophysectomized rats resulted in restoration of the levels of hepatic cyp7a activity to normal levels. Administration of growth hormone to normal rats fed a diet enriched in Chol (2%) and sodium cholate (0.5%) was associated with a marked increase in the levels of hepatic cyp7a activity.

Administration of Chol or of cholestyramine increases the levels of cyp7a activity, its mRNA, and transcriptional activity in rat liver, whereas feeding of bile acids, in general, reduces these parameters (449, 564, 753, 755, 756). However, feeding of deoxycholic acid at a high level (1% in diet for 10 days) not only did not result in a decrease in cyp7a mRNA levels but caused a very substantial increase in the levels of mRNA for this protein (513). A large fraction of this increase appeared to be related to decreased food consumption (associated with decreased body weight). The increase in mRNA levels by deoxycholic acid feeding appeared to be largely due to posttranscriptional processing.

Several studies (753, 755, 965, 1137) have indicated repression of cyp7a by taurocholate at the level of gene transcription. Pandak et al. (756) reported that feeding of cholic acid (1% in diet), chenodeoxycholic acid (1% in diet), or deoxycholic acid (0.25% in diet) to male rats resulted in marked reduction of the levels of enzyme activity, mRNA, and transcriptional activity for cyp7a in liver. The levels of specific activity of the 7α-hydroxylase were suppressed more than the levels of transcriptional activity, leading to the suggestion of additional posttranscriptional regulation. Shefer et al. (966) reported that feeding deoxycholic acid (0.4 or 1.0% in diet) to male rats resulted in a marked lowering of cyp7a activity in liver, which was associated with a very marked increase in the level of mRNA for the enzyme. In contrast, deoxycholate was reported to have no effect on the levels of hepatic 27-hydroxylase activity or on the levels of mRNA for this enzyme. Administration of cholestyramine (5% in diet) to male rats resulted in a marked elevation of the levels of activity, mRNA, and transcriptional activity for cyp7a in liver as well as an elevation of the levels of HMG-CoA reductase activity. In another study (318), cholestyramine administration (2% in diet for 2 wk) to male rats was associated with a substantial increase in the levels of hepatic microsomal cyp7a activity and 7α-OH-Chol in serum. Horton et al. (400) reported that dietary administration of psyllium (7.5% in diet) or cholestyramine (1% in diet) to hamsters resulted in increases in the levels of hepatic cyp7a activity and mRNA. Hayashi et al. (377) reported that administration of cholestyramine (2% in diet for 14 days) to Syrian golden hamsters resulted in 600% increase in the levels of cyp7a activity in liver.

Maeda et al. (601) observed that the levels of cyp7a activity and protein (determined by immunochromical assay) were higher in liver microsomes from patients (n = 3) treated with cholestyramine than in those from untreated patients (n = 6). However, increases in enzyme activity and enzyme content were not proportional. In the treated patients, the levels of enzyme protein in liver microsomes were reported to be “approximately twofold higher than those of liver microsomes from untreated patients” (P < 0.02). However, the level of enzyme activity was “approximately sixfold higher” in liver microsomes from patients treated with the resin than the activity in microsomes from untreated patients. These findings were
interpreted as suggesting, in addition to transcriptional control, “a posttranslational mechanism.”

Sudjana-Sugiaman et al. (1062) introduced the cDNA for human cyp7a into COS cells and observed the production of 7α-hydroxylase protein and the accumulation within the cells of 7α-OH-Chol. The mean level of cyp7a activity in the transfected cells was 0.26 ± 0.05 pmol/min·mg protein⁻¹ (cf. 0.03 ± 0.02 in control cells). The transfected cells showed low but significant levels of 7α-OH-Chol (from 11 to 67 ng/mg cell protein). A high correlation between the level of cyp7a activity in the transfected cells and the basal level of 7α-OH-Chol in the cells was reported. The levels of 7α-OH-Chol were based on GC-MS methodology (although no characterization data were presented). It was stated that no 7β-OH-Chol was detected. The level of HMG-CoA reductase activity was reported to be higher (+58%) than that in control cells. The free Chol levels in the transfected COS cells were not different from control values.

A number of investigations have indicated that cyp7a activity can be modified in vitro by phosphorylation-dephosphorylation processes (209, 214, 256, 347, 397, 530, 709, 893, 903, 1093). Other investigators (60, 274, 578) have not obtained results consistent with this potential in vitro modulation of cyp7a activity, perhaps due to differences in experimental conditions (709). Whereas phosphorylation-dephosphorylation changes of the enzyme may modulate cyp7a in vitro, this author is unaware of any studies demonstrating the importance of such mechanisms in the control of cyp7a activity in intact cells or in vivo in animals. Cytosolic proteins have been reported to affect cyp7a activity in vitro (237, 238, 529 and references cited therein). Danielsson et al. (238) purified a protein (Mr 25,000) from rat liver cytosol that stimulated cyp7a activity. Stimulation was observed only in the presence of reduced glutathione or reduced thioredoxin. The activation of cyp7a activity by the cytosolic protein did not appear to be affected by ATP, MgCl₂, or sodium fluoride.

Bensch et al. (59) reported that acute (3 or 24 h) oral administration of ML-236B (compactin; 20 mg/kg) to male Sprague-Dawley rats had no significant effect on the levels of cyp7a activity in liver. Endo et al. (281) found that oral administration of ML-236B twice a day at 250 mg/kg for 11 days to male Wistar-Imamichi rats caused a modest (−31%) reduction in the levels of cyp7a activity in liver and a decrease (−53%) in fecal bile acids. Björkhem (82) reported that dietary administration of mevinolin (0.1% in a “commercial fat-free diet”) for 3 days to male Sprague-Dawley rats caused a decrease (−36%) in the levels of cyp7a activity when assayed for the 7α-hydroxylation of exogenous labeled Chol. However, no significant change in cyp7a activity was observed when the enzymatic activity was assayed by determination of the 7α-hydroxylation of endogenous Chol in liver microsomes. Mevinolin administration had no effect on the levels of free Chol in liver microsomes. Björkhem (86) reported that dietary administration of mevinolin (0.1% in diet) to male Syrian golden hamsters for 6 days had no significant effect on hepatic cyp7a activity. They also reported that dietary administration of mevinolin (0.1%) to male Sprague-Dawley rats decreased the rise in hepatic cyp7a activity induced by the administration of cholestyramine (5% in diet) or Chol (2% in diet). More recently, Jones et al. (458) reported that intravenous administration of mevinolin to rats with chronic biliary cannulation resulted in lowering of liver microsomal cyp7a specific activity and a lowering of enzyme mass, mRNA, and transcriptional activity for the 7α-hydroxylase. In contrast, intravenous administration of mevalonate resulted in increases in cyp7a specific activity, mRNA levels, and transcriptional activity. The same laboratory (1173) also reported that short-term biliary diversion in rats resulted in a substantial increase in cyp7a (+259% at 48 h and +827% at 96 h). Treatment with mevinolin prevented the increase in 7α-hydroxylase activity induced by the biliary diversion. In contrast, administration of mevalonic acid caused a marked increase in cyp7a activity over that induced by biliary diversion alone. Under the conditions of this study, i.e., short-term biliary diversion, the results suggested that regulation of the 7α-hydroxylase was under the control of both “bile acids and newly synthesized Chol or its metabolites” (which were suggested to be oxysterols). Atorvastatin, an inhibitor of HMG-CoA reductase, had no significant effect on hepatic microsomal cyp7a activity after dietary administration of the drug in a high-fat diet to guinea pigs for 3 wk (213). Pravastatin, another competitive inhibitor of HMG-CoA reductase, had no effect on the levels of cyp7a activity in livers of Syrian golden hamsters under the conditions studied (377). Pravastatin administration to normal male subjects had little or no acute effect on bile acid synthesis, at least as indicated by its effects on the levels of 7α-hydroxycholest-4-en-3-one in plasma (1217).

Zaragozic acid A (squalenstatin 1), a squalene synthetase inhibitor, at a concentration of 1 μM, caused a very marked lowering (−98%) of cyp7a activity and a marked lowering of steady-state mRNA levels for the 7α-hydroxylase in primary cultures of rat liver cells (258). Direct addition of squalenstatin 1 (150 μM) to liver microsomes (from male rats fed a diet containing cholestyramine) had no effect on cyp7a activity (258). The marked lowering of cyp7a activity and mRNA levels by squalenstatin 1 in rat hepatocytes was reversed by the addition of Chol (200 μM in β-cyclodextrin). The effect of administration of zaragozic acid A on the levels of liver mRNA for cyp7a has been studied in rats (706). Subcutaneous administration of zaragozic acid A (1, 2, or 5 mg/kg body wt 9 h before death) was reported to result in decreases in the levels of cyp7a mRNA. In the same experiment, substantial increases in mRNA for the LDL receptor were observed. The levels of mRNA in liver for HMG-CoA reduc-
tase, squalene synthetase, and HMG-CoA synthase increased (with maximum values observed at ~6 h) after a single subcutaneous injection of zaragotic acid A.

Bezafibrate administration (200 mg 3 times/day for 4 wk) to human subjects caused a lowering (~60%) of the levels of cyp7a activity in liver (1033).

Administration of phenobarbital to different strains of rats has been reported to give variable acute effects on the levels of cyp7a, with increases in some strains and decreases or no changes in others. Sudjana-Sugiaman et al. (1061) reported that three of nine rat strains (Wistar F, Lewis, and Brown Norwegian) showed increases of cyp7a activity in liver microsomes after daily intraperitoneal administration of phenobarbital (100 mg/kg body wt). The increases in 7α-hydroxylase activity were maximal at 48 h and decreased to control levels at 96 h. In one typical set of experiments, the specific activity of cyp7a was 62 ± 13 pmol·min⁻¹·mg protein⁻¹ in Wistar F rats treated with phenobarbital for 48 h and 29 ± 5 pmol·min⁻¹·mg protein⁻¹ in the corresponding control rats. The levels of mRNA for cyp7a also showed increases that appeared to be maximal at 48 h. It is interesting to note that whereas the Wistar F, Lewis, and Brown Norwegian rats showed increases in cyp7a activity after phenobarbital administration, three other rat strains showed decreases in 7α-hydroxylase activity after phenobarbital administration for 48 h. Gerbils and three other rat strains showed no significant changes in cyp7a activity under the same conditions. Strain differences in hepatic cyp7a activity have also been reported in mice. C57BL/6 mice (a strain reported to have increased susceptibility to the development of atherosclerosis on a high-Chol diet) were reported to show higher levels of hepatic cyp7a activity and mRNA than control rats when the animals were on a chow diet (264). Administration of a high-Chol diet increased cyp7a activity in both strains. Addition of taurocholate to the high-Chol diet markedly lowered cyp7a activity, significantly more so in the C57BL/6 mice. Kirk et al. (494) reported a high degree of variation in the levels of hepatic cyp7a mRNA in different strains of mice. Moreover, different strains of mice showed different effects of feeding safflower oil or safflower oil plus Chol on the levels of cyp7a mRNA in liver. Three of nine strains showed increased levels upon safflower oil administration in a rodent chow diet. One of the nine strains showed a significant decrease. Feeding of Chol plus safflower oil in the rodent chow diet showed a further increase (relative to safflower oil diet alone) in one strain. One strain of mice showed no change in cyp7a mRNA on either the safflower oil diet or the safflower oil plus Chol diet. Poorman et al. (814) reported increased levels of cyp7a activity in livers of an inbred strain of NZW rabbits in which there was little or no elevation of serum Chol levels upon Chol feeding. The Chol-resistant rabbits also showed increased levels of cyp7a mRNA in liver (relative to that observed in normal rabbits). The increased level of the 7α-hydroxylase activity (and of mRNA) correlated with the increase in excretion of bile acids observed in the Chol-resistant rabbits upon Chol feeding (relative to that observed in normal rabbits).

Dietary administration of 7-keto-Chol (0.1% in chow diet) to rats for 6 days resulted in an increase (+170%) in the levels of liver microsomal cyp7a activity (1091). Administration of the 7-ketosterol was associated with significant decreases in the levels of free Chol (~30%), Chol esters (~64%), and total Chol (~34%) in liver microsomes and marked increases in the levels of 7-keto-Chol (+771%) and 7β-OH-Chol (+2,922%) in liver microsomes. No significant effect on the level of 7α-OH-Chol in microsomes was observed. The increased hepatic microsomal cyp7a activity after dietary administration of 7-keto-Chol as reported by Tamasawa et al. (1091) is in contrast to reported in vitro inhibitory effects of 7-keto-Chol on cyp7a activity of liver microsomes (965, 1152). Doerner et al. (258) studied the effects of the addition of several oxysterols on the levels of cyp7a mRNA in primary rat hepatocytes. 5α-Cholestane-3β,6α-diol, but not 7α-OH-Chol, 5α-cholestane-3β,5α,6β-triol, or (25R)-26-OH-Chol, had a significant effect (an increase) on the levels of cyp7a activity. The oxysterols were added in 2.5% β-cyclohextrin at a single concentration (not specified). None of the above oxysterols (at 50 μM; added in β-cyclohextrin) reversed the effect of squalestatin 1 (a marked lowering of cyp7a activity). Feeding an oxysterol mixture (0.5% in a Chol-free diet) resulted in lower levels of cyp7a activity in livers of male rats (735). A limitation of this study was the lack of a pair-fed control group to deal with the decreased food consumption caused by the administration of the oxysterol mixture. Feeding the same mixture of oxygenated derivatives of Chol to rats at a level of 0.2% in a basal Chol-free diet for 20 days lowered the level of cyp7a activity in liver (739). Osada et al. (740) reported on the levels of cyp7a activity in livers from 4-wk-old rats fed one of three diets: a basal Chol-free diet, the basal diet containing added Chol (0.5%), or the basal diet supplemented with Chol (0.5%) and a mixture of oxidized Chol species (0.5%). The major component in the oxysterol mixture was 7-keto-Chol (22%), which was accompanied by a number of other Chol oxidation products and unidentified material. Because administration of the diet supplemented with Chol plus the oxidized Chol mixture suppressed food consumption (data not shown), the animals receiving the basal diet or that supplemented with Chol were given food only in the amount consumed by the animals receiving the diet supplemented with both Chol and the oxidized Chol mixture. The levels of cyp7a activity in liver were elevated in the Chol-fed animals. This elevation was partially blocked by the inclusion of the oxidized Chol mixture in the Chol-supplemented diet.

Bile duct ligation has been reported to elevate cyp7a
activity in rat liver (236, 364). Selective portal vein ligation did not affect the levels of 7α-hydroxylase activity in liver of male Wistar rats (608). In these studies, the branch of the portal vein supplying the left lateral and median lobes of the liver was ligated, and liver samples from the left lateral and right lobes were obtained for analysis of cyp7a activity. On the basis of these and other findings, the authors concluded that the intrahepatic concentrations of Chol and bile acids had no regulatory effect on cyp7 under the conditions studied.

Shefer et al. (967) reported decreased levels (~39% lower than those of control subjects) of cyp7a activity in liver microsomes of human subjects with sitosterolemia. Assay of cyp7a activity using acetone-treated microsomes (to remove endogenous sterols) showed no difference in cyp7a activity between the sitosterolic and control subjects. The levels of hepatic HMG-CoA reductase activity were markedly lowered in the human subjects with sitosterolemia (relative to controls).

The levels of cyp7a undergo dramatic developmental changes in the rat (999). Very low levels (“near the limits of detectability”) of cyp7a activity were observed in fetal liver (1 or 4 days before birth). By 18 h after birth, the level of 7α-hydroxylase activity “increased to about 40% of the adult level.” The levels of enzyme activity then fell so that on days 3 and 6 the activity was almost undetectable. The values then increased [days 12, 17, 18, 19, 21 (weaning), and 28]. The highest value was on day 21. In general, the changes in the levels of cyp7a protein followed that of activity except for the day −1, day +1 (18 h) values when the protein values were very much higher than the values for enzyme activity. Day 1 (18 h) to day 19 corresponded to suckling, and day 21 corresponded to weaning. Oren et al. (733) reported that hepatic microsomal cyp7a activity in piglets was not detectable (~1.0 pmol·min⁻¹·mg protein⁻¹) during gestation or at birth. 7α-Hydroxylase activity increased after birth to 6.8 ± 2.6 pmol·min⁻¹·mg protein⁻¹ at 3 wk and 18.2 ± 2.5 pmol·min⁻¹·mg protein⁻¹ at 7 wk. Fasting (12 h) decreased cyp7a activity. Bertolotti et al. (71) found a lower level of 7α-hydroxylation in older human subjects (above 62 yr). The total study included 18 males and 10 females in which 7α-hydroxylation was assayed by a method involving a tritium-release assay after intravenous injection of [7α-3H]Chol. Spady et al. (1022) reported that, in short-term experiments (2 days), infection of hamsters with a recombinant adenovirus encoding rat cyp7a increased microsomal cyp7a activity in liver and a lowering of plasma total Chol and LDL Chol. The magnitudes of these changes were related to the amounts of the administered recombinant adenovirus. The reduction in LDL Chol levels could also be affected, in short-term experiments, by overexpression of cyp7a in LDL receptor −/− mice (1024). Moore et al. (670) reported the expression of human cyp7a in atherosclerossis-susceptible mice using an adenovirus vector. At 2 wk after infection, mice receiving the adenovirus vector containing the human cyp7a cDNA showed total plasma Chol and LDL-VLDL Chol levels that did not appear to differ from those of mice infected with the adenovirus vector alone. Partial transfection of liver with a synthetic cyp7a gene using a nonviral delivery vector has been reported to affect plasma Chol levels in mice in short-term experiments (5).

Ishibashi et al. (426) reported the production of a mutant strain of mice affecting cyp7. Newborn homozygous (Cyp7α−/−) mice appeared normal at birth but showed high mortality (~85%) in the first 18 days of life. The mortality appeared to occur in two phases, one in the period from days 1 to 4 that could be suppressed by the addition of a vitamin mixture to the drinking water of nursing mothers and another in the period from days 11 to 18 that could be suppressed by the addition of cholic acid to the chow consumed by nursing mothers. Liver microsomal preparations from the Cyp7α−/− mice showed no cyp7a activity. The mutant mice born to Cyp7−/− mothers fed normal chow showed severely depressed gain in body weight, indications of lowered liver cytochrome P-450 content, elevated levels of conjugated bilirubin in liver, lowered motor activity, and changes in skin (dry scaly appearance accompanied by thickening of stratum corneum and granular layers) and in eye (delayed opening). Schwarz et al. (942) obtained additional results on this important mutant mouse. Stool fat levels of Cyp7α+/+ mice were clearly elevated during the first 3 wk of life but by 4 wk returned to the level of wild-type mice. The mutant mice showed suggestions of lowered levels of vitamin D in serum on days 6, 16, and 23 (based on analyses of pooled serum samples from mutant and wild-type groups). Little or no differences between mutant and wild-type mice were observed on days 34–38 and 120–180. In contrast to comments by the authors, provision of a vitamin mixture appeared to have no consistent effects on the levels of vitamin D in serum. The levels of vitamin E in samples of fat (epididymal or ovarian fat pads) appeared to be clearly lowered in mutant mice (relative to wild-type animals) on days 16, 23, 34–38, and 120–180. Supplementation with a vitamin mixture appeared to have no consistent effects on the levels of vitamin E. Supplementation with vitamins and cholic acid increased the levels of vitamin E in the fat pads of the mutant mice. It is important to note that the levels of vitamin E in fat samples were expressed relative to triglyceride and that information of the effects of the mutation on epididymal or ovarian triglyceride levels in the ovarian and epididymal fat pads was not presented. The mutant mice showed little or no differences from wild-type animals in the levels of Chol and triglycerides in serum at various points of development (6, 15, 23, 60–90, and 150–180 days). Analyses of bile acid composition and levels in bile and feces from adult Cyp7α−/− mice and wild-type mice were also
reported. The concentration of total bile acids in bile did not appear to differ in the mutant and wild-type mice (although the analyses were limited to two each of the mutant and wild-type mice). However, lower levels of total bile acids in feces from the mutant mice were reported. Cholic acid, the major bile acid in bile of wild-type mice, appeared to be reduced in the mutant. In contrast, chenodeoxycholic acid, \( \alpha \)-muricholic acid, and hyodeoxycholic acid were not detected in bile from wild-type animals but were present at significant levels in bile from the Cyp7\( \alpha \) \(-/-\) mice. As noted, the mean level of total bile acids in the mutant mice was lower than that of wild-type mice. \( \alpha \)-Muricholic acid (\( \beta \alpha \beta \alpha ,7\alpha \)-trihydroxy-5\( \beta \)-cholanic acid), which was not detected in feces from any of the mutant mice, was present at significant levels in feces from wild-type animals. This is in notable contrast to the case of bile, in which this acid was not detected in wild-type mice but was present at substantial levels in the bile from mutant animals. An important finding was the observation of substantial levels of 7\( \alpha \)-hydroxylated bile acids in the Cyp7\( \alpha \) \(-/-\) mice. A possible explanation for the occurrence of the 7\( \alpha \)-hydroxy bile acids in the mutant mice was provided by the demonstration of the presence of 7\( \alpha \)-hydroxylase activity for 25-OH-Chol in liver microsomes of the mutant mice. The product was characterized by its TLC and by MS (data not shown). Very low levels of the enzyme activity were present through the first 3 wk of life in both mutant and wild-type animals. Thereafter, the levels of activity increased substantially in both mutant and wild-type mice. The induction of this activity corresponded to the timing of the decrease of elevated levels of fecal fat in the mutant mice. The authors considered that the occurrence of substantial levels of the 7\( \alpha \)-hydroxy bile acids in the adult mutant mice arose from an alternative mode of bile acid formation not involving initial 7\( \alpha \)-hydroxylation of Chol but involving initial mitochondrial hydroxylation to give 26-OH-Chol followed by its \( \alpha \)-hydroxylation via the induced (at \( \approx \)4 wk) microsomal \( \alpha \)-hydroxylase and then subsequent metabolism of the 3\( \beta \),7\( \alpha \),26-trihydroxysterol to bile acids. Schwarz et al. (943) subsequently reported on the developmental course of the levels of cyp7b mRNA, protein, and enzyme activity (assayed by TLC with \[^{3}H\]25-OH-Chol as substrate) in wild-type mice. After appearance at about day 18, the levels of each of the above increased thereafter. Assays were made on pooled samples obtained from livers of mice (3–10/time point). The combined results with the mutant Cyp7\( \alpha \) \(-/-\) mice demonstrate the critical importance of cyp7a in the newborn state and also indicate the importance of \( \alpha \)-oxysterol 7\( \alpha \)-hydroxylase (cyp7b) activity in bile acid formation (and relief of consequences of cyp7a absence) after its induction in ~1-mo-old mice. Further studies in 3-mo-old male cyp7a \(-/-\) mice (944) showed a reduction in bile acid synthesis, a decrease in the intestinal bile acid pool, a very marked decrease in Chol absorption (which was reversed by feeding of cholic acid but not by administration of hyodeoxycholic acid), and increased sterol synthesis in liver and small intestine (as measured by incorporation of \(^{3}H\) into DPS 1 h after intraperitoneal injection of \(^{3}H_{2}O\)).

Gene expression for cyp7a has been reported to be related to changes in plasma lipoprotein Chol levels. However, the associations appear to vary in different animals and their derived genetic variants. The selectively bred hypercholesterolemic RICO rat, with elevated levels of HDL Chol in serum, showed reduced levels of hepatic microsomal cyp7a activity (684). However, reductions in the fecal excretion of bile acids or Chol were not observed. An elevated level of cyp7a expression (814) and increased fecal bile acid excretion (744) has been reported for a genetic variant of a NZW rabbit that is resistant to the effect of dietary Chol on the level of blood Chol. In short-term (3 days) experiments, hamsters infected with a cyp7a adenoviral construct showed 10- to 15-fold higher levels of hepatic cyp7a mRNA than hamsters infected with a control virus (1022). Plasma levels of LDL Chol decreased 60%. HDL Chol levels also decreased but to a lesser extent, and VLDL Chol levels were unchanged. Very recent studies in inbred strains of female mice have indicated a coordinate regulation of cyp7a gene expression in liver and plasma between the loci for HDL Chol in plasma (599). Three of the five loci for cyp7a mRNA levels on chromosomes 3, 5, and 11 were reported to be coincident with the loci for HDL Chol levels on an atherogenic diet containing Chol (1.25%) and cholic acid (0.5%). This coincidence was observed only when the animals were on the atherogenic diet. That the observed coincidence on the atherogenic diet was due to chance was considered to be remote, since the loci for the cyp7 and the loci for HDL Chol “covered only a few percent or less of the mouse genome.” In another recent study, a correlation was observed between the levels, in liver, of cyp7a mRNA and apoA-I mRNA in female mice of two strains (265). In C57BL/6 mice, a direct relationship was observed between the levels of cyp7a mRNA in liver and the levels of HDL Chol in serum. This relationship was not observed in BALB/c mice, an atherosclerosis-resistant mouse strain. Other recent studies indicated genetic linkage between cyp7a and high plasma LDL Chol levels in humans (1179) and genetic relationships between alleles for cyp7a and plasma total Chol levels in the pig (817).

C. Formation of 5,6-Epoxides of Cholesterol and Cholestane-3\( \beta \),5\( \alpha \),6\( \beta \)-triol

Aringer and Eneroth (20) reported important early studies of the conversion of labeled Chol and \( \beta \)-sitosterol to the corresponding 5,6-epoxides, the corresponding 3\( \beta \),5\( \alpha \),6\( \beta \)-triols, and to material with the chromatographic

Downloaded from http://physrev.physiology.org/ by 10.220.33.3 on August 12, 2017
behavior of the corresponding 7α-hydroxysterols and 7β-hydroxy plus 7-ketosterols upon incubation with subcellular fractions of rat liver. Their study, as well as a prior study by Danielsson (233), indicated the complexities involved in these matters and the existence of significant nonenzymatic formation of the 5,6-epoxides and the 7-oxygenated sterols in such investigations. Aringer and Eneroth (20) also reported important early studies of the enzymatic conversion of the 5α,6α- and 5β,6β-epoxides of Chol to 5α,6β-dioH-Chol by subcellular fractions of rat liver. Essentially no activity was observed with the 100,000-g supernatant fraction of liver. The 7-oxygenated sterols, 7β-OH-Chol and 7-keto-Chol, were reported to inhibit the 5,6-epoxysterol hydrolase activity in the 18,000-g supernatant fraction of rat liver using a mixture of the 5α,6α- plus 5β,6β-epoxides of Chol (or of sitosterol). They also reported that the 5α,6α- and 5β,6β-epoxides of Chol, but not 5α,6β-dioH-Chol, inhibited the conversion of labeled Chol to 7α-OH-Chol by the 18,000-g supernatant fraction of liver homogenates.

Watabe et al. (1186) reported the conversion of both the 5α,6α-epoxide and 5β,6β-epoxide of Chol, pregnenolone, 3β-hydroxy pregn-5-ene, and 3-deoxChol to the corresponding 5α,6α-glycols upon incubation of the epoxides with washed bovine liver microsomes. The 5α,6β-glycols were characterized by GC-MS. The conversions of the 5α,6α- and 5β,6β-epoxides of Chol to the 5α,6β-glycol were inhibited by the aziridines 5α,6α-imino-5α-cholestan-3β-ol (1 mM) and 5β,6β-imino-5β-cholestan-3β-ol (1 mM) but they were stimulated by 3,3,3-trichloro-1-propane oxide (1 mM), commonly used as an inhibitor of the enzymatic hydrolysis of epoxides of xenobiotics. The imino derivatives of Chol (1 mM) had no effect on the hydrolysis of styrene oxide and safrole oxide, whereas the trichloro-propene oxide resulted in a complete inhibition of the hydrolysis of styrene oxide and safrole oxide. Nashed et al. (696) showed the presence of Chol 5,6-epoxide hydrolase activity in microsomes obtained from livers of rats, hamsters, mice, rabbits, and humans. The 5α,6α-epoxide was a better substrate than the 5β,6β-epoxide. With the rat liver microsomes, 5α,6β-dioH-Chol was reported to be “virtually the exclusive product.” The 5α,6α-iminocholestan-5-one has been reported to be a potent competitive inhibitor of 5,6-epoxy-Chol hydrolase in liver microsomes of the rat (696) and mouse (1188). The 5β,6β-epoxide of 7-dehydrocholesterol (5β,6β-epoxy-cholest-7-en-3β-ol) also inhibits the enzyme activity in rat liver microsomes (697), and it was suggested that this inhibitor acts by covalent modification of the active site of the enzyme.

Watabe et al. (1186, 1187) reported detailed studies of the formation of 5,6-epoxides of several Δ⁵-steroids and their subsequent conversion to the corresponding 5α,6β-glycols in a system containing bovine liver microsomes, an NADPH-generating system, ferrous ion, and ADP. Incubations were carried out for 40 min at 37°C (after a 5-min preincubation of the steroids with microsomes in buffer alone) at a level of 2.82 mM (in the case of Chol, 9.26 mg/8.5 ml of final incubation mixture). Under these conditions, the conversion of 14C-labeled Chol to labeled 5α,6α-epoxy-Chol, 5β,6β-epoxy-Chol, and 5α,6β-dioH-Chol was reported. From the data supplied, the calculated extents of formation of the 5α,6α-epoxide, the 5β,6β-epoxide, and the 5α,6β-glycol were 0.38, 1.69, and 0.25%, respectively. It was reported (1187) that “the microsomal formation of 5,6-oxygenated steroids from all of the Δ⁵-steroids examined was negligible either when boiled microsomes were used or when ferrous ion was omitted or scavenged with EDTA (1 mM), indicating the double bond oxidation processes to be enzymatic and dependent on ferrous ion.” Other steroidal substrates studied under the same conditions were 3-deoxyChol (cholest-5-ene), pregnenolone, and 3β-hydroxy pregn-5-ene. In each case, the β-epoxide was reported as the major product, a finding similar to that reported earlier by Aringer and Eneroth (20) with Chol and β-sitosterol. Characterization of the products was based on HPLC and GC-MS of the free sterols. In evaluation of this work, the very high concentration (~2.8 mM) of the sterols in the aqueous incubation mixtures is noteworthy as are the relatively low extents of metabolism. The highest extent of conversion was observed with the 3-deoxyChol (5α,6α-epoxide, 0.88%; 5β,6β-epoxide, 3.27%; and 5α,6β-glycol, 0.42%) which could reasonably be expected to have minimal solubility in the incubation medium. The conversion of [4,14C]Chol to the 5α,6α-epoxide, the 5β,6β-epoxide, and 5α,6β-dioH-Chol with bovine liver microsomes was suppressed by the use of boiled microsomes, the omission of Fe²⁺ and ADP, and by the addition of EDTA (1 mM) but was unaffected by CO. The combined data were interpreted as indicating an enzymatic peroxidative epoxidation of the Δ⁵-steroids not involving a cytochrome P-450 system.

Relatively little is known about the enzyme that catalyzes the formation of 5α,6β-dioH-Chol from either 5α,6α-epoxy-Chol or 5β,6β-epoxy-Chol. Only very slight enrichment of the enzyme from rat liver has been achieved despite apparently substantial effort (683, 1189). Nonetheless, the results of a number of varied studies indicate that hepatic microsomal Chol epoxide hydrolase is clearly distinct from much more extensively studied epoxide hydrolases of liver that act on xenobiotics and other substrates (561, 683, 697, 723, 1188, 1189).

Sevanian et al. (953) reported the presence of 5,6-epoxy-Chol in rat lung tissue. The reported level of the epoxide was extraordinarily high, i.e., 6.5 ± 0.66 mg/lung (with Chol reported at a level of 3.95 ± 0.20 mg/lung). The reported level of the epoxide may represent a typographical error, since an abstract from the same group (957) in the same year noted a level of 5.0 μg/g lung tissue. The epoxide was considered to be mostly the 5α,6α-isomer.
Although the mechanism of formation of the 5,6-epoxysterol in lung was not studied, its high levels in lung tissue were ascribed to autoxidation of Chol. Lung tissue apparently contains 5,6-epoxy-Chol hydrolase activity (953, 958), but at a lower level than in liver (958). In contrast to more extensive studies with liver (see above), the enzyme catalyzing the hydration of 9,10-epoxystearate in lung was considered to be the same as that catalyzing the hydration of the sterol epoxide (958), and 5β,6α-epoxy-Chol was reported to competitively inhibit the hydration of 9,10-epoxystearate.

5β,6β-Epoxy-Chol, 5α,6α-epoxy-Chol, 5α,6β-dihydroxy-Chol, and 5α,6α-dihydroxy-Chol have been reported to be formed upon oxidation of LDL in the presence of CuSO₄ or upon in vitro oxidation of LDL with soybean lipoxygenase (124). Identification of the novel 3β,5α,6α-triol was based on GC-MS comparisons with a synthetic sample (for which the detailed reaction conditions and characterization of the product were not presented). The mechanism involved in the formation of the 3β,5α,6α-triol was not established.

D. Formation of 5,6-Chlorohydrins of Cholesterol

Heinecke et al. (380) reported that incubation of Chol, incorporated into multilamellar vesicles containing phosphatidylcholine, with myeloperoxidase gave a mixture of products that included 5α,6α-epoxy-Chol, 5β,6β-epoxy-Chol, 6β-chloro-5α-cholestan-3β,5-diol, and another sterol chlorohydrin of undetermined structure. In the absence of the enzyme, hypochlorous acid was reported to give the same distribution of products. Identiﬁcations of the products were based on TLC and MS, and the TLC behavior of the chlorohydrins 5β,6β-chloro-5α-cholestan-3β,5-diol and 5-chloro-5α-cholestan-3β,6β-diol (more polar than Chol on TLC) and two other less polar components, one of which appeared to be a chlorohydrin. Similar products were observed upon incubation of HOCl with red cell lipids, red cell membranes, intact red blood cells, neutrophils, and a breast carcinoma cell line (MCF7). Studies of the chlorohydrins by GC-MS or LC-MS were compromised by the instability of the chlorohydrins, with the resulting formation of products with the properties of 5,6-epoxysterols. In a subsequent study by the same group, three chlorohydrins of Chol were formed upon incubation of Chol-lecithin (dipalmitoyl phosphatidylcholine (1:1) with hypochlorous acid (163). The three chlorohydrins were 6β-chloro-5α-cholestan-3β,5-diol, 5-chloro-5α-cholestan-3β,6β-diol, and 5α-chloro-5β-cholestan-3β,5-diol. The latter compound was reported to be the major product formed upon treatment of the Chol-lecithin liposomes with HOCl or with the myeloperoxidase-hypochlorite system. The structure of the compound was established by NMR. Complete 13C assignments were provided as well as those for the other two chlorohydrins of Chol. The structures of the three chlorohydrins are shown in Figure 2.

Hazen et al. (378) reported on the in vitro oxidation of LDL Chol with myeloperoxidase. Products, characterized by TLC and MS, included 6β-chloro-5α-cholestan-3β,5-diol, 5-chloro-5α-cholestan-3β,6β-diol, and another chlorohydrin for which the stereochemical orientations of the chlorine and hydroxyl functions were not established. In addition, a nonpolar (on TLC) dichlorosterol was also isolated. The orientation of the chlorine atoms in the dichlorosterol was not established. Carr et al. (162) presented a nice discussion of the question of the possible in
vivo physiological significance of reactions involving HOCl leading to halogenated sterols. They noted that competing reactions with free amino groups in proteins and phospholipids (to give chloramine derivatives), with SH groups in proteins and glutathione, and with unsaturated fatty acids in various lipids have to be taken into account in considerations of this matter. In contrast, Hazen et al. (378) suggested that the action of myeloperoxidase may be important in the oxidation-halogenation of LDL Chol in the artery wall, and they further suggested that these modifications of Chol occur in acidic cell compartments. Their results indicated that, at acidic pH and at concentrations of Cl− found in plasma, the formation of chlorinated derivatives of Chol proceeds in high yield. It is also important to note that myeloperoxidase has been reported to be present in atherosclerotic lesions and absent in normal arteries (241). Heinecke et al. (380) suggested that the chlorohydrins might prove to be useful “as markers for lipoproteins damaged by activated phagocytes.” However, no information is available on the occurrence of the chlorohydrins in LDL modified by cellular oxidation or in atherosclerotic lesions. The in vitro effects of hypochlorite, generated by myeloperoxidase, on HDL3 have been shown to result in predominant modification of apoA-1 and to a lesser extent modification of the unsaturated fatty acids in HDL3 (762). Products resulting from action on Chol were not identified. Treatment of HDL3 with NaOCl reduced its capability to accelerate Chol efflux from mouse peritoneal macrophages.

E. Formation of 24,25-Epoxy sterols

The enzymatic formation of 24,25-epoxy sterols has been shown to occur via cyclization of 2,3,22,23-diepoxy squalene (910). The results of subsequent studies have indicated that a partially purified squalene epoxidase from pig liver preferentially catalyzes the conversion of squalene to 2,3-epoxysqualene relative to the conversion of 2,3-epoxysqualene to 2,3:22,23-diepoxy squalene (45). In contrast, rat liver microsomes and a partially purified enzyme from pig liver have been reported to preferentially catalyze the conversion of 2,3(S):22(S)-diepoxy squalene to 24,25-epoxylanosterol [relative to the conversion of 2,3(S)-epoxysqualene to lanosterol] (110). A key discovery in this area was the demonstration that incubation of the 10,000-g supernatant fraction of rat liver homogenate with [2,14C]mevalonate in the presence of 4,4,10β-trimethyl-trans-decalin-3β,7β-diol inhibited Chol formation and led to the accumulation of 2,3-epoxysqualene and 2,3:22,23-diepoxy squalene (699) (as judged by TLC). Similar findings were also made upon incubation of CHO cells with labeled acetate in the presence of the decalin derivative (699). Subsequent studies (700) with the same compound inhibited the incorporation of [3H]acetate into Chol by Hep G2 cells and showed accumulation not only of labeled 2,3-epoxysqualene and 2,3:22,23-diepoxy squalene but also (24S)-24,25-epoxy-Chol. Sexton et al. (959) reported the formation of labeled materials with the TLC behavior of 2,3-epoxysqualene, 2,3:22,23-diepoxy squalene, and “polar compounds” (believed to be polar sterols) upon incubation of [3H]acetate with rat intestinal epithelial cells in the presence of 3β-[2-(diethylamino)ethoxy]androst-5-en-17-one (U-18666A). No definitive characterization of these labeled materials was presented.

Nelson et al. (701) reported the formation of (24S)-24,25-epoxy-Chol upon aerobic incubation of [3H]labeled 2,3:22,23-diepoxy squalene with a rat liver homogenate preparation. No conversion of either squalene, 2,3(S):22(S),23-diepoxy squalene, or (24S)-24,25-epoxy-Chol to 25-OH-Chol was detected upon their incubation with rat liver homogenate preparations. Nelson et al. (700) reported that small amounts of material with the properties of 24,25-epoxy-Chol are formed upon incubation of rat liver homogenerate preparations with [14C]acetate in the absence of an inhibitor of 2,3-epoxysqualene cyclase. Steckbeck et al. (1039) reported that labeled (24R)-24,25-epoxy-Chol was not detected upon incubation of a rat liver homogenate preparation with [2-3H](24R),25-epoxylanosterol. However, labeled materials with the properties of (24R)-24-hydroxylanost-8-en-3β-ol and (24R)-24-OH-Chol were reported. Taylor et al. (1102) reported the conversion of (24S),25-epoxylanosterol to (24S)-24,25-epoxy-Chol in both mouse L cells and Chinese hamster lung cells. The same workers reported that (24R),25-epoxylanosterol was converted to (24R)-24,25-epoxy-Chol in mouse L cells and, in Chinese hamster lung cells, to (24R)-24-OH-Chol. Saucier et al. (997) reported the formation of [14C]-labeled 24,25-epoxylanosterol upon incubation of CHO-K1 cells with [1-14C]acetate in the presence of ketoconazole (15 μM). Full characterization of the labeled product was not presented. Panini et al. (758) reported the formation of labeled (24S)-24,25-epoxylanosterol after incubation of rat intestinal epithelial (IEC-6) cells with [3H]acetate in the presence of U-18666A (to cause an accumulation of 2,3,22,23-diepoxy squalene) followed by further incubation of the cells with fresh medium containing ketoconazole (an inhibitor of sterol 14α-demethylation). The product was characterized by chromatography. Panini et al. (759) reported that incubations of rat intestinal epithelial (IEC-6) cells with [3H]acetate in the presence of progesterone (10 μg/ml) caused
an accumulation of materials with the chromatographic behavior (reverse-phase HPLC) of desmosterol (cholesta-5,24-dien-3β-ol) and (24S)-24,25-epoxy-Chol.

Dollis and Schuber (259) found that incubation of Hep G2 cells with [2,14C]acetate in the presence of a substituted azadecalin (N-[1,5,9-trimethyldecyl]-4α,10-dimethyl-8-aza-trans-decal-3β-ol), an inhibitor of 2,3-epoxysqualene-lanosterol cyclase, resulted in the accumulation of 2,3-epoxysqualene, 2,3,22,23-diepoxysqualene, and 24,25-epoxy-Chol (characterized by TLC). The same workers also reported the conversion, by Hep G2 cells, of added 2,3,22,23-diepoxysqualene to 24,25-epoxy-Chol (characterized by GC and GC-MS). Mark et al. (617) described the chemical synthesis of a new inhibitor (BIBX 79) of 2,3-epoxysqualene formation. The potency of this compound was reported to be considerably higher than that reported previously for inhibitors of this enzyme. The IC50 value for inhibition of the incorporation of [2,14C]acetate into DPS by Hep G2 cells was reported to be 3.8 × 10−9 M. The IC50 value for the conversion of 14C-2,3-epoxysqualene to lanosterol or epoxylanosterol by the Hep G2 cells was very similar (6 × 10−9 M). BIBX 79 was reported to cause the accumulation of significant amounts of 2,3-[14C]epoxysqualene and diepoxysqualene after incubation of Hep G2 cells with labeled acetate. Lesser amounts of 14C were observed to be associated with “epoxycholesterol” and “desmosterol.” These results were based on reverse-phase HPLC. This chromatographic system almost certainly is incapable of separating various C27 sterols from each other (869) and provided only slight separation of standards of Chol and lanosterol. Thus, a number of studies indicate the formation of 24,25-epoxylanosterol (and of 24,25-epoxy-Chol formed from it) via enzymatic cyclization of 2,3,22,23-diepoxysqualene. 24,25-Epoxysterols could also arise from direct enzymatic epoxidation of Δ24-sterols, although this process has not, to my knowledge, been described in mammalian systems. 24,25-Epoxysterols could also result from autoxidation of Δ24-sterols or via epoxidation secondary to peroxidation of other lipids.

F. Formation of 32-Oxygenated Sterols

Tabacik et al. (1083) reported the formation of labeled “lanost-3β-ol-32-al” by human lymphocytes incubated with sodium [2,14C]mevalonate. The evidence for the structural assignment, while suggestive, was limited. A cytochrome P-450 involved in the removal of the 14α-methyl group of sterol precursor of Chol has been purified to varying extents from rat liver microsomes (947, 1124) and pig liver microsomes (1016). Trzaskos and co-workers (1126, 1127) reported incubation conditions favoring the accumulation of lanost-8-ene-3β,32-diol and 3β-hydroxylanost-8-en-32-al (not resolved in the reverse-phase HPLC system used, Ref. 1126) upon incubation of [24,25-3H]lanost-8-en-3β-ol with rat liver microsomes. These included limiting levels of NADPH in the presence of NADH (1126), elevated pH (1127), limiting enzyme protein levels (1127), and incubation in the presence of miconazole or ketoconazole (1127). Saucier et al. (898) presented evidence indicating the formation of 32-oxolanosterol and 32-hydroxylanosterol in Chinese hamster lung cells incubated in the presence of a very high level (23 mM) of sodium mevalonate. Although neither sterol was detected in control cells, the levels of the 32-oxo and 32-hydroxy-sterols in the treated cells were reported to be, in two experiments, 0.058 and 0.084 μg/mg protein for the 32-oxosterol and 0.0088 and 0.0120 μg/mg protein for the 32-hydroxyxymethylsterol. Structure assignments were based on HPLC and limited mass spectral and 1H-NMR data along with chromatographic studies of the product of borohydride reduction of the putative 32-oxo sterol.

A number of inhibitors of the 14α-demethylase have been described. Included are ketoconazole (361, 1016, 1127, 1133), miconazole (430, 734, 1127), 14α-ethyl-5α-cholest-7-ene-3β,15α-diol (430, 679, 805, 860), 14α-methyl-5α-cholest-7-ene-3β,15α-diol (679), lanost-7-ene-3β,15α-diol (17, 947), 14α-alkyl-substituted lanosterol analogs (1132, 1133), and 3β-hydroxylanosta-8,15-diene-3-carboxylic acid (627). The latter synthetic compound was reported to be highly active in the inhibition of 14α-demethylase activity in Hep G2 cells, with an IC50 value of 2 nM (627).

The cloning of the cDNA encoding human and rat liver 14α-demethylase has been reported (16, 868, 996, 1054). Approximately 93% homology in the deduced amino acid sequences for the rat and human enzymes was observed (16, 1054). Approximately 38–42% homology was observed between the rat sequence and those reported previously for the protein from fungal sources. Strömstedt et al. (1054) reported that the human 14α-demethylase gene was expressed in a variety of tissues with the highest levels observed in testes, ovary, adrenal, prostate, liver, kidney, and lung. Transfer of either human adrenocortical (H295R) cells or human hepatoma (Hep G2) cells from medium containing 10% bovine serum to medium containing 10% delipidated bovine serum resulted in increased levels of mRNA for the 14α-demethylase, suggesting regulation by Chol (or other lipid constituents of serum). The increase was more substantial in the adrenal cells. 25-OH-Chol (12.4 μM) resulted in substantial decreases in the levels of mRNA for the 14α-demethylase. Thus transcription of the 14α-demethylase gene may be regulated by other oxysterols. No other oxygenated sterols were studied in this regard.

The levels of the enzyme catalyzing the oxidation of the 14α-methyl group of Chol precursors appear to be under physiological regulation. Chol feeding (3% in diet for 1 or 4 wk) to male Wistar rats (150–160 g) lowered the...
level of lanosterol 14α-demethylase (cyp51) activity and cyp51 content in liver microsomes (1017). After 1 wk of Chol feeding, 14α-demethylase activity and P450Δ7 content were reduced 38 and 33%, respectively. After 4 wk of Chol feeding, cyp51 activity and cyp51 protein were reduced 45 and 44%, respectively. Chol feeding (1 and 4 wk) had no effect on the levels of microsomal NADPH-P450 reductase activity or total cytochrome P450. The level of activity of cyp51 was increased in ovary (but not liver) after subcutaneous administration of gonadotrophin (pregnant mare’s serum) (1219). Germ cells from mature rats showed higher cyp51 activity than in those from prepubertal animals (1055).

The human 14α-demethylase gene has been localized to chromosome 7 (7q21.2-q21.3) and has been shown to contain 10 exons (867). The mRNA levels for the 14α-demethylase (ERG11) of the yeast Saccharomyces cerevisiae increased upon incubation with glucose or heme and increased under low oxygen growth conditions (1136). Interestingly, cytochrome P450 reductase and the 14α-demethylase of the yeast appeared to be coordinately regulated. The nucleotide sequence for lanosterol 14α-demethylase from Candida albicans has been reported (536).

Whereas the formation of 14α-hydroxymethyl and 14α-formyl sterols has been demonstrated in mammalian tissues, the occurrence of 14α-carboxylic acid derivatives of sterols has not been shown in animals, with the exception of certain sponges. Cheng et al. (195) reported the occurrence of a 14α-carboxylic acid derivative of lanosterol in an Okinawan sponge (Penares sp.). This new sterol, penasterol (Fig. 3A), was reported to show in vitro antitumor activity (IC50 7.9 μM) against L1210 mouse leukemia cells. Shoji et al. (977) reported the occurrence of two other 14α-carboxylic acid derivatives of lanosterol in an Okinawan sponge (Fig. 3, B and C).

Sonoda et al. (1018) described the chemical synthesis of the Δ7-analog of penasterol, which was reported to show in vitro antitumor activity against L1210 mouse leukemia cells (IC50 2.3 μM) and human epidermoid carcinoma KB cells (IC50 7.2 μM). The methyl ester derivative showed little or no activity under the conditions studied. Other oxygenated derivatives of 24,25-dihydrolanosterol were also reported to show in vitro antitumor activity, most notably the (24R)-24,25-epoxide and the 24-keto derivatives.

It is noteworthy that several substituted ent-5α,14β-androstane derivatives with a 14α-carboxymethyl function have been isolated from a Penicillium species and shown to have in vitro inhibitory activity against partially purified farnesyltransferase from human cells (732, 1141). The structures of three new compounds, termed andrastatins A, B, and C, are shown in Figure 4. The potencies (IC50) of andrastatins A, B, and C against farnesyltransferase were 24.9, 47.1, and 13.3 μM, respectively.

G. Formation of 4-Hydroxysterols

The presence of 4β-OH-Chol in human blood (121, 128) and in rat liver has been reported (121). The origin of this oxysterol has not been established.

H. Formation of 19-Hydroxysterols

19-OH-Chol has been reported by two groups (520, 1108) to be present in membranes of erythrocytes from patients with sickle cell anemia. The reported occurrence of 19-OH-Chol in animal tissues is, to the knowledge of the reviewer, without precedent. Hydroxylation of the C19 methyl group of C19 steroids is known to occur in the series of reactions involved in the formation of estrogens. However, enzymatic 19-hydroxylation of C27 sterols has not been described in mammalian systems. Nonetheless, it should be noted that Gustafsson et al. (366) suggested the presence of a 19-hydroxylated bile acid as a metabolite of labeled lithocholic acid by microsomes from human fetal liver. Furthermore, Kurosawa et al. (525) reported the occurrence of a 19-hydroxylated bile acid, i.e., 3α,7α,12α,19-tetrahydroxy-5β-cholan-24-oic acid in the urine samples from 2- to 9-day-old healthy human infants. This bile acid was present at 0.1–1.5 μg/ml and corresponded to an estimated 1.5 to 7.0% of total urinary bile acids. The 19-hydroxylated bile acid was character-
ized by GC-MS studies with comparisons with an authentic standard prepared by chemical synthesis. The 19-hydroxylated bile acid was not detectable in the urine of older healthy children or adults. In an earlier study, Gustafsson et al. (366) reported evidence indicating 19-hydroxylation of lithocholic acid by microsomes of fetal human liver. [24-14C]3α-hydroxy-5β-cholane-24-oic acid (lithocholic acid) was incubated with liver microsomes of fetal (14–24 wk) human subjects in the presence of NADPH. The products were subjected to TLC, and one radioactive zone was found to contain material which, as its TMS derivative, showed a MS that was compatible with the TMS derivative of a primary alcohol. The structure 3α,19-dihydroxy-5β-cholane-24-oic acid was suggested. An authentic sample was not available for comparison. Kimura et al. (491) reported low levels of the 19-hydroxy derivative of cholic acid in the urine of newborn infants. Even lower levels were reported for urine obtained from women in late pregnancy and shortly after delivery. Identification of the 19-hydroxylated bile acid was based on GC-MS of the dimethylethylsilyl ether derivative of the methyl ester. Full MS data were not presented.

I. Formation of 15-Oxygenated Sterols

3β-Hydroxy-5α-cholesta-8(14)-en-15-one has been reported to be present in rat skin and rat hair (278). The mode of formation of the 15-ketosterol is not known. However, in view of the demonstration of the occurrence of 5α-cholesta-8(14)-en-3β-ol in rat skin (551, 597), it is possible that the 15-ketosterol in rat skin and hair might have arisen from autoxidation of the Δ8(14)-sterol present in skin (and presumably in hair). The results of a control experiment, including the addition of Δ8(14)-sterol to hair before processing of the sample, demonstrated that the 15-ketosterol isolated was not formed by autoxidation of the Δ8(14)-sterol during the procedures utilized in the purification and analysis of the 15-ketosterol. However, the possibility exists that some or all of the 15-ketosterol found to be present in rat skin and hair was formed by autoxidation of the Δ8(14)-sterol before the death of the rats. It is possible that 5α-cholesta-8(14)-en-3β-ol served as a precursor of the 15-ketosterol, either by enzymatic action or by autoxidation.

J. Bioorganic Syntheses of Oxygenated Sterols

The development of conditions for large-scale incubations of 2,3-epoxysqualene with baker’s yeast (144) provided the opportunity to explore this system for the preparation of various analogs of lanosterol using appropriately substituted 2,3-epoxysqualenes as substrates. With the use of the synthetic methyl ester as substrate, the methyl ester of ganoderic acid Z was obtained (Fig. 5).

The same approach was utilized for the preparation of the 4α-hydroxyethyl analog of lanosterol (634). Exploiting this approach further, Medina et al. (633) provided a novel method for the preparation of C-32 oxygenated sterols. They observed the cyclization of the appropriately vinyl-substituted analog of 2,3-epoxysqualene to give the 14α-vinyl analog of lanosterol (Fig. 6).

Xiao and Prestwich (1206) utilized the same approach to prepare 21-hydroxylanosterol and 19-hydroxylanosterol from appropriately substituted analogs of 2,3-epoxysqualene.

Chol oxidase has been used, on a preparative scale, to synthesize the 3-keto derivatives of a number of oxygenated sterols from the corresponding 3β-hydroxysterols (769, 779, 924, 931, 935, 938). The use of this commercially available enzyme provided a powerful approach for the selective oxidation of the 3β-hydroxy function in sterols with multifunctional substituents. Recent results of Teng and Smith (1109) indicate that careful attention to product identification cannot be bypassed since, with Chol oxidase of Pseudomonas fluorescens, oxidation of Chol gave 6β-hydroperoxycholest-4-en-3-one as the major product, instead of the expected cholesta-4,6-en-3-one. Oxidation of 25-OH-Chol under the same conditions gave material with the expected chromatographic and 1H-NMR spectral properties of 6β-hydroperoxycholesta-25-hydroxycholest-4-en-3-one. Oxidation of 19-OH-Chol by the enzyme from P. fluorescens was reported to give four products, the identity of which was not established. In an earlier study, Liu et al. (581) reported that incubation of rabbit LDL with commercial Chol oxidase (from P. fluorescens) resulted in the conversion of Chol in LDL to 20α-OH-Chol and 25-OH-Chol. Identification of the latter compounds was based solely on HPLC analysis, and these assignments of structure should be viewed with caution. A more recent study from the same laboratory (836) claimed the formation of 25-OH-Chol and 20α-OH-Chol from Chol present in rat...
K. Formation of Oxysterols In Vivo

Marco de la Calle and co-workers (614, 615) studied the incorporation of tritium into hepatic sterols of liver of male Wistar rats at 1 h after the intraperitoneal injection of $^3$H$_2$O. Significant incorporation of the labeled hydrogen male Wistar rats at 1 h after the intraperitoneal injection of $^3$H$_2$O into hepatic sterols of liver of K. Formation of Oxysterols In Vivo

Chol, 0.56. 1.29; 24-keto-Chol, 1.32; 25-OH-Chol, 0.61; and (25
that such precursors (e.g., 32-hydroxylanosterol and the
labeled polar sterols to oxysterols and suggested that Chol feeding (or ketoconazole treatment) resulted in the accumu-
lar sterols or even the establishment that the concerned
vations were reported (615) between the accumulation of
polar sterols to oxysterols and suggested that Chol feed-
ated with polar sterols (679). The authors ascribed the
increase in the percentage of $^3$H of NSL that was associ-
duced cholesterol during sample processing.” To deal with
this, a second experiment was carried out in which the
animal was exposed to normal air for 5 min after the
exposition to $^{18}$O$_2$ for 178 min, after that $^{18}$O$_2$ was introduced
for 25 min. Blood samples were taken at 0, 178, and 205
min. The livers were homogenized in phosphate buffer
containing butylated hydroxytoluene (BHT). The plasma
and liver homogenate were frozen and stored for 20 days
at $-20^\circ$C. One control rat, exposed to $^{16}$O$_2$, was studied
similarly. Oxysterols were analyzed after mild alkaline
hydrolysis and workup as described previously by the
authors (273) except that TBDMSi ethers were analyzed
by GC-MS. The $^{18}$O content of various oxysterols, Chol,
and 5a-cholest-7-en-3b-ol was reported. With the exception
of some of the studies with liver in one experiment, it appears that all of the results reflect a single analysis of
samples from one animal. No data on the levels of the
various sterols were provided. This may be relevant to
some observations made since two sterols of particular
interest with regard to $^{18}$O incorporation, i.e., 7b-OH-Chol
and 25-OH-Chol, were reported in another study from the
same laboratory (273) to be present in human plasma at
very low levels, i.e., 0.008 ± 0.12 and 0.005 ± 0.007 $\mu$M,
respectively. The authors noted that with the design used
in the first experiment “there is a theoretical risk that $^{18}$O$_2$
 dissolved in plasma or bound to hemoglobin might oxi-
dize cholesterol during sample processing.” To deal with
this, a second experiment was carried out in which the
animal was exposed to normal air for 5 min after the
period of exposure to an atmosphere containing $^{18}$O$_2$.

“After 183 min of $^{18}$O$_2$ exposure, $^{16}$O$_2$ was delivered to the
cage for 22 min. The cage was then opened wide at 210
min to allow equilibration with room air for 5 min.” Blood
samples were taken at 0 and 210 min. The liver was also
removed at 210 min. In both experiments, very little or
none of the Chol in plasma or liver contained $^{18}$O. In
the first experiment, plasma Chol contained 1% $^{18}$O species
at 178 and 205 min and 2% $^{18}$O species in Chol of liver at 205
min. In the second experiment, plasma and liver Chol
contained 3 and 5% of $^{18}$O species at 210 min, respectively.
In contrast, 5a-cholest-7-en-3b-ol contained substantial
amounts of $^{18}$O in both experiments. In the first experi-
The results with the oxysterols are less amenable to simple interpretation. The authors concluded that “in vivo formation of oxysterols, indicated by enrichment in $^{18}$O, was established for cholest-5-ene-3β,7α-diol, cholest-5-ene-3β,7β-diol, 7-oxocholesterol, cholest-5-ene-3β,24-diol, cholest-5-ene-3β,25-diol, and cholest-5-ene-3β,27-diol. Additionally, it seems likely that cholest-5-ene-3β,4β-diol is formed in vivo. The $^{18}$O labeling pattern suggests that there is incomplete equilibration between liver and plasma pools of cholest-5-ene-3β,27-diol. No evidence for the in vivo formation of 5,6-oxygenated oxysterols was obtained.” Because these results have been cited and discussed in later publications (121, 591), a close inspection of these results is warranted. Despite the conclusions made by the authors, the interpretation of the observed data appears to be less than simple. Substantial $^{18}$O incorporation was observed with most, but not all, of the oxysterols studied.

Two of the oxysterols studied, 26-OH-Chol and 24-OH-Chol, are known as products of P-450-dependent hydroxylation of Chol and are not considered to be significant autoxidation products of Chol. Interestingly, the $^{18}$O content of these two sterols differed markedly. In both experiments, the presence of two atoms of $^{18}$O was not detected in 24-OH-Chol from plasma. $^{18}$O was detected in 24-OH-Chol from plasma but only as a mono-$^{18}$O species.

The levels of mono-$^{18}$O species in plasma were 17% at 178 min and 11% at 205 min in the first experiment and 9% at 210 min in the second experiment. No detectable $^{18}$O was found in 24-OH-Chol in liver in either the first or second experiments. In a subsequent report from the same laboratory (596), the level of mono-$^{18}$O species in brain at 210 min in the second experiment was reported as 11%. No mention of species containing two atoms of $^{18}$O was made. In another subsequent report from the same laboratory (94), the much lower $^{18}$O content of 24-OH-Chol (relative to 26-OH-Chol and 7α-OH-Chol) in plasma was ascribed to the lower rate of synthesis of the 24-OH-Chol in relation to its pool size. In contrast to these results with 24-OH-Chol, 26-OH-Chol in plasma was substantially enriched with $^{18}$O species, most of which were present as mono-$^{18}$O species (in both experiments). For example, in the first experiment, 26-OH-Chol of plasma contained 47 and 8% as mono- and di-$^{18}$O species at 178 min, respectively, and 50 and 4% as mono- and di-$^{18}$O species at 205 min, respectively. In the second experiment, 26-OH-Chol from plasma at 210 min contained 47 and 4% as mono- and di-$^{18}$O species, respectively. The $^{18}$O content of 26-OH-Chol in plasma was substantially higher than that of 26-OH-Chol in liver. This difference was ascribed by the authors to “an incomplete equilibration between the liver and plasma pools” of this sterol.

4β-OH-Chol has been reported to occur in human plasma (121, 128). However, its origin (i.e., enzymatic or via autoxidation) has not been established. The $^{18}$O content of 4β-OH-Chol in plasma and liver was relatively low, and all of the $^{18}$O appeared to be mono-$^{18}$O species. No $^{18}$O enrichment was observed with either 5α,6α-epoxy-Chol or 5α,6β-dioOH-Chol. 25-OH-Chol and 7α-OH-Chol can arise from both enzymatic and autoxidation of Chol. The 25-OH-Chol and 7α-OH-Chol in plasma contained mostly mono-$^{18}$O species (in both experiments). The same was reported for these two sterols from liver. In the first experiment, the 7α-OH-Chol in plasma contained 21 and 19% mono-$^{18}$O species at 178 and 205 min, respectively, and 41% mono-$^{18}$O species at 210 min in the second experiment. The 25-OH-Chol in plasma contained 57 and 50% mono-$^{18}$O species at 178 and 205 min, respectively, in the first experiment and 50% mono-$^{18}$O species at 210 min in the second experiment. 7α-OH-Chol in liver contained 11 and 25% mono-$^{18}$O species in the first and second experiments, respectively. The $^{18}$O in liver 7α-OH-Chol from one experiment was reported to be mostly located at C-7. The 25-OH-Chol in liver was reported to contain 54 and 42% mono-$^{18}$O species in the first and second experiments, respectively. 7-Keto-Chol is a known product of autoxidation of Chol and of lipid peroxidation. 7-Keto-Chol from the plasma and liver showed moderate levels of $^{18}$O incorporation, and all of this $^{18}$O was in mono-$^{18}$O species.

In contrast to all of the oxysterols noted above, a very substantial fraction of the $^{18}$O in 7β-OH-Chol was in di-$^{18}$O species. This was true for samples from both plasma and liver. For example, in the second experiment, the 7β-OH-Chol from plasma contained 59 and 23% of mono- and di-$^{18}$O species, respectively, and the sample from liver contained 29 and 24% of mono- and di-$^{18}$O species, respectively. The 7β-OH-Chol from liver, when analyzed by GC-MS as its dTMS ether derivative, showed (based on M+) 29% mono-$^{18}$O species and 22% di-$^{18}$O species. Based on M-90 (with the authors’ assumption that this ion represents specific loss of O at C-3), only mono-$^{18}$O species (34%) were observed. The unique high incorporation of two atoms of $^{18}$O in the 7β-OH-Chol prompted the following statement from the authors “... it is tempting to suggest that cholest-5-ene-3β,7β-diol is predominantly formed from newly synthesized Chol, or a Chol.
precursor, by enzymatic reactions in vivo.” It should be noted that substantial di-^{18}O species were observed only with 7β-OH-Chol. With all other oxysterols the estimated levels of di-^{18}O species were, in almost all samples, between −2 and +4%. In view of the fact that oxidation of Chol with ^{18}O_2 in the presence of Cu^{2+} gave ^{18}O-labeled 7β-CHol with 6% di-^{18}O species, it might be surmised that, within the apparent errors of the experiment, essentially no di-^{18}O species were observed for the various oxysterols other than 7β-CHol. It seems reasonable to assume that, in these oxysterols, the ^{18}O was not at C-3 but was at the site of the oxygen substitution. However, of these oxysterols, evidence on this point was provided only for one sample of 7α-OH-Chol from the liver obtained in one experiment. Also, it is assumed that the presence of ^{18}O in the oxysterols establishes their enzymatic formation in vivo. The second experiment was carried out in such a manner as to make it unlikely that residual ^{18}O_2 dissolved in plasma or bound to hemoglobin could oxidize the Chol during sample preparation. However, direct evidence on this point was not provided. In addition, the possibility that significant levels of ^{18}O might be present in a fatty acid hydroperoxide (594) (or a similar species with a potentially longer biological and chemical half-life) existed at the end of the experiment, which could provide a source of ^{18}O for subsequent autoxidation of Chol, was not excluded. In addition, because the level of 7β-OH-Chol in plasma has been reported to be very low (121, 273, 521), any ^{18}O-labeled 7β-OH-Chol would undergo very little dilution with endogenous unlabeled 7β-OH-Chol (relative to other oxysterols).

Johnson et al. (456) reported on the formation of labeled oxysterols in liver 1 h after the intraperitoneal injection of [5RS,-^3H]mevalonolactone to one control rat and to one Chol-fed (5% in Chol-free diet) rat. The rats were fasted for 6 h before feeding for 1 h of Chol (in a Chol-free diet) or, in the case of the control rat, the Chol-free diet alone. The livers were frozen in liquid nitrogen and then saponified with treatment with a solution containing 50% aqueous KOH (2 parts) and 95% ethanol (3 parts) “at reflux temperature for 3 h in the dark in an atmosphere of nitrogen.” Under these conditions, essentially quantitative decomposition of any 7-keto-Chol would be anticipated (273, 521, 603, 771). A small amount of BHT was added as an antioxidant. The NSL were recovered by extraction with hexane. After separation of polar oxysterols from Chol by reverse-phase flash chromatography on a silica gel (C_{18} bonded-phase) column, the labeled polar sterols were subjected to normal-phase HPLC. In the liver from the control rat, two major labeled components with the reported mobility of 7-keto-Chol and 7α-OH-Chol were observed. The mobilities of unlabeled standards corresponding to the labeled sterols were not shown (nor those of other oxysterols). In the liver of the rat, which received the Chol-containing meal for 1 h, three major peaks were observed that were said to correspond to 25-OH-Chol, 7-keto-Chol, and 7α-OH-Chol. No further characterization of the labeled materials was made. The reported substantial formation of material with the chromatographic mobility of 7-keto-Chol is noteworthy in view of the saponification conditions employed (see above). The authors claimed that the peaks corresponding to 7-keto-Chol and 7α-OH-Chol were labeled with tritium “to a greater extent” in the Chol-fed rat than in the control rat and that this was “consistent with a stimulation of bile acid formation in response to the Chol challenge.” These claims must be viewed with caution in view of the lack of complete characterization of the labeled materials and the use of an experimental design involving the use of only one experimental rat and one control rat. Johnson et al. (456) also reported the incorporation of labeled hydrogen of water into several oxysterols of livers from rats fed a Chol-containing meal. The rats (number not clearly stated for this particular experiment) were given D_{2}O (33% in distilled water) ad libitum as drinking water for 3 days before death. Control animals received no D_{2}O. The animals were fasted for 6 h (5–11 A.M.). The experimental rats were fed for 1 h with a Chol-containing (5%) diet; the control group was fed the same diet containing purified Chol (5%). After an additional hour, the rats were killed, and the pooled liver samples were then analyzed for deuterium incorporation into various sterols. The incorporation of deuterium into the following oxysterols was reported: 7α-OH-Chol, 26-OH-Chol, and 25-OH-Chol. The extents of incorporation of the deuterium into 7α-OH-Chol and 25-OH-Chol were similar, whereas that into 26-OH-Chol was lower. It was reported that no incorporation into 7β-OH-Chol was observed. No other oxysterols were mentioned. The incorporation of deuterium was studied in a special way, based only on the enrichment of the M+1 ion on GC-MS.

Lund et al. (593) reported increases in the levels of 24-OH-Chol and 26-OH-Chol in liver after administration of Chol (2% in diet for 4 days). Nonetheless, the authors concluded that “neither 24-hydroxylation nor 27-hydroxylation are critical for the cholesterol-induced downregulation of HMG-CoA reductase in mouse liver.” This conclusion was based on the results of in vivo experiments in which deuterium-labeled analogs of 26-OH-Chol or 24-OH-Chol were fed to mice. The effects of feeding either Chol, [23,23,24,24,25-^2H_5]Chol, or [26,26,26,27,27,27-^2H_6]Chol at levels of 0.05% by weight in diet to mice on the levels of liver microsomal HMG-CoA reductase activity were studied. The Chol samples (0.05% by weight in diet) were fed in diet supplemented with peanut oil (10% by weight in diet). The mice were fed individually 6 g of food for 24 h; then “the mice were again fed individually in the morning with 6 g of the sterol-containing diet and killed 24 h later.” No information was provided as to whether or not the animals were on a normal light-dark cycle or on the actual times at which the
bulk of the food was consumed. The results indicated that the animals fed Chol or the deuterated Chol samples each showed mean levels of HMG-CoA reductase activity that were ∼50% that of control animals (no added Chol). The authors anticipated a reduced suppression of HMG-CoA reductase activity in animals fed the 26-OH-Chol and 24-OH-Chol (if the formation of these sterols from Chol was important in the lowering of reductase activity) due to the presence of isotope effects observed in their formation from Chol in mouse liver mitochondrial incubations. The absence of an isotope effect in the lowering of HMG-CoA reductase activity in liver (i.e., the lack of a reduced suppression of reductase activity in animals receiving the deuterated Chol samples) was presented as major evidence “that 24- and 27-hydroxylation are not involved in the Chol-induced down-regulation of HMG-CoA reductase activity in mouse liver.”

Lund et al. (593) reported an isotope effect in the formation regulation of HMG-CoA reductase activity in mouse liver. The absence of an isotope effect in the lowering of HMG-CoA reductase activity in liver (i.e., the lack of a reduced suppression of reductase activity in animals receiving the deuterated Chol samples) was presented as major evidence “that 24- and 27-hydroxylation are not involved in the Chol-induced down-regulation of HMG-CoA reductase activity in mouse liver.”

Lund et al. (593) reported an isotope effect in the formation of 26-OH-Chol from [26,26,26,27,27,27-2H6]Chol and [25,26,26,26,27,27,27-2H7]Chol and in the formation of 24-OH-Chol from [23,23,24,24,25-2H5]Chol and [24,24-2H2]Chol by mouse liver mitochondria in the presence of isocitrate and NADPH. The GC-MF analyses were also made of the molecular ion region of the TMS ether derivative of the 26-OH-Chol formed from a 1:1 mixture of [6,7α,7β-2H2]Chol and [26,26,26,27,27,27-2H6]Chol or [25,26,26,26,27,27,27-2H7]Chol during a 1-h incubation with the mouse liver mitochondria. The GC-MF analyses were also made of the ion corresponding to M-TMSOH-43 (isopropyl function) region of the MS of the TMS derivative of the 24-OH-Chol formed from a 1:1 mixture of [6,7α,7β-2H2]Chol and [24,24-2H2]Chol or [23,23,24,24,25-2H5]Chol during a 1-h incubation with the mouse liver mitochondria. From these 1-h incubations, the authors estimated a $K_D$ of 2.5 for the formation of the 26-hydroxysterol and a $K_D$ of 4.5 or 4.3 for the formation of the 24-hydroxysterol. Whereas these results strongly indicate the presence of isotope effects, the estimates of the magnitude at $K_D$ based on a single time determination of the amount of product formed from the precursor labeled with deuterium at the concerned carbon atom and its analog lacking the deuterium substitution is subject to criticism. This follows from the simple consideration that the ratios of products from the deuterated and nondeuterated precursors can be expected to vary considerably with the extent of conversion of the precursors to Chol. Moreover, in the case of the 26-OH-Chol, the extent of its further metabolism (conversion to the corresponding aldehyde and carboxylic acid) by the mitochondria under the conditions studied, as well as the presence or absence of isotope effects in the concerned reactions, should be considered. In the case of the experiments involving the in vivo feeding of the deuterated Chol samples, the experimental system should be recognized as considerably more complex. The lack of observation of isotope effects in the in vivo experiments does not, for this reviewer, justify any conclusion with regard to the involvement of 26-OH-Chol or 24-OH-Chol in the regulation of hepatic HMG-CoA reductase.

Interesting results on the side-chain oxidation of deuterium-labeled Chol by highly purified cytochrome P-45026 from pig liver mitochondria were reported by Lund et al. (592). The purified hydroxylase was incubated with the Chol samples (added in acetone) in the presence of adrenodoxin, adrenodoxin reductase, and NADPH for 30 min in Tris acetate buffer containing 20% glycerol and 0.1 mM EDTA. Under these conditions, the reconstituted cytochrome P-450 system was reported to catalyze the formation of 26-OH-Chol, 25-OH-Chol, and 24-OH-Chol. In contrast to the case of the studies with mouse mitochondria, no significant isotope effects were observed for the 26-hydroxylation (or the 25-hydroxylation). However, a marked (and much higher than for the mouse system) isotope effect $K_D$ of >10 was reported for 24-hydroxylation of Chol. These results were based on analyses of sterol products after a 30-min incubation period. 26-Hydroxylation was assayed by GC-MS of the TMS derivative after incubation of a 2:1 mixture of [6,7,7-2H3]Chol and [25,26,26,26,27,27,27-2H7]Chol. The products were monitored at $m/z$ 540 and 552, corresponding to molecular ions of the bis-TMS derivatives. 25-Hydroxylation was assayed after incubation of a 1:1 mixture of [6,7,7-2H3]Chol and [25,26,26,26,27,27,27-2H7]Chol. The 25-OH-Chol products were monitored at $m/z$ 534 and 537, corresponding to the M-57 ion of the 3β-TMS, 25-TBDMS derivative of 25-OH-Chol. 24-Hydroxylation was assayed after incubation of a 1:1 mixture of [23,23,24,24,25-2H5]Chol and [25,26,26,26,27,27,27-2H7]Chol. These analyses ignore possible effects of secondary isotope effects and, in the case of the 26-hydroxylation, of primary isotope effects in the further metabolism of 26-OH-Chol to the 26-acid.

L. Oxysterol Formation in Membrane Preparations

Girotti et al. (339) studied lipid peroxidation in resealed human erythrocyte ghosts. Treatment of the erythrocyte ghosts with xanthine oxidase and xanthine was reported to give two hydroperoxides of Chol which, after treatment of the lipid extract with sodium borohydride, gave two polar sterols, ascribed to the 7α-hydroxy and 7β-hydroxy derivatives of Chol on the basis of TLC. An additional component of higher $R_f$ was observed but not characterized. The addition of either catalase or superoxide dismutase was reported to prevent the formation of the Chol oxidation products when added to the xanthine oxidase plus xanthine-treated preparations. These materials were not observed in incubations lacking either xanthine oxidase or xanthine. Photo-oxidation of the erythrocyte ghosts in the presence of either rose bengal or protoporphyrin IX was reported to yield 5-hydroperoxy-5α-cholest-6-en-3β-ol, which was detected on the basis of
the TLC behavior of the product of the borohydride reduction of the lipid extract of the oxidized membranes. In a subsequent study by the same laboratory, Bachowski et al. (44) studied the oxidation products of \([4-^{14}C]\)Chol incorporated into erythrocyte membrane preparations. Photo-oxidation of the labeled Chol was carried out in the presence of a hematoporphyrin derivative. Under these conditions, the major product was reported to be 5-hydroperoxy-5\(\alpha\)-cholest-6-en-3\(\beta\)-ol for which characterization was limited to TLC and the mobility of the corresponding 5\(\alpha\)-hydroxysterol obtained upon borohydride reduction. After photo-oxidation, further incubation with ascorbate (1 mM) and FeCl\(_3\) (10 \(\mu\)M) for 1 h in the dark led to a much more complex mixture of Chol oxidation products [including material ascribed to the 7\(\alpha\)- and 7\(\beta\)-hydroperoxides of Chol (plus possibly 7-keto-Chol) and the 7\(\alpha\)- and 7\(\beta\)-hydroxy derivatives of Chol], a complexity that was prevented by the addition of EDTA (50 \(\mu\)M).

Sevanian and McLeod (952) studied the oxidation of \([4-^{14}C]\)Chol in unilamellar liposomes containing Chol and phospholipids as induced by incubation under common autoxidation conditions, by oxidation with cumene hydroperoxide, or by \(\gamma\)-ray irradiation. The formation of the 5\(\alpha\),6\(\alpha\)- and 5\(\beta\),6\(\beta\)-epoxides of Chol, 7-keto-Chol, the 7-hydroperoxides of Chol, and the 7-hydroxy derivatives of Chol, 5\(\alpha\),6\(\beta\)-diOH-Chol, and cholesta-3,5-diene-7-one was reported. It should be noted that control preparations of the liposomes contained significant amounts of Chol oxidation products, which presumably arose during the preparations of the liposomes. The formation of oxidation products of Chol was lowest in liposomes prepared from a phospholipid containing saturated fatty acids (i.e., dipalmitoylphosphatidylcholine). Lang and Vigo-Pelfrey (542) studied the oxidation of Chol in small unilamellar vesicles and multilamellar vesicles composed of Chol and phosphatidylcholines (of variable degrees of unsaturation, i.e., native, egg PC, partially hydrogenated egg PC, or fully hydrogenated egg PC) after incubation at 50°C for 3 mo. As might be anticipated, oxidation of Chol was markedly suppressed or absent in the vesicles prepared from fully hydrogenated egg PC. The major oxidation product of Chol detected by HPLC was 7-keto-Chol, which was accompanied by lesser amounts of 7\(\alpha\)-OH-Chol and 7\(\beta\)-OH-Chol. Significant amounts of unidentified products were noted in some experiments. Van Amerongen et al. (1150) studied the transfer of oxysterols from a mixed monolayer to acceptor vesicles in the subphase. With the use of labeled sterols, the transport of Chol was negligible, whereas notable transport (in order of increasing rate of transport) of 7-keto-Chol, 7\(\alpha\)-OH-Chol, and 25-OH-Chol was observed. Addition of the nonspecific lipid transfer protein (sterol carrier protein 2) increased the rate of transfer of Chol and of each of the oxysterols.

### IV. OCCURRENCE AND LEVELS OF OXYSTEROLS

#### A. Oxysterols in Plasma

The occurrence and levels of oxysterols in plasma are topics of considerable potential importance in considerations of their potential physiological importance. Studies of this matter are complicated by difficulties in the separation and identification of the various oxysterols, especially in the absence of a comprehensive set of authentic standards. Quantitation of the sterols requires attention to recoveries in extractions, completeness of derivatization reactions, and the stability of the compounds to reactions used in the isolation and analytical procedures. Because a substantial fraction of a number of oxysterols is esterified to fatty acids, conditions of saponification are critical, especially in the case of 7-keto-Chol, which has been shown to undergo substantial decomposition under usual saponification conditions (273, 521, 603, 771). The ease of autoxidation of Chol, present at relatively high levels in plasma of normal human subjects, provides an additional problem, i.e., artificial generation of oxysterols from Chol during sample storage, processing, and analysis. Certain oxysterols of biomedical interest (e.g., 26-OH-Chol, 24-OH-Chol, and 22-OH-Chol) are generally considered to not represent significant products of the autoxidation of Chol. Others (including 7-keto-Chol, 7\(\beta\)-OH-Chol, 7\(\alpha\)-OH-Chol, 25-OH-Chol, 5\(\beta\),6\(\beta\)-epoxy-Chol, 5\(\alpha\),6\(\alpha\)-epoxy-Chol, and 5\(\alpha\),6\(\beta\)-diOH-Chol) are recognized products of Chol autoxidation. More or less rigorous studies (see below) have indicated that some oxysterols of biomedical interest occur in fresh normal human plasma at levels of \(\sim 0.010\)–\(0.100 \) \(\mu\)M. To obtain valid estimates of the levels of such compounds, very major suppression or total elimination of autoxidation of Chol (present in plasma samples of normocholesterolic subjects at \(\sim 5,000 \) \(\mu\)M) is essential. Formation of an oxysterol by autoxidation of Chol to the extent of only 0.001% could severely compromise a valid assay of a given oxysterol. Autoxidation of Chol can be reduced by analysis of fresh samples clearly free of hemolysis, inclusion of antioxidants, use of peroxide-free degassed solvents (and avoidance of solvents prone to peroxide formation such as ethyl ether), conduct of saponification and extraction steps under conditions involving rigorous exclusion of oxygen, and isolation (free of Chol) at an early stage in the analysis of the oxysterols. Even with such efforts to suppress autoxidation of Chol, an internal standard should be included in the assay of each sample to detect and quantitate the artifactual formation of each oxysterol from Chol during sample processing and analysis. To our knowledge, only one study (521) included such controls in the analysis of oxysterols in plasma.
In 1989, Kudo et al. (521) developed methodology based on the ability to separate the acetate derivatives of an number of oxysterols by the combination of reverse-and normal-phase HPLC. Fresh plasma samples were saponified and extracted with methyl t-butyl ether (an ether relatively resistant to peroxide formation) in a closed all-glass system under argon. The NSL were subjected to reverse-phase MPLC to isolate the polar sterols free of Chol. Acetylation of the polar sterols with [3H]acetanhydride permitted not only following the separation of the oxysterols but also, with knowledge of the specific activity of the acetanhydride, quantitation of the individual oxysterols. A critical feature of the study was the use of an internal standard of highly purified (immediately before use) [14C]Chol of high specific activity that was added to the plasma sample before any processing. Thus, by determination of the amounts of 3H and 14C associated with the acetate derivative of a given oxysterol, the amount of the oxysterol could be calculated as well as the amount formed artifactualy from Chol during sample processing. The results of these studies demonstrated (after correction for artifactual generation of oxysterols by autoxidation during sample processing or analysis) the presence of significant levels of 26-OH-Chol, (24RS)-24-OH-Chol, and 7α-OH-Chol (Table 1). After correction for their formation by autoxidation of Chol, essentially none of the following sterols was observed in plasma: 7β-OH-Chol, 5α,6α-epoxy-Chol, 5β,6β-epoxy-Chol, 5α,6β-dioOH-Chol, and the 25-, 22R-, 20α-, and 19-hydroxy derivatives of Chol. The levels of 26-OH-Chol (0.158 and 0.246 μM) and of (24S)-24-OH-Chol (0.074 and 0.107 μM) in the plasma of the two normal human subjects studied were considerable. The stereochemistry at C-25 of the 26-OH-Chol was not established. It is important to note that (24RS)-24-OH-Chol (469), (25R)-26-OH-Chol (290, 1103), and (25S)-26-OH-Chol (1103) have been shown to be highly active in lowering the levels of HMG-CoA reductase activity in cultured mammalian cells. For example, the (24RS)-24-OH-Chol caused a 50% suppression of HMG-CoA reductase activity in mouse L cells at 0.3 μM (469), and the 25R- and 25S-isomers of the 26-OH-Chol caused a comparable lowering of reductase activity at 0.26 and 0.16 μM, respectively, in the L cells (1103). The results of Kudo et al. (521) and studies by others (273, 447, 505, 1011) indicate that the levels of 26-OH-Chol in plasma (or serum) are comparable to those shown to cause a substantial reduction in the levels of HMG-CoA reductase activity in cultured cells.

Other studies, none of which employed controlled for autoxidation during processing of the samples, have reported the presence of the following oxysterols in human plasma: 5,6-epoxy-Chol (one or both isomers) (86, 122, 273, 348, 956), 7α-OH-Chol (51, 97, 121, 122, 273, 505, 722, 1011, 1156), 7β-OH-Chol (97, 122, 273, 505, 956, 1068, 1156), 7-keto-Chol (81, 122, 273, 446, 883, 956), 24-OH-Chol (121, 128, 273, 1011), 25-OH-Chol (121, 122, 273, 1011), 26-OH-Chol (121, 122, 273, 375, 446, 447, 505, 1011, 1156), 4β-OH-Chol (121, 128), and 5α,6β-dioOH-Chol (122, 273, 956). A review of earlier published studies on this matter is included in the article of Kudo et al. (521) on oxysterols in human plasma. In one early study, Smith et al. (1011) reported the presence of six oxygenated oxysterols in the sterol esters of pooled human plasma. The concentrations of the individual oxidized sterols of relatively fresh plasma were estimated to be from 5 to 106 ng/ml. Analysis of stored samples of plasma indicated that the concentrations of 4 of the 6 sterols were markedly higher, 83 and 91 times higher for the cases of 7-keto-Chol and 7β-OH-Chol. Smith et al. (1011) reported the presence of 25-OH-Chol in the ester fraction of fresh (5 ng/ml) and stored (20 ng/ml) samples of human plasma. In the study of Kudo et al. (521), which employed controls on autoxi-

### Table 1. Reported mean levels of oxysterol in plasma (or serum) in normal human subjects

<table>
<thead>
<tr>
<th>Oxysterol</th>
<th>Mean Levels of Oxysterols, mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ref. 446 (n = 8)</td>
<td>Ref. 521 (n = 2)</td>
</tr>
<tr>
<td>Ref. 122 (n = 19)</td>
<td>Ref. 273 (n = 31)</td>
</tr>
<tr>
<td>Ref. 121 (n = 8)</td>
<td>Ref. 800 (n = 1)</td>
</tr>
<tr>
<td>Ref. 41 (n = 7)</td>
<td>Ref. 375 (n = 13)</td>
</tr>
<tr>
<td>Ref. 856 (n = 7)</td>
<td>Ref. 375 (n = 7)</td>
</tr>
<tr>
<td>Ref. 524 (n = 18)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Oxysterol</th>
<th>Limits</th>
<th>Limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chol, cholesterol</td>
<td>n, no. of subjects.</td>
<td>n, no. of subjects.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Oxysterol</th>
<th>Mean Levels of Oxysterols, mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>26-OH-Chol</td>
<td>0.417 (0.202, 0.199)</td>
</tr>
<tr>
<td>25-OH-Chol</td>
<td>&lt;0.008 (0.019, 0.005)</td>
</tr>
<tr>
<td>24-OH-Chol</td>
<td>0.091 (0.159, 0.177)</td>
</tr>
<tr>
<td>22-OH-Chol</td>
<td>&lt;0.005 (0.000, 0.000)</td>
</tr>
<tr>
<td>20-OH-Chol</td>
<td>&lt;0.005 (0.000, 0.000)</td>
</tr>
<tr>
<td>19-OH-Chol</td>
<td>&lt;0.003 (0.000, 0.000)</td>
</tr>
<tr>
<td>7α-OH-Chol</td>
<td>0.027 (0.286, 0.107)</td>
</tr>
<tr>
<td>7β-OH-Chol</td>
<td>0.001 (0.008, &lt;0.0007)</td>
</tr>
<tr>
<td>7-Keto-Chol</td>
<td>0.175 (0.055, 0.030)</td>
</tr>
<tr>
<td>5α,6α-Epoxy-Chol</td>
<td>0.003 (0.059, 0.015)</td>
</tr>
<tr>
<td>5β,6β-Epoxy-Chol</td>
<td>0.003 (0.189, 0.065)</td>
</tr>
<tr>
<td>5α,6β-dioOH-Chol</td>
<td>&lt;0.006 (0.048, 0.004)</td>
</tr>
<tr>
<td>4β-OH-Chol</td>
<td>0.000 (0.000, 0.000)</td>
</tr>
</tbody>
</table>

Chol, cholesterol; n, no. of subjects.
dation during sample processing and analysis, 25-OH-Chol was not detected in the plasma of two human subjects (with limits of detection of less than 3.2 and 0.4 ng/ml in the two cases). Other workers (121, 273, 375, 447, 505) also noted the absence or extremely low levels of 25-OH-Chol in human serum or plasma. Kou and Holmes (509) were unable to detect 25-OH-Chol in normal rat plasma; however, the estimated detection limit (~10 ng/ml) was quite high.

Harik-Khan and Holmes (375) reported on the levels of 26-OH-Chol in plasma of normal subjects [7] and those [18] with “angiographically proven atherosclerosis.” The normal subjects were reported to show a mean value of 0.44 ± 0.15 (SD) μM with a range of 0.31–0.73 μM. The levels of 26-OH-Chol in 16 of the patients were reported to be within the range of “normal values” with a range of 0.18–0.65 μM. The levels of 26-OH-Chol (0.79 and 0.87 μM) in two of the subjects were considered to be elevated. The authors interpreted their results as “indicating that high 26-OHC levels cannot be a major factor in the development of atherosclerosis” (since most of the patients had “normal” levels of 26-OH-Chol). The authors reported a positive correlation between the serum levels of 26-OH-Chol and Chol. In these studies, an internal standard of biosynthetic 26-OH-{[1,2-3H]Chol (1,000 dpm}; 931 mCi/mmol) was added before saponification. The NSL were passed through a C18 Sep-Pak cartridge and then subjected to reverse-phase HPLC and then normal-phase HPLC (with detection at 210 nm). The 26-OH-Chol peak was collected, and its radioactivity was estimated. It should be noted that no other standards were used and that no precautions to avoid autoxidation were employed. It was stated that “the identity and purity of the peak in three different samples was confirmed by electron impact mass spectrometry and a comparison of the spectra with authentic 26-hydroxycholesterol. The absence of any molecular ions greater than m/z 402 and the similarity of the fragmentation patterns of the standard and the samples confirmed the purity of the peak.” GC-MS was done using a short (7 m) DB-1 column. The major weakness of this work is the lack of presentation of results demonstrating the ability of the chromatographic system employed to resolve 26-OH-Chol from other oxygenated sterols. The labeled internal standard was prepared by incubating [1,2-3H]Chol (of unstated purity) with rat liver mitochondria followed by purification of the product by HPLC. No detailed evaluation of the radiopurity was presented (or the ability of the HPLC system used to provide separations of 26-OH-Chol from other oxygenated sterols).

Breuer and Björkhem (122) described methodology for the simultaneous analysis of a number of oxysterols (7α-OH-Chol, 5α,6α-epoxy-Chol, 5β,6β-epoxy-Chol, 7-keto-Chol, 5α,6β-diol-Chol, 25-OH-Chol, and 26-OH-Chol) in serum as their TMS derivatives by GC-MS using deuterium-labeled internal standards for each of the oxy-

sterols. Their approach involved addition of BHT to blood before processing, addition of deuterium-labeled internal standards to serum, saponification (55°C for 45 min), and extraction of NSL with CHCl₃-methanol (2:1), reverse-phase column chromatography (which partially resolved the oxysterols from Chol), and finally GC-MS of the TMS derivatives of the oxysterol fraction with selective ion monitoring. Mean values for 19 normal subjects are presented in Table 1. It should be noted that the levels of the oxysterols reported in Reference 122 are erroneously presented as milligram per milliliter (rather than ng/ml) as can be noted by discussion of the results by the authors. Whereas the mean value for 26-OH-Chol was similar to that of Kudo et al. (521), the mean values for 7β-OH-Chol, 7α-OH-Chol, 5β,6β-epoxy-Chol, 5α,6α-epoxy-Chol, 25-OH-Chol, and 5α,6β-diol-Chol were considerably higher. The higher values for the latter oxysterols most probably are a consequence of their artificial generation from Chol during sample processing. Dzletovic et al. (273) subsequently reported improvements and extension to cover 24-OH-Chol and 7β-OH-Chol in the GC MS analysis of oxysterols in plasma over that described by Breuer and Björkhem (122). In this modification, BHT and the deuterium-labeled internal standards for each of the oxysterols were added to plasma. After saponification under mild conditions (22°C for 2 h), the NSL were subjected to solid-phase extraction to remove the bulk of the Chol and then analyzed by GC-MS as TMS ether derivatives. The modified procedure was applied to 31 normocholesterolemic subjects. The mean values for the various oxysterols are presented in Table 1. The mean level of 26-OH-Chol was higher in males (0.445 ± 0.100 μM) than in females (0.326 ± 0.077 μM). No effect of sex on the levels of other oxysterols or differences in the levels of Chol in males and females was noted. Some aspects of the methodology used in this study warrant further comment. The saponification was carried out at 22°C since it was found that 7-keto-Chol decomposed (under the same conditions) at 38 or 55°C. Furthermore, “when the hydrolysis time was increased from 1 to 14 h an almost 50% loss in 7-oxocholesterol was observed.” Furthermore, it was stated that “the hydrolysis procedure was always performed under an argon atmosphere since higher oxysterol concentrations were obtained when the hydrolysis was performed in air.” The quantitation of the various oxysterols was based on GC-MS analyses (involving selected ion monitoring) of the TMS ether derivatives and the use of deuterium-labeled internal standards that were added to the plasma before sample processing. No data were provided on the identity and purity of the various synthetic deuterium-labeled sterols with the exception of the statement that “the internal standards were pure as determined by GC-MS after the workup described unless otherwise stated.” The latter qualification applied to a sample of [26,26,26,27,27,27-2H₆]-5α,6α-epoxy-Chol which was re-
ported to contain ~5% [26,26,26,27,27,27-2H6]-5β,6β-epoxy-Chol “as determined by GC-MS.” No data were provided as to the determination of the location of the label in the sterols. These matters appear to be particularly important with regard to two of the deuterium-labeled oxysterols. The preparation of [23,23,24,25,25,25-3H3]cholest-5-ene-3β,24-diol was described (273). It should be noted that there can only be one hydrogen at C-25 in this sterol. No characterization of the product was reported. No data on the localization of the deuterium or the extent of deuteration were presented. The procedure described could be expected to yield [22,23,24,25-3H3]-24-OH-Chol. The selected ion monitoring of the TMS derivative of the unlabeled and labeled 24-OH-Chol was carried out at m/z 413 and 416, respectively. The ion at m/z 413 corresponds to an anticipated loss of C6H5 (corresponding to loss of C-25, C-26, and C-27) plus the loss of TMSOH. However, the origin of the loss of the TMSOH was not established (from C-3 or C-24). In the case of the deuterium-labeled 24-OH-Chol, the number and location of the deuterium atoms become important in interpretation of the GC-MS data. The location of deuterium label at or near the site of the concerned major EI-induced fragmentations is undesirable. Moreover, evidence for the precise origin of the ion at m/z 416 in the deuterated 24-OH-Chol was not presented. Potential problems also exist with regard to the nature of the d5-26-OH-Chol internal standard. It was stated that “this compound was prepared by Clemmensen reduction of kryptogenin in deuterated medium as described.” The references cited were to the basic method, as reported by Scheer et al. (905), for the synthesis of 26-OH-Chol by Clemmensen reduction of kryptogenin. The second reference was to Wachtel et al. (1175), which deals with the preparation of 3H-labeled 26-OH-Chol by Clemmensen reduction of kryptogenin in the presence of tritiated water. The paper by Dzeletovic et al. (273) does not deal with the results of an important reinvestigation of the Clemmensen reduction of kryptogenin (479) and later of the Clemmensen reduction of diosogenin (710), which demonstrated the complexity of this procedure and of previously undescribed by-products. For example, Kluge et al. (498) reported that both Clemmensen reduction of kryptogenin and Wolff-Kishner reduction of the resulting 22-ketosterol by-product led to epimerization at C-20 and C-25. As noted elsewhere (711), use of this approach requires careful attention to the purity of intermediates and products and the localization of the deuterium in the concerned sterols. It should also be noted that no assignment of the location of the deuterium was made. The authors noted that “the maximum theoretical number of deuterium atoms that can be incorporated in this molecule is 10 (in positions 15, 16, 17, 20, 22, and 23). However, molecular species with 5 atoms were most abundant. Probably these are a mixture of molecular species with 5 deuterium atoms in different positions.” Also, in the previous publication (122) on the d5-26-OH-Chol, a partial MS (m/z 400 to m/z 500) of its TMS ether derivative was presented along with comparable data on the undeuterated compound. No analysis or comparison was made; however, it is very clear that the deuterated sample gave a number of significant, discrete ions in the range m/z 470 to m/z 500, a region in which no ions were presented for the undeuterated sterol. Similarly, the deuterated sterol also gave a number of significant, discrete ions in other regions in which analogous ions were not present in the undeuterated sterol. These data raise question as to the identity and purity of the d5-26-OH-Chol.

Breuer (121) reported on the levels of several oxysterols in plasma of normal human subjects. Notable was the indication of the presence of significant levels of 4β-OH-Chol in plasma. The methodology was reported to be the same as in Reference 273. The identification of the 4β-OH-Chol was based on results of GC-MS studies of its TMS derivative and that of a synthetic standard. The mean level of the 3β,4β-dihydroxysterol in eight normal human subjects was reported to be 0.090 ± 0.010 μM. The mean levels of other oxysterols observed (i.e., 26-OH-Chol, 25-OH-Chol, 24-OH-Chol, 7α-OH-Chol, and 7β-OH-Chol) are presented in Table 1. Significant levels of 26-OH-Chol (0.410 μM), 24-OH-Chol (0.177 μM), and 7α-OH-Chol (0.092 μM) were observed. Especially notable are the very low levels of 25-OH-Chol and 7β-OH-Chol, similar to that observed by Kudo et al. (521). Brooks and Cole (128) had previously noted, in abstract form, the presence of 4β-OH-Chol in human serum. It was reported that “in some sera, 4β-OH-Chol was detected in significant amounts (~20–600 ng/ml).” This would correspond to 0.05–1.49 μM. Full details were not presented. The general procedure involved extraction, saponification, “chromatographic isolation of minor sterol fractions,” derivative formation, and GC-MS.

Lütjohann et al. (596) studied the levels of 24-OH-Chol in plasma obtained from eight human subjects. Sampling was made from brachial artery and internal jugular vein. The mean level of the 24-hydroxysterol in the arterial samples (0.147 ± 0.019 μM) was reported to be lower (P < 0.02) than that of the venous samples (0.165 ± 0.029 μM). Inspection of the graphical data presented showed major differences in two subjects, no difference in three subjects, and relatively slight differences in three subjects. The variation in individual subjects was not studied. The authors interpreted their results as demonstrating “a net flux” of 24-OH-Chol “from the brain into the circulation.” A similar arterial-venous difference was not observed in the case of 7α-OH-Chol, 7β-OH-Chol, 7-keto-Chol, 25-OH-Chol, 26-OH-Chol, 5α,6α-epoxy-Chol, 5β,6β-epoxy-Chol, or 5α,6β-dioH-Chol (although specific data on these sterols were not presented). Björkhem et al. (95) extended this research to include four additional subjects,
one of which showed no arterial-venous difference for 24-OH-Chol. However, three of the four new subjects showed a substantially higher level of 24-OH-Chol in the peripheral artery than in the internal jugular vein. For the 12 total subjects, the mean arteriovenous difference was 10.2 ± 2.8 ng/ml \( (P = 0.004) \). Sampling was also made from the hepatic vein and the brachial artery of 12 other normal subjects. The mean levels of 24-OH-Chol in the arterial samples was found to be slightly higher than that for the venous samples (mean arteriovenous difference of 7.4 ± 2.2 ng/ml; \( P < 0.006 \)), suggesting a net uptake of 24-OH-Chol by liver.

Lütjohann et al. (596) observed that the 24-OH-Chol in plasma was largely found as esters, i.e., ~71% as fatty acid esters and 11% as sulfate esters. The sulfate ester(s) was not directly determined but was estimated by determination of the sterol after treatment under conditions for the solvolysis of sulfate esters. The 24-OH-Chol in plasma (and in liver) was reported to be only the 24S-isomer on the basis of capillary GC studies. No diurnal variation in the levels of 24-OH-Chol in plasma was observed in two human subjects. The ratios of 24-OH-Chol to Chol in plasma were higher in children (157 ± 74 ng/mg) than in adults (35 ± 9 ng/mg). In contrast, the ratios of 26-OH-Chol to Chol were lower in children than in adults. It was stated that “there was little or no correlation between age and the levels of other circulating oxysterols.”

Babiker and Diczfalusy (41) reported on the levels of oxysterol in plasma from seven normal human subjects in the fasting state. The oxysterol levels were determined by GC-MS methodology as described previously from the same laboratory (273). Mean levels of nine oxysterols in plasma were reported (Table 1). 26-OH-Chol, 24-OH-Chol, and 7α-OH-Chol represented the major oxysterols present in plasma. Much lower levels were observed for oxysterols known to arise from autoxidation of Chol (e.g., 7-keto-Chol and 7β-OH-Chol).

Sevanian et al. (956) reported on plasma levels of certain oxysterols of normocholesterolemic human subjects (age, sex, and health status not specified). Methodology involved modified Folch extraction, solid-phase extraction, saponification, treatment with diazomethane, TMS ether formation, and GC-MS with selected ion monitoring. BHT was added before extraction of the plasma but not before its storage (up to 2 mo at \(-70^\circ\)C). Whereas the range of plasma Chol levels was from 171 to 222 mg/dl, the mean value was reported as 109 mg/dl. The reported mean levels of oxysterols were very high (Table 1). The number of subjects studied was not given. The only expression of variation presented was the range of values, which, for most of the oxysterols, was quite small. The levels of 7-keto-Chol, 7β-OH-Chol, 5β,6β-epoxy-Chol, 5α,6α-epoxy-Chol, and 5α,6β-dioxygen-Chol were very much higher than those reported by others. In addition to the oxysterols presented in Table 1, a high mean value (3.4 \( \mu \)M) was reported for cholesta-3,5-dien-7-one. The authors stated that “analysis of cholesterol, freshly purified by HPLC and collected under argon and then subjected to the isolation procedure described for plasma samples, indicated that formation of cholesterol oxides from cholesterol during sample work-up was below the limits of quantitation.” The representative chromatograms of standards presented were not impressive; the TMS derivative of 7α-OH-Chol was incompletely separated from the TMS ether of Chol, and the TMS derivative of 7β-OH-Chol was incompletely separated from the TMS ether of 5β,6β-epoxy-Chol. However, selective ion monitoring was used for these oxysterols. A representative chromatogram for human plasma was also not impressive. No peaks ascribable to 26-OH-Chol or 24-OH-Chol were reported.

Mol et al. (662) also studied the levels of oxysterols in plasma from normal human subjects. Mean values for 13 subjects are presented in Table 1. No values were reported for 7α-OH-Chol, 5β,6β-epoxy-Chol, 24-OH-Chol, 26-OH-Chol, or 4β-OH-Chol. Methodology cited included Folch extraction, saponification of the total lipids, silica gel column chromatography, and GC-MS analyses of the TMS derivatives of the sterols. No labeled internal standards were used in the GC-MS studies. BHT was added to the plasma before storage. Samples were stored “at \(-80^\circ\)C for up to 12 months” before analysis. It should be noted that the mean level of 7β-OH-Chol reported in this study is higher than those reported by others (41, 121, 273, 521). The reported mean level of 7-keto-Chol is considerably higher than that reported by Dzeletovic et al. (273) and Babiker and Diczfalusy (41). It is very likely that the levels of oxysterols reported by Mol et al. (662) are artifically elevated due to autoxidation of Chol during sample storage and/or processing. Treatment of the subjects with vitamin E for 4 wk was reported to have no effect on the levels of the oxysterols studied. Non-insulin-dependent diabetic subjects \( (n = 10) \) showed no differences in the levels of the oxysterols studied relative to those in the control subjects. Higher mean levels of 5α,6α-epoxy-Chol and 7-keto-Chol were reported in cigarette smokers than in the control subjects.

One recent study (890) claimed that the levels of 7β-OH-Chol were significantly associated with progression of carotid atherosclerosis. However, another study (1160) noted no association between the degree of coronary stenosis and the levels of unesterified 7β-OH-Chol (or any other oxysterol studied) in plasma. These studies are considered in more detail in section IX.

Emanuel et al. (277) investigated the levels of “Chol oxidation products” (i.e., the combination of 5α,6α-epoxy-Chol, 5β,6β-epoxy-Chol, 7β-OH-Chol, and 7-keto-Chol) in human plasma and in chylomicrons at various early times after the administration of a test meal of spray-dried powdered eggs (0.7 g/kg body wt) in the form of scrambled eggs. The Chol oxidation products and their levels
5β,6β-epoxy-Chol, 50; 5α,6α-epoxy-Chol, 90; 7β-OH-Chol, 60; and 7-keto-Chol, 30. The methodology was apparently as follows. Plasma was subjected to Folch extraction. The extracts were apparently saponified to give NSL (conditions not given) that were silylated and then analyzed by capillary GC. An illustrative chromatogram obtained from plasma samples obtained from one individual showed very poor resolution. Assignment of peaks (in order of elution) were Chol, 5β,6β-epoxy-Chol, 5α,6α-epoxy-Chol, 7β-OH-Chol, and 7-keto-Chol. However, a number of other peaks were present for which no comments were made. The separations were not such as to provide confidence for quantitation. The authors claimed increases in the “total Chol oxidation products” in plasma after ingestion of the test meal. Little or no increases were observed in plasma after the ingestion of a comparable test meal prepared from fresh eggs. In most (but not all) of the subjects, apparent peaks of “total Chol oxidation products” in plasma and in chylomicrons isolated from plasma occurred at between 2.75 and 4 h after administration of the test meal prepared from the powdered egg material. It is important to note that data beyond 4 h were not presented. In some individuals, the levels of total “Chol oxidation products” rose as much as ~15 mg/l. With the assumption of a molecular weight of 402, this would correspond to ~37 μM.

Very high levels of 26-OH-Chol in plasma from baboons have been reported in three studies from one laboratory (376, 526, 527). Hasan and Kushwaha (376) reported on the plasma levels of 26-OH-Chol in baboons on a chow diet; the animals had previously been established to be “high responders” (n = 6) or “low responders” (n = 6) to dietary Chol. The mean levels (±SE) of 26-OH-Chol in the two groups on the chow diet were reported to be 26.6 ± 9.2 and 27.9 ± 5.2 μM, respectively. The same authors also reported on the levels of 26-OH-Chol in plasma from other baboons classified as high responders (n = 9) and low responders (n = 9) to dietary Chol while maintained on a high-fat (40% of calories from lard), high-Chol (1.7 mg/kcal) diet. Even higher levels of 26-OH-Chol in plasma were reported for animals on the high-fat, high-Chol diet. The mean level in low responders (167 ± 62 μM) reported to be higher than those of the high responders (42.5 ± 2.4 μM). From the data presented, the percentages of 26-OH-Chol in Chol plus 26-OH-Chol in the low responders and high responders were 43 and 0.6%, respectively. In a subsequent study using the same methodology, Kushwaha et al. (526) studied the levels of 26-OH-Chol in young male baboons on a chow diet (0.03 mg Chol/kcal) and upon administration of a high-fat (40% of calories from coconut oil), high-Chol (1.35 mg/kcal) diet. On the basis of the response to the high-Chol, high-fat diet with respect to total plasma Chol and the levels of LDL + VLDL Chol, the animals were divided into three groups: high responders (n = 5), intermediate responders (n = 4), and low responders (n = 5). Plasma levels of 26-OH-Chol were determined at 0, 1, 3, 6, 10, 18, 26, 36, 52, 78, and 104 wk. The results were presented in graphical form only with comments in the text. The levels of 26-OH-Chol in plasma of the low responders were significantly higher than those in the high responders at 1, 3, 6, and 10 wk. The levels of 26-OH-Chol on the high-fat, high-Chol diet were higher than the mean value on the chow diet at weeks 3, 6, and 10. On changing from the chow diet to the high-fat, high-Chol diet, the level of 26-OH-Chol in plasma of low responding baboons increased and reached a maximal level (~100 μM) at 3–10 wk and thereafter decreased to ~50 μM for the remainder of the period of study. In the high responders, changing from the chow diet to the high-fat, high-Chol diet resulted in little or no effect on the levels of 26-OH-Chol in plasma, with mean values of ~30–40 μM throughout the period of study. In another study (527) from the same laboratory, the mean levels of 26-OH-Chol in the plasma of six ovariectomized baboons on a high-Chol (1.7 mg/kcal), high-fat (40% of calories from lard) diet were reported to be 9.9 ± 5.7 μM. Although the mean plasma level of 26-OH-Chol of progesterone-treated animals was not significantly different from controls, treatment with estrogen (estradiol cypionate) or the combination of estrogen and progesterone was associated with increased levels of 26-OH-Chol in plasma (21.0 ± 2.5 and 34.9 ± 6.2 μM, respectively). Plasma levels of 26-OH-Chol were reported to show a negative correlation with total plasma Chol and LDL Chol levels.

The reported levels of 26-OH-Chol in plasma from baboons in these studies are very much higher than those reported in credible studies for human subjects (Table 1). The methodology used in the baboon studies was stated to be based on the HPLC method of Harik-Khan and Holmes (375). Plasma samples were “saponified with a mixture of NaOH/methanol (1:9) for an hour at a constant pressure of 15 psi” (376). The temperature was not specified; however, in a subsequent publication (526), mention of heating under reflux was made. The saponification conditions of Harik-Khan and Holmes (375) involved methanol-3 M NaOH (9:1) at 37°C overnight with shaking. In the latter study (375), an internal standard of 26-OH-[3H]Chol was employed. In contrast, the baboon studies employed 7-keto-Chol as an internal standard that was added to the saponified mixture along with hexane and water. The mixture was centrifuged, and the hexane layer containing the 26-OH-Chol was “collected and dried under nitrogen.” The resulting sample was dissolved in methanol and “injected to a C18 reverse-phase silica column” (precise column and dimensions not given) using methanol-water (90:10) as the solvent; components were detected by absorbance at 210 nm. The identification of 26-OH-Chol was based solely on its retention time (7–8 min). However, only two other standards were mentioned: 7-keto-Chol (15 min) and Chol (60 min). The use of
7-keto-Chol as an internal standard presents notable problems. First, 7-keto-Chol could be present in the plasma sample or formed from Chol during sample storage and/or processing. Second, the addition of the 7-keto-Chol to the strongly alkaline solution could lead to its decomposition (273, 521, 603, 771). The estimation of the level of the 7-keto-Chol is of critical importance, since the quantitation of the 26-OH-Chol is based on an accurate and valid determination of the 7-keto-Chol. Also, as noted above, the mobilities of other oxygenated sterols potentially present in the sample were not established, and the chemical nature of the material with the retention time of 26-OH-Chol in this HPLC system was not established. It should also be noted that the only chromatography employed was simple reverse-phase HPLC that did not correspond to the procedures utilized by Harik-Khan and Holmes (375), i.e., solid-phase extraction, reverse-phase HPLC, and normal-phase HPLC.

In a subsequent study (190) from the same laboratory, substantially lower plasma levels of 26-OH-Chol were reported (without comment). The oxysterol levels were stated to have been measured by the same HPLC methodology as used previously (520) with the exception of a modification in which esterified oxysterols were hydrolyzed with Chol esterase. No details on this modification were provided. In this study, the high-Chol, high-fat diet contained 0.45 mg Chol/kcal and 40% of calories from coconut oil. The basal diet was low in Chol (0.03 mg/kcal) and fat (10% of total calories). The levels of 26-OH-Chol in the plasma of low responding (n = 6) and high responding (n = 6) baboons on the basal, low-Chol diet were reported as 0.130 ± 0.030 μM for the low responders and 0.126 ± 0.013 μM for the high responders. Upon changing to the high-Chol, high-fat diet, the levels of 26-OH-Chol in plasma of the low responders at 3, 6, 10, and 18 wk were reported as 0.237 ± 0.019, 0.251 ± 0.036, 0.180 ± 0.014, and 0.202 ± 0.018 μM, respectively. The levels of 26-OH-Chol in plasma from the high responders at the same time points were reported as 0.162 ± 0.011, 0.142 ± 0.008, 0.148 ± 0.019, and 0.177 ± 0.009 μM. On the high-Chol, high-fat diet, the levels of plasma 26-OH-Chol in the high responders did not differ significantly from the mean values on the basal (low-Chol, low-fat diet). In the low responders, the mean levels of 26-OH-Chol at 3, 6, and 18 wk were significantly higher than that on the basal diet. On the high-Chol, high-fat diet, the mean levels of 26-OH-Chol in the low responders were significantly higher than the high responders at 3 and 6 wk, but not significantly different at 10 and 18 wk. On the basal diet, no correlation was reported between plasma 26-OH-Chol levels and plasma lipoprotein Chol levels. On the high-Chol, high-fat diet, a significant negative correlation was observed at 3 and 6 wk between the plasma levels of 26-OH-Chol and LDL + VLDL Chol levels.

The results of recent studies of the levels of 26-OH-Chol in baboons fed either a chow diet or a high-fat, high-Chol (1.7 mg/kcal) diet by reverse-phase HPLC of the NSL followed by GC-MS (using an internal standard of deuterium-labeled 26-OH-Chol) gave results quite different from those described above (unpublished data). On the chow diet, mean plasma 26-OH levels (±SD) were 0.188 ± 0.025 μM (n = 7) in females and 0.142 ± 0.047 μM (n = 16) in males. On the high-fat, high-Chol diet, mean plasma levels for plasma 26-OH-Chol were 0.262 ± 0.070 μM (n = 6) for females and 0.294 ± 0.033 μM (n = 4) for males. From the results of all of the animals on each of the diets, a positive correlation was observed between the levels of 26-OH-Chol and Chol in plasma.

A study by Hodis et al. (389) concerned the levels of a number of oxysterols in the plasma and aorta of NZW rabbits fed either a standard rabbit chow diet or one enriched (1%) with Chol. Baseline plasma levels of a number of the oxysterols in two groups of animals on the chow diet were extraordinarily high, with total Chol oxides amounting to 12–13% (on a molar basis) of the levels of total plasma Chol. The baseline levels of individual oxysterols in plasma were ~1,000 times higher than those found by Kudo et al. (521) in plasma of normal human subjects. Extraordinarily high levels of the following sterols were reported: 7α-OH-Chol, 7β-OH-Chol, cholesta-3,5-dien-7-one (presumably, although not stated by the authors, arising from treatment of 7-keto-Chol with base), 5α,6α-epoxy-Chol, and 5α,6β-diol-Chol. Either we are dealing with an unprecedented, extraordinary species difference or a case of major artificial generation of oxysterols during processing and analysis of the samples. The distribution of the oxysterols in the plasma of the chow-fed animals was also interesting. Extraordinarily high levels of the Δ3,7-keto compound, the 5α,6α-epoxide, 7α-OH- and 7β-OH-Chol, and 5α,6β-diol-Chol (all of which can be derived from autoxidation of Chol) were observed, whereas curiously none of the 5β,6β-epoxide was detected. In another study reported by the same group (388), very high levels of oxysterols in plasma samples from NZW rabbits were reported. Mean baseline values of oxysterols for two groups of chow-fed rabbits were very similar. However, the mean values of total and individual oxysterols differed considerably from those reported previously by the same laboratory (389) for two groups of chow-fed rabbits. Hodis et al. (389) also reported that Chol feeding for 6 wk (389) or 9 wk (388) resulted in even more extraordinary levels of the same oxysterols (plus the 5β,6β-epoxide) in plasma. In one study (389), the mean level of total “oxides of Chol” was 415 μM (as compared with 81 μM for chow-fed control animals). Probucol administration was reported (388) to lower the levels of 7α-OH-Chol, 7β-OH-Chol, 5α,6α-epoxy-Chol, and 5β,6β-epoxy-Chol (but not of 5α,6β-diol-Chol) in Chol-fed rabbits. These extraordinarily high values of oxysterols in the plasma of control and Chol-fed rabbits must...
be viewed with caution since this study did not provide
determination of the extent of artifactual generation of
individual oxysterols during the storage, processing, and
analyses of the samples. Moreover, in the case of the
Chol-fed animals, it should be noted that the study in-
volved the use of unpurified Chol (USP) which was
sprayed on the diet in ethyl ether (itself very prone to
peroxide formation). The diet was allowed to dry at room
temperature and was changed only every 3 days during
the course of the study. In a third study from the same
laboratory (956), lower plasma levels of selected oxys-
sterols were reported for adult male NZW rabbits on a
rabbit chow diet. Nonetheless, the mean levels of the
following oxygenated sterols were still quite high (i.e.,
7β-OH-Chol, 0.25 μM; 7-keto-Chol, 1.0 μM; 5α,6α-epoxy-
Chol, 0.50 μM; 5β,6β-epoxy-Chol, 3.0 μM; and 5α,6β-
diOH-Chol, 0.95 μM), with a total of the above oxysterols
of 5.7 μM. Rabbits fed Chol (1% in diet) for 6 wk were
reported to have very substantially higher mean levels of
the same oxysterols, i.e., 7β-OH-Chol, 13.9 μM; 7-keto-
Chol, 29.8 μM; 5α,6α-epoxy-Chol, 28.6 μM; 5β,6β-epoxy-
Chol, 67.4 μM; and 5α,6β-diOH-Chol, 4.3 μM, with a total
oxysterol level of 144 μM. As noted above, the very high
levels of oxysterols reported by this laboratory (388, 389,
956) in rabbits on a chow diet could represent a species
difference between rabbits and humans. However, using
the same methodologies, researchers from this laboratory
also reported very high levels of oxysterols in plasma of
normcholesterolemic human subjects (956). When the
levels of oxysterols in plasma were expressed as a per-
centage of the Chol level, there appeared to be little or no
difference between humans and rabbits.

Freyschuss et al. (316) studied the levels of oxys-
sterols in serum obtained from male NZW rabbits fed
either chow diet or a chow diet supplemented with Chol
(1%) for 10 wk. The purity of the Chol or its mode of
addition to the diet were not specified. The levels of
oxysterols in serum were estimated by the GC-MS method
of Dezelovic et al. (273) as described previously. Their
results were presented as “7-oxygenated sterols” (i.e.,
combination of 7α-OH-Chol, 7β-OH-Chol, and 7-keto-
Chol) and “5,6-oxygenated sterols” (5α,6α-epoxy-Chol,
5β,6β-epoxy-Chol, and 5α,6β-diOH-Chol). Reported
values for 7-oxygenated sterols in control and Chol-fed rab-
bits were 0.16 ± 0.06 and 6.7 ± 2.6 μg/ml, respectively.
(With the assumption of a molecular weight of 400, these
mean values would roughly correspond to 0.40 and 16.8
μM, respectively.) Reported values for 5,6-oxygenated
sterols in the control and Chol-fed rabbits were 0.13 ±
0.05 and 5.9 ± 2.9 μg/ml, respectively. With the assump-
tion of a molecular weight of 402, these mean values
would roughly correspond to mean values of 0.33 and 14.7
μM, respectively. The values for the two classes of oxy-
sterol in serum from control animals appear to be con-
siderably higher than those for human plasma (Table 1)
but considerably lower than those reported by Sevanian
et al. (956) for the same strain of rabbits on a chow diet.
For example, the levels of 5,6-oxygenated sterols reported
by Sevanian et al. (956) are roughly 13 times higher than
those reported by Freyschuss et al. (316). Similarly, the
values reported for the two classes of oxysterols in serum
from Chol-fed NZW rabbits (1% Chol for 10 wk) are con-
siderably lower than those reported by Sevanian et al.
(956) for the same strain of rabbits fed Chol (1% in diet for
6 wk). For example, the mean values of the 5,6-oxygen-
ated sterols reported by Sevanian et al. (956) were ~7
times higher than those reported by Freyschuss et al.
(316). In the study of Freyschuss et al. (316), administra-
tion of ascorbic acid (500 mg/day) in the drinking water
was reported to have no effect on the levels of the two
classes of oxysterols in either control (chow-fed) animals
or Chol-fed animals.

Björkhem et al. (96) noted that while 26-OH-Chol is
present at relatively high levels in human blood, they
observed lower levels in rats and rabbits. However, no
data on this matter were presented. In a subsequent pub-
lication by Crisby et al. (225), some of the same workers
reported that the mean level of 26-OH-Chol in the serum
obtained from male NZW rabbits (3.0 kg) on a chow diet
was 3.1 ± 0.3 (SE) ng/ml (n = 12) or 7.71 ± 0.75 nM. The
value is very considerably less than that observed in
humans. Mattsson Hultén et al. (626) also reported on
the plasma levels of oxysterols in two NZW rabbits that were
fed a chow diet supplemented with Chol (1%) for 12 wk.
The source and purity of the Chol and the mode of addi-
tion to the chow diet were not presented. Control animals
(chow-fed alone) were not included in the study. The
levels of oxysterols in the two Chol-fed rabbits were
reported as follows: 7α-OH-Chol, 0.83 and 0.92 μM; 7β-
OH-Chol, 4.80 and 6.00 μM; 7-keto-Chol, 2.27 and 3.83 μM;
5α,6α-epoxy-Chol, 0.30 and 0.48 μM; 5β,6β-epoxy-Chol,
1.87 and 3.09 μM; 5α,6β-diOH-Chol, 1.59 and 2.36 μM;
24-OH-Chol, 0.12 and 0.17 μM; 25-OH-Chol, 0.17 and 0.29
μM; and 26-OH-Chol, 0.21 and 0.30 μM. It is interesting
that the levels of 24-OH-Chol, 25-OH-Chol, and 26-OH-
Chol in the Chol-fed rabbits were much higher than ob-
served in the WHHL rabbits in the same study (see below).

Stalenhoef et al. (1034) studied the levels of oxy-
sterol in plasma of three 6-mo-old WHHL rabbits fed a
“Chol-free” chow diet for 6 mo and of four 6-mo-old
WHHL rabbits fed the same chow diet containing probu-
col (10 mg/kg−1·day−1) for 6 mo. The reported levels of
oxysterols in the untreated WHHL rabbits were as fol-
loows: 7α-OH-Chol, 5.04 ± 0.33 μM; 7β-OH-Chol, 0.69 ±
0.23 μM; 7-keto-Chol, 1.41 ± 0.39 μM; 5α,6α-epoxy-Chol,
0.78 ± 0.44 μM; 5α,6β-diOH-Chol, 0.88 ± 0.10 μM; and
25-OH-Chol, 0.35 ± 0.13 μM. In the probucol-treated
animals, significantly lower levels for 7α-OH-Chol, 7-keto-
Chol, 5α,6β-diOH-Chol, and 25-OH-Chol were reported.
No significant differences were observed for 7β-OH-Chol
and 5α,6α-epoxy-Chol. The mean levels of serum Chol at 6 mo and after 6 additional months on the chow diet were 18.6 ± 1.3 and 19.0 ± 1.9 μM, respectively. The mean levels of serum Chol in the treated group before and after administration of probucol for 6 mo were 17.7 ± 2.4 and 14.2 ± 1.8 μM, respectively. The levels of 5β,6β-epoxy-Chol were not reported (nor were those for 26-0H-Chol and 24-0H-Chol). In the paper by Stalenhof et al. (1034), only a brief description of methodology was provided. Blood was collected in vacutainer tubes containing EDTA and reduced glutathione and BHT. Chol oxidation products were said to have been “determined by a modification of our previously described methodology” (1154). The latter method (1154) involved Folch extraction of total lipids that were then saponified (2 M ethanolic KOH at room temperature overnight or with heating under reflux for 30 min). The solvent for extraction of the NSL was not given. In some cases, saponification of samples was not carried out. Neutral lipids (elution with CHCl3) and sterols (elution with acetone) were separated on a silica gel column. The sterols were then fractionated by flash chromatography on a silica gel column (elution with mixtures of CHCl3 and acetone). Fraction A contained Chol. Fraction B contained 5α,6α-epoxy-Chol, 25-0H-Chol, “and the major part of 7β-0H-Chol.” Fraction C contained “the minor part of 7β-0H-Chol and 5α,6β-di0H-Chol.” Fractions B and C were analyzed by capillary GC in the form of TMS ethers (after addition of internal standards of cholestane and betulin). Mattsson Hultén et al. (626) also studied the levels of oxysterols in plasma from four WHHL rabbits maintained on a rabbit chow diet. Plasma was obtained from blood collected in the presence of EDTA and BHT. Oxysterol levels were determined by GC-MS by a published method from the same laboratory (125). Their results indicated the presence of a number of oxysterols not reported by Stalenhof et al. (1034), i.e., 5β,6β-epoxy-Chol, 24-0H-Chol, and 26-0H-Chol. The mean levels of oxysterols were as follows: 7α-0H-Chol, 0.61 μM; 7β-0H-Chol, 1.36 μM; 7-keto-Chol, 0.52 μM; 5α,6α-epoxy-Chol, 0.11 μM; 5β,6β-epoxy-Chol, 0.63 μM; 5α,6β-di0H-Chol, 0.04 μM; 24-0H-Chol, 0.01 μM; 25-0H-Chol, 0.02 μM; and 26-0H-Chol, 0.04 μM (total oxysterol, 3.34 μM). The mean levels of a number of the oxysterols in the WHHL rabbits differed in the two studies. Considerably lower levels were observed by Mattsson Hultén et al. (626) for the following sterols: 7α-0H-Chol, 7-keto-Chol, 5α,6α-epoxy-Chol, 5α,6β-di0H-Chol, and 25-0H-Chol, possibly due to a lower level of autoxidation of Chol during sample processing.

Osada et al. (735) reported on the levels of oxysterols in serum from young (4-wk-old) and adult (8-mo-old) male Sprague-Dawley rats fed either a Chol-free diet, a Chol (0.5%)-containing diet, or an oxysterol (0.5%)-containing diet for 21 days. The major component in the oxysterol mixture was 7-keto-Chol (27%) accompanied by a number of other oxidation products of Chol and unidentified materials. In the young rats on the Chol-free diet, the oxysterols detected in serum were 7α-0H-Chol (2.5 μM) and 7β-0H-Chol (29.9 μM). In the Chol-fed young rats, the same oxysterols were detected in plasma (7α-0H-Chol, 7.5 μM; 7β-0H-Chol, 19.9 μM). In the young rats on the oxysterol-containing diet, the following oxysterols were observed: 7α-0H-Chol, 24.9 μM; 7β-0H-Chol, 29.9 μM; 7-keto-Chol, 3.5 μM; 5α,6α-epoxy-Chol, 19.9 μM; 5β,6β-epoxy-Chol, 17.4 μM; and 5α,6β-di0H-Chol, 7.1 μM. In the adult rats on the Chol-free and Chol diets, the only oxysterol detected was 7α-0H-Chol at 7.5 and 14.9 μM, respectively. In the adult rats fed the oxysterol-containing diet, oxysterols observed in plasma were 7α-0H-Chol, 17.4 μM, 5α,6α-epoxy-Chol, 5.0 μM, 5β,6β-epoxy-Chol, 7.5 μM, and 5α,6β-di0H-Chol, 9.5 μM. The levels of 7α-0H-Chol in serum from rats fed the Chol-free diet appear to be very high in both young and adult rats. The level of 7β-0H-Chol in the young rats (29.9 μM) was extraordinarily high. In contrast, 7β-0H-Chol was not detected in adult rats on the Chol-free diet. In the young rats fed the oxysterol-containing diet, increased levels (relative to animals on the Chol-free diet) of the oxysterols were observed (except for 7β-0H-Chol). In the adult rats fed the oxysterol-containing diet, high levels of 7α-0H-Chol, 5α,6α-epoxy-Chol, 5β,6β-epoxy-Chol, and 5α,6β-di0H-Chol were reported. In contrast, 7β-0H-Chol and 7-keto-Chol were not detected (as was also the case for the animals on the Chol-free diet and the Chol-containing diet). It should be noted that no controls for autoxidation of Chol were included. Also, the saponification conditions would presumably destroy substantial amounts of the 7-keto-Chol. Characterization of the oxysterols was based on capillary GC. Subsequently, Osada et al. (739) reported on the levels of oxysterols in serum from young and adult male rats fed either a Chol-free diet, a Chol (0.2%)-containing diet, or an oxysterol (0.2%)-containing diet. The oxysterol mixture used was similar to that described above. In this study, the animals fed the Chol-free diet and the Chol-containing diet were pair-fed to the animals receiving the diet containing the oxysterol mixture. The mean levels of oxysterols in serum on day 20 in young rats on the Chol-free diet were as follows: 7α-0H-Chol, 3.5 μM and 7β-0H-Chol, 17.2 μM. In the young rats on the Chol diet, 7α-0H-Chol (6.5 μM) and 7β-0H-Chol (31.3 μM) were observed. Mean values of the rats on the oxysterol-containing diet were as follows: 7α-0H-Chol, 24.6 μM; 7β-0H-Chol, 24.6 μM; 7-keto-Chol, 11.8 μM; 5α,6α-epoxy-Chol, 9.2 μM; 5β,6β-epoxy-Chol, 31.3 μM, and 5α,6β-di0H-Chol, 26.4 μM. In the adult rats on the Chol-free diet, 7α-0H-Chol (1.2 μM) and 7β-0H-Chol (35.8 μM) were observed in plasma. In the adult rats on the Chol diet, 7α-0H-Chol (2.2 μM) and 7β-0H-Chol (22.6 μM) were reported. For the rats on the oxysterol-containing diet, the following oxysterols were reported: 7α-0H-Chol...
(37.3 μM), 7β-OH-Chol (23.1 μM), 7-keto-Chol (10.8 μM), 5α,6α-epoxy-Chol (9.0 μM), 5β,6β-epoxy-Chol (73.6 μM), and 5α,6β-diOH-Chol (7.8 μM). The levels of 7α-OH-Chol and especially 7β-OH-Chol in serum from rats fed the Chol-free diet appear to be quite high, and very much higher than those observed in humans in studies with attempts at control of autoxidation during sample processing. With the exception of 7β-OH-Chol, the levels of the oxysterols in serum were very much higher in animals fed the diet containing the oxysterols. The same workers (740) also reported on the levels of oxysterols in serum from 4-wk-old rats fed one of three diets: a basal Chol-free diet, the basal diet containing added Chol (0.5%), or the basal diet supplemented with Chol (0.5%) and a mixture of oxidized Chol species (0.5%). Another similar study (740) from the same laboratory gave similar results.

Nakano et al. (693), as part of a study of the effect of partial hepatectomy on the levels of 7α-OH-Chol in serum (and on levels of cyp7a activity in liver), reported levels of 7α-OH-Chol of male Fischer rats on a chow diet. The mean level of serum 7α-OH-Chol, measured by GC-MS of its TMS derivative, in nine rats was 0.178 ± 0.019 μM. This value was substantially lower than those reported by Osada et al. (739) for male Sprague-Dawley rats on Chol-free or Chol-containing (0.2%) diets, i.e., 3.5 ± 1.7 and 6.5 ± 3.0 μM, respectively. Dowling and Devery (260) studied the levels of 7α-OH-Chol in serum of control rats and rats treated with cholestyramine (3% in diet for 5 days). The nature of the diet and the strain of rats studied were not specified. The levels reported for the control and treated rats (n = 6) were 0.259 ± 0.064 and 0.591 ± 0.143 (SD) μM, respectively. BHT was added before the following general approach: Folch extraction, alkaline hydrolysis, and enzymatic conversion to 7α-hydroxycholest-4-en-3-one followed by reverse-phase HPLC (with detection at 240 nm). One HPLC result was shown that claimed the presence of 7α-hydroxycholest-4-en-3-one, cholest-4-en-3-one, and 20α-hydroxycholest-4-en-3-one. The peak for the 7α-hydroxysterol was very small, the peak for the 20α-hydroxysterol was very substantial, and the peak for the cholestenone was huge. No separation of authentic compounds was presented. No other characterizations of the materials were made. If correct, the results would indicate a very substantial level of 20α-OH-Chol in rat serum.

Thus only a very few studies have been made of the oxysterols present in plasma or serum from rats, and the only oxysterols reported to be present were known autoxidation products of Chol. None of the studies employed rigorous methodology to suppress autoxidation of Chol and/or internal controls to detect and quantify artificial generation of oxysterols during sample processing and analysis.

Rosen et al. (862) presented data on selected oxysterols in serum from C57BL/6J wild-type (n = 5) mice using the GC-MS methodology of Dzeletovic et al. (273). The levels of 7α-OH-Chol, 24-OH-Chol, and 26-OH-Chol were 1.17, 0.050 ± 0.000, and 0.199 ± 0.025 (SE) μM, respectively. Corresponding values for knockout mice (n = 6) with a disrupted sterol 26-hydroxylase gene (cyp26 −/−) were 4.98 ± 2.49, 0.075 ± 0.075, and <0.0025 μM, respectively. It should be noted that the mean levels of 7α-OH-Chol in the wild-type and mutant mice are very considerably higher than that observed in normal human subjects.

Toda et al. (1117) reported on the levels of 7-keto-Chol in plasma from female chicks on a basal diet. A mean value of 1.23 μM was reported; however, this value should be regarded with caution in view of the lack of detailed description of the methodology employed and the lack of description of precautions to suppress autoxidation of Chol during sample processing and the lack of methodology to detect and quantitate same.

Calf serum is very widely employed in a large variety of cell culture studies. In view of the actions of oxygenated sterols (and their high potencies) on a variety of cell processes, the levels of oxysterols in commercial preparations of FCS and newborn calf serum represent important matters. In an extremely large number of investigations, mammalian cells are maintained in media containing variable levels of FCS. Under these conditions, normal cells show very low levels of sterol synthesis and have low levels of activity of key regulatory enzymes in Chol biosynthesis. For example, CHO-K1 cells maintained in media containing FCS show low levels of activity of microsomal HMG-CoA reductase and of cytosolic HMG-CoA synthase and acetocacetyl-CoA thiolase (180, 650, 748). Transfer of the CHO-K1 cells to media containing delipidated FCS results in very marked increases in the levels of activity of these enzymes (180, 650, 748). Moreover, oxygenated sterols, e.g., 25-OH-Chol (180) or 3β-hydroxy-5α-cholest-8(14)-en-15-one (650, 748), at very low concentrations have been shown to block the increases in enzyme activity induced by transfer of the cells to media containing delipidated FCS. A number of other oxysterols have also been shown to be active in this respect (486, 1073, 1197, 1201). It is very probable that oxygenated steroids present in FCS are at least partially responsible for the low levels of activity of key regulatory enzymes involved in Chol biosynthesis observed in cells grown in media containing FCS. In view of this situation and the reported effects of oxysterols on a variety of cellular processes, knowledge of the chemical nature and levels of oxygenated sterols present in various preparations of fresh FCS as well as commercial preparations stored under varying conditions for varying periods of time is very important. Also worthy of study are the effects of the multiple filtrations (as well as the heat treatment in the case of certain types of serum) used in the processing of FCS by the commercial suppliers on the levels of oxysterols. In 1984, Chen (185) reviewed the role
of Chol in cellular growth and noted the variability in Chol levels in commercial samples. More importantly, he noted that TLC analysis of fresh bovine serum indicated the presence of at least one oxysterol, believed to be 7β-OH-Chol, and that prolonged storage of serum led to increases in the amounts of polar sterols. In 1990, Pie and Seillan (802) investigated the oxysterols present in commercial FCS. Their approach (801, 802) involved extraction of total lipids under Folch conditions in the presence of BHT (after addition of internal standards of cholesterol and 19-OH-Chol), evaporation of the solvent from the organic phase extracts, followed by saponification (overnight at room temperature with 1 N KOH in methanol), recovery of the NSL by extraction with diethyl ether, washing of the ether extracts with water, evaporation to dryness “under vacuum,” separation into Chol and oxysterol fractions by TLC (using hexane-diethyl ether, 70:30, as solvent), extraction of the silica gel zones with diethyl ether, evaporation of the solvent under nitrogen, and formation of TMS ether derivatives and capillary GC. Their results indicated the presence of the following oxysterols in the sample of FCS: 7α-OH-Chol (1.00 μM), 7β-OH-Chol (1.00 μM), 7-keto-Chol (4.45 μM), 5α,6α-epoxy-Chol (0.75 μM), 5β,6β-epoxy-Chol (0.20 μM), 20-OH-Chol (0.17 μM), and 25-OH-Chol (0.15 μM), with a total oxysterol of 7.72 μM. Their results suggest the presence of significant levels of oxysterols in commercial FCS. However, the high reported levels (i.e., 7.7 μM total oxysterol or ~0.95% of total sterols) should be viewed cautiously in view of the lack of an internal control to assess autoxidation of Chol, the use of procedures which could be expected to promote autoxidation, and assignments of structure based solely on GC retention times. Also, no data were presented relating to the probable decomposition of 7-keto-Chol under the saponification conditions employed. Nonetheless, their results should prompt expanded, more rigorous studies of FCS and newborn calf serum, since these materials are so extensively used in a wide variety of tissue-culture experiments. If oxysterols are present in these materials at significant levels, this situation should be noted by the large number of investigators routinely employing these materials in their research. It should be recognized that the commercial serum preparations are routinely sterilized by passage through multiple filters under conditions under which oxygen may not be excluded. Furthermore, the effect of conditions and duration of storage on oxysterol levels in these preparations should be investigated. It is very likely that various lots of serum from different suppliers may vary with regard to oxysterol composition and levels. In view of the high potencies of oxysterols on a variety of cellular processes, including cellular growth, sterol and isoprenoid synthesis, etc., knowledge of the levels of oxysterols in the serum preparations may be critical to successful, reproducible research and a meaningful interpretation of the results.

B. Possible Physiological Regulation of Levels of Oxysterols in Plasma

Relatively little is known about physiological regulation of the levels of the various oxysterols in plasma. In one study (97) the levels of unesterified 7α-OH-Chol in serum of six patients with gallstone disease were reported to be increased after treatment with cholestyramine. The levels of unesterified 7α-OH-Chol in serum of eight other patients with gallstone disease were found to be lower after treatment with chenodeoxycholic acid. The levels of unesterified 7α-OH-Chol in serum were also reported to be about five times higher in two subjects with ulcerative colitis after partial ileal resection. Thus the levels of unesterified 7α-OH-Chol appear to vary significantly under conditions known to perturb Chol and bile acid homeostasis. A limitation of this important study was the lack of controls to monitor artifactual generation of the 7α-OH-Chol by autoxidation of Chol during sample storage, processing, and analysis. For example, a typical tracing of the GC-MF analyses of the TMS derivative 7α-OH-Chol showed not only a peak corresponding to 7α-OH-Chol but also a very substantial peak attributed to its 7β-hydroxy epimer. The presence of this material in significant amounts suggests its formation from autoxidation of Chol and raises the possibility that a substantial portion of the 7α-OH-Chol in serum arose by autoxidation of Chol.

Another study (51) reported extraordinarily high levels of total (free plus esterified) 7α-OH-Chol in sera of human subjects treated with cholestyramine. The levels of 7α-OH-Chol in the subjects treated with the resin were reported to be higher in hypercholesterolemic subjects than in normal subjects. After treatment with the resin, the levels of 7α-OH-Chol varied from 0.65 to 4.98 μM in the hypercholesterolemic individuals and from 0.40 to 1.6 μM in the normocholesterolemic subjects. These values are to be compared with the mean levels of free 7α-OH-Chol in serum of gallstone patients of 0.075 ± 0.010 μM before resin treatment and 0.32 ± 0.05 μM after cholestyramine treatment. This study noted the problem of autoxidation of Chol. However, no internal controls were employed to quantitate autoxidation during sample processing (which involved saponification of plasma samples followed by extraction of the NSL with ethyl ether) and analysis.

Oda et al. (722) reported on the levels of 7α-OH-Chol in serum from patients with cholelithiasis and in patients without hepatobiliary disease (but who had gastric cancer). There were no significant differences between the two groups with regard to serum levels of total, unesterified, or esterified 7α-OH-Chol. The mean levels of 7α-OH-
Chol in cholelithiasis patients (n = 12) were as follows: total, 0.246 ± 0.106 μM; unesterified, 0.048 ± 0.020 μM; and esterified, 0.198 ± 0.090 μM. Cholelithiasis patients treated with chenodeoxycholic acid (400 mg/day) showed lower levels of 7α-OH-Chol in serum (total, free, and ester), whereas cholelithiasis patients treated with ursodeoxycholic acid (600 mg/day) showed levels of 7α-OH-Chol in serum that were not different from the untreated patients. The methodology of Oda et al. (722) involved extraction of serum after the addition of 5α-cholestan-3β,7β-diol as an internal standard and processing of the extracted sterols (with and without saponification) by solid-phase extraction and GC-MS of the TMS derivative with selected ion monitoring at m/z 456 (M-TMSOH) for 7α-OH-Chol and at m/z 458 (M-TMSOH) for the internal standard. No internal controls were used to monitor or quantitate autoxidation of Chol. The serum was stored at −20°C until analysis. No antioxidants were added. It was stated that “serum 7α-OH-Chol levels did not change significantly during storage at −20°C for at least 2 months.” Axelson et al. (30) developed an HPLC method for the determination of the levels of 7α-hydroxycholesterol-4-en-3-one in plasma. The median concentration in normal human subjects was found to be 0.030 μM with a range of 0.0075 to 0.10 μM. Much higher levels were observed in patients with ileal resection (median, 0.99 μM; range, 0.32–1.88 μM) or subjects receiving cholestyramine (median, 0.47 μM; range, 0.14–1.19 μM). Lower levels than those of normal subjects were observed in patients with extrahepatic cholestasis and in those with cirrhosis. As noted by the authors, 7α-hydroxycholesterol-4-en-3-one is not anticipated as an autoxidation product of Chol; therefore, the measurement of this oxysterol should provide more reliable plasma levels than is the case of 7α-OH-Chol with which plasma levels can be artifactualy high due to autoxidation of Chol during sample processing. In a subsequent study, Axelson et al. (31) showed a very strong positive correlation between the levels of 7α-hydroxycholesterol-4-en-3-one in plasma and the levels of liver microsomal cyp7a activity in human subjects. Yoshida et al. (1218) also reported on the levels of 7α-hydroxycholesterol-4-en-3-one in plasma of human subjects. The sterol was extracted from plasma with acetonitrile, purified by solid-phase extraction, and then treated with O-methylhydroxylamine hydrochloride followed by formation of the dimethylthelylsilyl ether derivative. The resulting methylxime-dimethylthelylsilyl derivative was analyzed by GC-MS. Essentially complete resolution of the syn- and anti-isomers of the oxime was achieved. The authors reported a correlation between the levels of the 7α-hydroxy-3-ketosterol in plasma and the levels of cyp7a activity in liver of human subjects. A correlation between the levels of the 7α-hydroxy-3-ketosterol in plasma and those of unesterified 7α-OH-Chol was also observed. However, no significant correlation was observed between the levels of the 7α-hydroxy-3-ketosterol and total 7α-OH-Chol or esterified 7α-OH-Chol. A circadian rhythm in humans for plasma levels of 7α-hydroxycholesterol-4-en-3-one was also reported. It was suggested that plasma levels of the 7α-hydroxy-3-ketosterol may be a highly reliable index of bile acid biosynthesis. Muhrbeck et al. (682) reported increased plasma levels of 7α-hydroxycholesterol-4-en-3-one in asymptomatic subjects with gallstones, with mean levels of 0.070 ± 0.009 μM in the gallstone subjects and 0.050 ± 0.007 μM in matched control subjects. Triglycerides were slightly higher in the gallstone group (1.6 ± 0.2 vs. 1.3 ± 0.1 μM); there were no differences in mean levels of total Chol, LDL Chol, HDL Chol, or 5α-cholestan-7-en-3β-ol in plasma of gallstone and control subjects. In the same study, no differences in the plasma levels of 26-Oh-Chol were observed, with mean values of 0.47 ± 0.02 μM in subjects with gallstones (n = 10) and 0.48 ± 0.03 μM in control subjects (n = 10). Kuroki et al. (524) reported that the levels of total 7α-OH-Chol in plasma are decreased in patients with cirrhosis but unchanged in patients with chronic hepatitis. The methodology used involved extraction of samples of serum stored at −20°C for unspecified periods of time followed by saponification, solid-phase extraction (to remove most of Chol), and GC-MS (with selective ion monitoring) of the TMS derivative. No antioxidants or other special precautions to suppress autoxidation of Chol were employed. The mean levels of 7α-OH-Chol in normals (n = 18) were 0.237 ± 0.097 (SD) μM. Cirrhosis patients and chronic hepatitis patients showed mean levels of 0.078 ± 0.059 μM (n = 23) and 0.262 ± 0.102 μM (n = 21). Nakano et al. (693) studied the levels of hepatic cyp7a activity and serum 7α-OH-Chol levels in male Fischer rats after partial (70%) hepatectomy. Male Fischer 344 rats were maintained on a chow diet, and sampling was done at 900 and 1030 h (on light-dark cycle). Cyp7 activity decreased on days 1 and 2, returned to basal levels on day 3, and was elevated (over basal level) on days 7 and 14. The values on days 0, 1, 2, 3, 7, and 14 were 28.3 ± 3.7, 8.5 ± 2.8, 13.3 ± 3.6, about same as day 0 (on day 3), 71.4 ± 10.9, and 51.3 ± 6.6 (SE) pmol·min⁻¹·mg protein⁻¹, respectively. The levels of 7α-OH-Chol in serum decreased from basal level (0.178 ± 0.010 μM) on days 1 and 2, about the same on day 3, and were elevated on days 7 and 14 (0.359 ± 0.066 and 0.258 ± 0.026 μM, respectively). There was a very high correlation (P < 0.0001) between the levels of hepatic cyp7a activity and 7α-OH-Chol in serum. Thus the levels of 7α-OH-Chol and of 7α-hydroxycholesterol-4-en-3-one in plasma have been employed as indicators of hepatic cyp7a activity for potential use in evaluating hepatic function and/or the effects of dietary or drug treatment on this enzyme activity as an indicator of bile acid synthesis in human subjects. Another approach toward these ends has been an assay of tritium release to body water after the intravenous administration of [7α-
underwent liver transplantation. On follow-up, 11 of 13 of the subjects either died or appeared to be an indicator of very severe liver disease; high levels of the doubly conjugated 24-OH-Chol in serum could be observed. Sulfation and glucuronidation of the 24-OH-Chol were also notable and ranged from 0.5 to 1.1 mU; respectively (assuming a molecular weight of 400). The authors noted that the presence of high levels of the doubly conjugated 24-OH-Chol in serum appeared to be an indicator of very severe liver disease; on follow-up, 11 of 13 of the subjects either died or underwent liver transplantation.

Sauter et al. (902) studied the levels of 7α-hydroxycholesterol-4-en-3-one in serum samples from human subjects with hypothyroidism (n = 19) and hyperthyroidism (n = 11). Treatment of hypothyroid patients with thyroid hormones (thyroxine, n = 8; triiodothyronine, n = 12) had no effect on the levels of the 7α-hydroxy-3-ketosterol (before and after treatment, 0.061 ± 0.045 and 0.054 ± 0.039 µM, respectively) in serum despite significant increases in the levels of LDL Chol and total Chol in serum. It should be noted that the levels of the 7α-hydroxy-3-ketosterol in serum of hyper- and hypothyroid patients did not appear to differ significantly due to the large standard deviations reported.

Gray et al. (348) reported that hypercholesterolemic individuals showed much higher serum levels of total (free plus esterified) 5α,6α-epoxy-Chol than did normal individuals. Subsequently, Björkhem et al. (86) reported that the levels of free 5α,6α-epoxy-Chol were not elevated in hypercholesterolemic individuals. However, the levels of the free epoxysterol in normal subjects were considerably higher than those reported for total 5α,6α-epoxy-Chol in other studies (122, 273) including subsequent studies from the same laboratory (122, 273).

Harik-Khan and Holmes (375) reported a positive correlation between the levels of Chol and 26-OH-Chol in serum samples of human subjects. In contrast, Kushwaha and co-workers (376, 526, 527) claimed an inverse relationship in the level of 26-OH-Chol in plasma and the level of total plasma Chol in baboons. However, another study of the plasma levels of 26-OH-Chol in baboons, using more credible methodology, showed a strong positive relationship between the plasma levels of 26-OH-Chol and Chol (unpublished results).

Björkhem et al. (95) reported a positive correlation (P < 0.001) between the levels of 24-OH-Chol and Chol in normal human subjects.

Shoda et al. (975) reported that patients with hyperlipoproteinemia (types IIa and IV) had increased levels of 7α-hydroxycholesterol-4-en-3-one in plasma relative to normolipidemic subjects. The mean levels of the 3-ketosterol in plasma from type IIa and type IV were 0.044 ± 0.002 and 0.057 ± 0.002 µM, respectively, whereas normal subjects showed a mean level of 0.034 ± 0.002 µM. As noted above, the levels of 7α-hydroxycholesterol-4-en-3-one in plasma have been taken as indicative of hepatic cyp7a activity. In the study of Shoda et al. (975), significant elevation of cyp7a activity was observed in liver biopsy samples from type IV subjects but not from type IIa subjects. Elevated levels of mRNA for cyp7a in liver biopsies were observed in both type IV and type IIa subjects.

Brooks et al. (132) noted the presence of the 5α,6α- and 5β,6β-epoxides of 24-ethyl-Chol in plasma of a patient with Waldenström’s macroglobulinemia. However, details of this work were not presented.

Arshad et al. (23) reported on the level of total “Chol oxides” in plasma of normal and diabetic human subjects. The mean levels of oxysterols in controls and diabetic subjects were 0.22 ± 0.19 and 7.70 ± 2.49 mg/l, respectively. These values would correspond to 0.55 and 19.2 µM, respectively (assuming a molecular weight of 400). The methodology was based on isolation of the NSL (using ethyl ether) followed by GC. No internal standard was...
used to detect or correct for autoxidation during sample processing. The GC conditions used were apparently capable of separating the following sterols: Chol, cholest-3,5-dien-7-one, 5β,6β-epoxy-Chol, 7α-OH-Chol, 25-OH-Chol, and 7-keto-Chol. No mention was made of other oxysterols. In a previous publication from the same laboratory (74), little or no separations between the 5β,6β-epoxide and the “5β,6α-epoxide” (presumably 5α,6α-epoxide) and between 25-OH-Chol and 7α-OH-Chol were observed.

As noted previously, the plasma levels of 24-OH-Chol in human subjects have been found to be higher in samples from the internal jugular vein than from peripheral artery (95, 596), suggesting a net flux of this oxysterol from brain. It was suggested that the level of 24-OH-Chol in blood might “serve as a marker for disturbed turnover of cholesterol in the brain.”

C. State of Esterification of Oxysterols in Plasma

Javitt et al. (446) found that ~68% of the 26-OH-Chol in serum from normal human subjects was esterified. Björkhem et al. (97) reported, on the basis of replicate analyses of one pool of human serum, that “no significant part of 7α-OH-Chol in serum is esterified under normal conditions.” Full experimental details were not presented. Oda et al. (722) subsequently observed that a substantial fraction of the 7α-OH-Chol in human serum was esterified (~75% in patients with no evidence of liver abnormalities). Whether or not the esterified 7α-OH-Chol was present as mono- or di-esters was not established. However, processing of the serum with either of two procedures to hydrolyze the steryl esters (either alkaline hydrolysis or enzymatic hydrolysis with Chol ester hydrolase) gave comparable results. With the assumption of specificity of the Chol ester hydrolase for esters at the 3β-hydroxyl moiety, these results would suggest that the bulk of the esters of 7α-OH-Chol were mono-esters (at C-3).

Very extensive esterification of an oxygenated sterol in plasma was observed in studies of the metabolism of 3β-hydroxy-5α-cholest-8(14)-en-15-one in nonhuman primates and rats. After oral administration of [2,4-3H]3β-hydroxy-5α-cholest-8(14)-en-15-one to a baboon, almost all of the labeled 15-ketosterol in plasma was found as fatty acid esters (920). The percent esterified at 4, 8, 12, 16, and 24 h after its administration was 96.1, 97.5, 98.6, 99.0, and 97.8%, respectively (920). Essentially identical results were observed in three other baboons (747). It is interesting to note that, in the same animals that also received a tracer dose of [4-14C]Chol, the extent of esterification of the labeled Chol in plasma was considerably less than that for the 15-ketosterol at all time points studied, and even at its maximum in each animal at 24 h (mean, 73.9%). After intravenous administration of [2,4-3H]3β-hydroxy-5α-cholest-8(14)-en-15-one to a baboon in pharmacological type vehicle (a mixture of PEG-400, ethanol, and saline), rapid formation of fatty acid esters of the 15-ketosterol was observed (917). By 4 h after the administration of the labeled 15-ketosterol, almost all of the compound in plasma was in the esterified state. The major ester of the 15-ketosterol in plasma at all time points studied (through 24 h) corresponded to the linoleate ester (as indicated by reverse-phase HPLC analysis). The second most abundant species of esters of the 15-ketosterol corresponded chromatographically with the oleate ester (plus palmitate ester). The predominance of the linoleate esters corresponds to the specificity of lecithin:cholesterol acyltransferase (LCAT) in this species. After intravenous administration of chyiomiconrons of intestinal lymph from rats (which received a mixture of [2,4-3H]3β-hydroxy-5α-cholest-8(14)-15-one and [4-14C]-Chol by intragastric administration) to groups of rats, almost all of the 15-ketosterol was found to be esterified (i.e., >98% at early time points of 3, 7, 10, and 20 min) (912). In contrast, the extent of esterification of the labeled Chol was less (80.4% at 3 min, 81.0% at 7 min, 82.5% at 10 min, and 70.4% at 20 min). At early time points (3, 7, and 10 min), the bulk of the 15-ketosterol was found as the oleate ester (99.5, 90.6, and 88.5%, respectively). Thereafter, the composition of the esters changed, and by 120 min, the major species of the 15-ketosteryl esters corresponded to the arachidonate (34%), linoleate (33%), and oleate (30%) species.

Dzeletovic et al. (273) presented important results with regard to the state of esterification of a number of oxysterols in plasma. In the case of the 5,6-epoxides of Chol, 5α,6β-diOH-Chol, and 7-keto-Chol, most of the oxysterols were found as free sterols. In contrast, most of the 7α- and 7β-OH-Chol, 24-OH-Chol, and 26-OH-Chol was found as esters. The form of the esters (i.e., mono- or diesters) was not studied. The percentages of the oxysterols in plasma that were found as esters were as follows: 26-OH-Chol, 91 ± 1.1%; 24-OH-Chol, 72 ± 4.4%; 7α-OH-Chol, 83 ± 5.0%; 7β-OH-Chol, 85 ± 13%; 7-keto-Chol, 38 ± 18%; 5α,6α-epoxy-Chol, 21 ± 31%; 5β,6β-epoxy-Chol, 22 ± 33%; 5α,6β-diOH-Chol, 14 ± 18%; and 25-OH-Chol, 59 ± 16%. Lüttjohann et al. (596) reported that the 24-OH-Chol in human plasma was largely found as esters, i.e., ~71% as fatty acid esters and 11 ± 2% (SD; n = 5) as sulfate esters. The methodology employed did not permit a distinction between mono- and diesters.

Smith et al. (1011) analyzed human aorta, liver, and plasma for oxysterol esters by fractionation of the total lipids on silica gel, followed by HPLC, TLC, and MS analyses in conjunction with authentic standards. Various palmitate, stearate, and oleate mono- and diesters of 7α-OH-Chol, 7β-OH-Chol, 25-OH-Chol, and 26-OH-Chol were
detected by HPLC (ultraviolet detection at 212 nm) and identified by coelution with authentic standards and/or MS (chemical ionization). Despite extensive additional analytical work, including comparisons of saponified and nonsaponified material, quantitation of the oxysterol ester levels was limited by the modest resolving power of HPLC, the complexity of the oxysterol ester mixture, and the potential presence of ultraviolet-absorbing nonsteroidal components.

D. Oxysterols in LDL

The important discovery by Brown and Goldstein that the binding of LDL to fibroblasts is defective in patients with homozygous familial hypercholesterolemia (FH) (139, 140) and the demonstration (137, 340) that LDL suppressed HMG-CoA reductase activity and sterol synthesis in fibroblasts from normal subjects but not in those from FH patients stimulated an incredible amount of research on the effects of the addition of LDL to a variety of mammalian cells. As noted in section IV A, certain oxysterols are present in plasma at physiologically relevant concentrations, and these oxysterols show high potencies in the suppression of sterol synthesis and HMG-CoA reductase activity in cultured mammalian cells. In view of this situation, knowledge of the levels of oxysterols in LDL (and other lipoprotein classes) is a subject of considerable fundamental and practical importance. This information is essential to considerations of the possibility that oxysterols present in LDL, rather than Chol, are responsible for the observed effects of the addition of LDL to cells, a subject discussed in detail previously (909). Presented below are the results of a number of studies attempting to define the oxysterol levels in native LDL from normal human subjects and a few animal species. These studies are complicated by possible autoxidation of Chol and Chol esters present in the LDL, during sample storage, processing and analysis, as noted previously for analyses of oxysterols in plasma. None of the studies on LDL reported to date has included rigorous procedures to detect and quantitate this artifactual generation of oxysterols. Moreover, determination of oxysterols in LDL also requires consideration of their artifactual formation during the procedures employed to isolate LDL for use in cell culture experiments. Chol in human LDL is present largely as its linoleate ester, a species notably susceptible to autoxidation, a process which could be markedly catalyzed by the presence of iron porphyrin components from hemolysis. Attempts to suppress autoxidation by addition of various antioxidants are dependent on their retention in LDL during the procedures used for the isolation of the LDL. These include extended ultracentrifugation (commonly in the presence of KBr), prolonged dialysis, and sterilization by filtration before use or storage. There have been no rigorous, systematic studies of the effects of these procedures on the artifactual generation of oxysterols. However, one recent study noted higher levels of certain oxysterols in LDL than in the plasma from which it was prepared (41). Moreover, the results of several studies have claimed levels of certain oxysterols in LDL that are very substantially in excess of the levels of the same oxysterols in total plasma as reported in independent, credible studies. Unfortunately, none of the former studies included rigorous internal controls to detect and quantitate the artifactual formation of the oxysterols after isolation of the LDL, during sample processing and analysis so that it is not possible to differentiate artifactual generation of oxysterols during analysis of the LDL from that arising during the isolation of the LDL. One approach that might be explored is immunoprecipitation of LDL under argon, which could provide critical information on autoxidation of Chol during individual steps in the isolation of the LDL, i.e., ultracentrifugation dialysis, and ultrafiltration.

Recently, attempts have been made to minimize oxidation of LDL (and its components) during its isolation. Kleinveld et al. (497) described a relatively short procedure for the isolation of LDL with the goal of suppressing its oxidation during sample processing. This method involved a shorter than usual ultracentrifugation time (but still involving 22 h of centrifugation), dialysis in the dark at 4°C in buffer containing EDTA which was degassed and purged with nitrogen, and, finally, ultrafiltration. Comparisons of the LDL isolated by this method and a more traditional method were essentially limited to lipid classes and fatty acid distribution. No comparisons of the levels of lipid peroxides, malondialdehyde, or oxygenated sterols were made. Viera et al. (1170) described a rapid method for the isolation and concentration of plasma LDL. The isolation of LDL was achieved by a discontinuous gradient density ultracentrifugation step (180 min) that was followed by a purification and concentration step (45 min) using ultrafiltration through a collodium bag under nitrogen. The latter step, in contrast to the usual dialysis techniques or filtration through short gel columns, was reported to suppress oxidation of the LDL. The isolated LDL showed low levels of lipid peroxides (<5 nmol/mg LDL protein) and very low levels of malondialdehyde (<0.1 nmol/mg LDL protein). Others had reported that using conventional methods for isolation of LDL, the levels of malondialdehyde in LDL as 3.6 ± 0.1 nmol/mg LDL protein. The rapid method resulted in the essentially complete loss of ascorbate and urate (water-soluble antioxidants present in plasma) but with no apparent loss of vitamin E of LDL. The levels of oxysterols in the isolated LDL were not studied.

In 1983, Brooks et al. (132) reported the presence of four oxysterols in LDL of plasma from one subject with type II familial hypercholesterolemia. 26-OH-Chol was re-
ported to be present at 0.51 μM. Notably high levels of 5α,6α-epoxy-Chol (1.23 μM), 5β,6β-epoxy-Chol (1.17 μM), and 7-keto-Chol (0.84 μM) were also reported. Subsequently, Addis et al. (3) reported the presence of six oxysterols in LDL of plasma from six subjects (Table 2).

TABLE 2. Reported mean levels of plasma (or serum) LDL oxysterols in native LDL from normal human subjects

<table>
<thead>
<tr>
<th>Oxysterol</th>
<th>Mean Levels of LDL Oxysterols, μM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ref. 446 (n = 2)</td>
</tr>
<tr>
<td>26-OH-Chol</td>
<td>0.105</td>
</tr>
<tr>
<td>25-OH-Chol</td>
<td>0.014</td>
</tr>
<tr>
<td>24-OH-Chol</td>
<td>0.082</td>
</tr>
<tr>
<td>20-OH-Chol</td>
<td>0.082</td>
</tr>
<tr>
<td>19-OH-Chol</td>
<td>1.030†</td>
</tr>
<tr>
<td>7α-OH-Chol</td>
<td>0.075</td>
</tr>
<tr>
<td>7β-OH-Chol</td>
<td>0.100</td>
</tr>
<tr>
<td>7-Keto-Chol</td>
<td>0.175</td>
</tr>
<tr>
<td>5α,6α-Epoxy-Chol</td>
<td>0.050</td>
</tr>
<tr>
<td>5β,6β-Epoxy-Chol</td>
<td>0.174</td>
</tr>
<tr>
<td>5α,6β-diOH-Chol</td>
<td>0.024</td>
</tr>
<tr>
<td>4β-OH-Chol</td>
<td></td>
</tr>
</tbody>
</table>

Oxysterol only. * Considered by authors to be erroneously high (see text). † Unesterified oxysterol only. ‡ Calculated from data of Sevanian et al. (944) as explained in text.

As part of a study of the oxidation of LDL by Cu²⁺ and lipoxygenase, Dzeletovic et al. (272) presented data on selected oxysterols in LDL in plasma (Table 2). The procedure for isolation of the LDL was a method presented by Kleinveld et al. (497). Data on 24-OH-Chol and 26-OH-Chol were not presented, but it was stated that these did not increase upon oxidation with Cu²⁺. These results on one sample of plasma can be compared with their previously published data (273) (using the same methodology) for whole plasma from 31 normal human subjects. As part of another study of the oxidation of LDL by various agents, the same laboratory (780) reported on the levels of oxysterols in a single LDL preparation. These results are also summarized in Table 2. In another study (41) from the same laboratory, mean levels of oxysterols in plasma and in LDL from seven normal human subjects were presented (Table 2). The levels of the oxysterols in LDL reported by Babiker et al. (41) are very substantially less than those reported by Addis et al. (3) and that reported for 7-keto-Chol in LDL by Dyer et al. (271). Moreover, Babiker and Diczfalusy (41) considered that only their values for 26-OH-Chol, 24-OH-Chol, and 5α,6β-diOH-Chol were valid since the sum of the levels of these oxysterols in various lipoprotein fractions was higher than the levels of the individual oxysterols in unfractonated plasma, a finding ascribed to their artifactual generation during lipoprotein isolation and/or processing.

Staprans et al. (1036) reported on oxysterol levels in β-VLDL and in LDL in fasted NZW rabbits fed a Chol-containing diet (0.33%) for 12 wk. The source and purity of the added Chol were not given, but it was reported that the diet (prepared weekly by adding the sterol in ether to the diet with evaporation of the solvent under nitrogen) contained “no detectable oxidized Chol during the feeding period.” Significant levels of the following oxysterols were reported for β-VLDL (in μg/mg Chol as read from graph): 7-keto-Chol (~3.0), 7β-OH-Chol (~2.6), 7α-OH-Chol (~1.6), 5α,6α-epoxy-Chol (~0.9), and 5β,6β-epoxy-Chol (~0.7). Significant levels of the following oxysterols were reported for LDL (in μg/mg Chol as read from graph): 7β-OH-Chol (~2.4), 7-keto-Chol (~1.6), 5β,6β-epoxy-Chol (~2.6), and 5α,6α-epoxy-Chol (~1.3). The values for the various oxysterols in β-VLDL and in LDL
appear to be quite high. For example, from the graphical presentation of the results for the level of \( \beta \)-VLDL Chol in serum (\( \sim 22 \mu M \)) and the level of 7-keto-Chol in \( \beta \)-VLDL (\( \sim 3.0 \mu g/mg \) Chol), the level of this oxysterol associated with \( \beta \)-VLDL can be calculated to be \( \sim 64 \mu M \). Similarly, from the graphical presentation of the levels of LDL Chol in serum (\( \sim 5 \mu M \)) and the level of 7\( \beta \)-OH-Chol in LDL, the level of this oxysterol associated with LDL can be calculated to be 11.8 \( \mu M \). These values were taken as indicating endogenous production of the oxysterols; however, no data were presented that preclude their artifactual generation from Chol during sample processing.

Apart from the possibility that oxygenated sterols in LDL are the important natural or artifactual constituents responsible for the effects on sterol synthesis noted above, it appears reasonable to consider that, as an excess of Chol is delivered into a cell, the enzymatic formation of oxygenated derivatives of Chol may be induced, and they then suppress HMG-CoA reductase activity and the synthesis of Chol. Reports of significant quantities of oxygenated derivatives of Chol in hepatic steryl esters in Wolman’s disease (25), the reported increased levels of (24S)-24-OH-Chol in liver after Chol feeding (889), and the effects of Chol administration on hepatic Chol 7-\( \alpha \)-hydroxylase (449, 564, 1048) are noteworthy in this respect. Moreover, recent results of Axelson and Larsson (33) with human fibroblasts have indicated the formation of 26-OH-Chol from Chol of FCS (and presumably of LDL).

A very recent study (579) claimed the first identification of 20\( \alpha \)-OH-Chol in plasma from coronary disease patients. Moreover, the authors claimed extraordinarily high levels of 20\( \alpha \)-OH-Chol in LDL and in VLDL of 23 normal human subjects and 23 individuals with proven coronary artery disease. The mean levels of 20\( \alpha \)-OH-Chol in LDL of normal subjects and patients were reported as 79.9 \( \pm 16.5 \) and 139 \( \pm 27 \mu g/mg \) protein, respectively, and those for Chol (presumably free) were 98.5 \( \pm 7.2 \) and 164 \( \pm 15 \mu g/mg \) LDL protein, respectively. The mean levels of 20\( \alpha \)-OH-Chol in VLDL of normal subjects and patients were reported as 123 \( \pm 23 \) and 112 \( \pm 22 \mu g/mg \) VLDL protein, respectively, and those for Chol (presumably free) were 76.5 and 75.2 \( \mu g/mg \) VLDL protein, respectively. The high values of the free 20\( \alpha \)-OH-Chol in LDL and VLDL in the normal subjects were reported to correspond to mean levels of 41.5 and 26.2 mg/dl, respectively (or the astounding concentrations of 1.032 and 0.652 mM, respectively). If correct, such levels of an oxysterol in LDL and VLDL would certainly warrant the most serious consideration. Assignment of structure was based on normal-phase HPLC of lipid extracts of the LDL and VLDL samples with ultraviolet detection at 208 nm. No comparisons with authentic standards were made, nor were any other spectral or chemical characterizations reported. The conclusions with regard to the identity and quantitation of the 20\( \alpha \)-OH-Chol should be regarded cautiously at best. As noted by the authors, significant levels of 20\( \alpha \)-OH-Chol in normal human plasma had not been reported by others. One detailed study of the nature of oxygenated sterols in plasma (521) of two normal subjects showed no material corresponding to 20\( \alpha \)-OH-Chol (with estimated limits of detection of \( <0.005 \) and \( <0.002 \mu M \)), observations in sharp contrast to the report of 1.032 and 0.652 mM of unesterified 20\( \alpha \)-OH-Chol in LDL and VLDL. Similarly, the claim of the presence of significant levels of cholest-4-en-3-one in LDL and VLDL of plasma of normal human subjects and the reported increase in the levels of this sterol in LDL of patients with coronary artery disease should be regarded cautiously in view of the methodology employed (simple normal-phase HPLC).

Mori et al. (673) reported on the levels of oxysterols in LDL from normal female subjects (\( n = 20 \)). Two oxysterols were detected in the LDL, 7\( \beta \)-OH-Chol and 7-keto-Chol. The mean level of the 7\( \beta \)-hydroxysterol was 0.84 \( \pm 0.09 \) nmol/mg LDL protein (or 0.21 \( \pm 0.02 \) nmol/\( \mu g \) LDL Chol). The mean level of the 7-ketosterol was 1.02 \( \pm 0.10 \) nmol/mg LDL protein (or 0.26 \( \pm 0.02 \) nmol/\( \mu g \) LDL Chol). These values correspond to mean levels of the LDL 7\( \beta \)-hydroxy sterol and 7-ketosterol of 74.3 \( \pm 7.1 \) and 92.0 \( \pm 7.1 \mu M \), respectively. The total oxysterol concentration in LDL was reported as 3.55 nmol/mg LDL protein. The nature of the other oxysterols was not presented. The methodology used in this study was as follows. Plasma (EDTA) was subjected to density gradient ultracentrifugation (NaCl). The isolated LDL was passed through a Sephadex column to remove excess salt and “the majority of the EDTA.” The LDL was stored at 4°C under nitrogen in the dark for an unspecified time and then saponified for 1 h at 45°C in the dark (with flushing with nitrogen). The sterols were silylated and subjected to GC-MS using selective ion monitoring.

Hodis et al. (390) reported the levels of oxysterols in native LDL isolated from hypercholesterolemic (mean plasma LDL Chol, 304 mg/dl) and normocholesterolemic (mean plasma LDL Chol, 52 mg/dl) cynomologus monkeys (Macaca fuscicularis). The hypercholesterolemic animals were maintained on an atherogenic diet containing 40% butter fat and Chol (0.5 mg/kcal diet; source and purity of Chol not specified) for 32 mo. The “native LDL” was separated from an electronegatively charged LDL fraction (LDL\( ^- \)) by HPLC on an ion-exchange column before analysis. Total lipids, extracted with a mixture of chloroform and methanol (containing 0.01% BHT), were subjected to solid-phase extraction, and the eluted sterols (and presumably their fatty acid esters) were subjected to cold alkaline saponification. After treatment of the resulting mixture with diazomethane, the solvent was evaporated and the residue was derivatized with N,O-bis(trimethylsilyl)trifluoroacetamide. The TMS ethers were then subjected to capillary GC analysis (no details provided as to column or conditions). The results of these analyses were
reported to show the presence of a number of oxysterols, with assignments apparently based solely on GC retention times. The results were expressed as percentages of the Chol in LDL. For the hypercholesterolemic monkeys, the mean total plasma LDL Chol level was given as 304 mg/dl, and it was stated that the native LDL represented ~95% of the total LDL. Thus the mean levels of oxysterols in native LDL should be 289 mg/dl or 7.48 μM. The values given by Hodis et al. (390) for “percent of Chol content” in LDL were used to calculate the levels of the oxysterols in native LDL. Similar calculations were made for native LDL from normocholesterolemic monkeys. The mean levels of the oxysterols in native LDL of the normocholesterolemic monkeys were as follows (in μM): 7α-OH-Chol, 1.0; 7β-OH-Chol, 8.0; 7-keto-Chol, 2.0; cholesta-3,5-dien-7-one, 9.9; 5α,6α-epoxy-Chol, 9.7; 5β,6β-epoxy-Chol, 8.5; 5α,6β-diOH-Chol, 4.8; and 25-OH-Chol, 0.6; with a total oxysterol level of 44.5 μM (3.5% of Chol level). Even more extraordinarily high levels were noted for oxysterols in native LDL of the hypercholesterolemic monkeys (in μM): 7α-OH-Chol, 62; 7β-OH-Chol, 49; 7-keto-Chol, 22; cholesta-3,5-dien-7-one, 405; 5α,6α-epoxy-Chol, 197; 5β,6β-epoxy-Chol, 70; 5α,6β-diOH-Chol, 101; and 25-OH-Chol, 28; with a total oxysterol level of 935 μM (12.5% of Chol level). The mean levels of these oxysterols reported by Hodis et al. (390) for native LDL of normocholesterolemic and hypercholesterolemic monkeys appear to be extraordinarily high. For example, it was reported that native LDL of the hypercholesterolemic animals contained the 5α,6α-epoxide, 5β,6β-epoxide, and 5α,6β-diOH-Chol at mean levels of 197, 70, and 101 μM, respectively. In the native LDL of the normocholesterolemic monkeys, the corresponding mean levels were 9.7, 8.5, and 4.8 μM. Dzeletovic et al. (273) reported that mean values for 5α,6α-epoxy-Chol, 5β,6β-epoxy-Chol, and 5α,6β-diOH-Chol in human plasma were 0.015, 0.065, and 0.064 μM, respectively. Even lower levels for the oxysterols in plasma of normal human subjects were reported by Kudo et al. (521). These values in total plasma of normal human subjects were obviously very much lower than the levels of the same sterols reported by Hodis et al. (390) to be present in the native LDL fraction of plasma from normocholesterolemic and hypercholesterolemic monkeys. It should be noted that the mean levels of 7-keto-Chol and of cholesta-3,5-dien-7-one, a decomposition product formed upon treatment of the 7-ketosterol with base, reported by Hodis et al. (390) for native LDL of normocholesterolemic monkeys were 2.0 and 9.9 μM, respectively. Thus the total level of the 7-ketosterol plus its decomposition product was 11.9 μM. The corresponding value for native LDL of hypercholesterolemic monkeys was 427 μM. These astounding high values should be compared with the levels of 7-keto-Chol in fresh samples of plasma collected (in the presence of antioxidant) from normal human subjects. For example, in a study by Dzeletovic et al. (273), the mean level of total 7-keto-Chol (free plus ester) in plasma of normal human subjects was reported to be 0.055 μM.

In another study, Sevanian et al. (951) reported on the mean levels of a number of oxysterols present in native LDL obtained from 11 normocholesterolemic human subjects (range of total plasma Chol, 160–210 mg/dl). “Native LDL” was obtained from total LDL after separation of native LDL and LDL – by anion-exchange chromatography as described above for LDL from monkeys (390). Oxysterols in LDL were estimated by capillary GC analysis of the TMS derivatives of the sterols. The results of these analyses of the native LDL were presented as percentage of total Chol (±SE): 7α-OH-Chol, 0.08 ± 0.02; 7β-OH-Chol, 0.06 ± 0.02; 7-keto-Chol, 0.47 ± 0.11; 5α,6α-epoxy-Chol, 0.28 ± 0.10; 5β,6β-epoxy-Chol, 0.33 ± 0.17; 5α,6β-diOH-Chol, 0.03 ± 0.02; and 25-OH-Chol, 0.02 ± 0.009. The authors did not present the levels of Chol in total LDL or in native LDL. The range of total plasma Chol was reported as from 160 to 210 mg/dl. If one assumes a mean value of total plasma Chol of ~185 mg/dl and a mean value of total LDL Chol of ~145 mg/dl and that 95.5% of the total LDL Chol is associated with native LDL, then the approximate mean levels of individual oxysterols in the native LDL can be estimated to be as follows (in μM): 7α-OH-Chol, 2.9; 7β-OH-Chol, 2.2; 7-keto-Chol, 16.9; 5α,6α-epoxy-Chol, 10.0; 5β,6β-epoxy-Chol, 11.8; 5α,6β-diOH-Chol, 1.1; and 25-OH-Chol, 0.7; with a total oxysterol level of 45.6 μM. It should be noted that these levels of oxysterols associated with native LDL are far in excess of the levels of the same oxysterols reported by others for total plasma (41, 273, 521) and for LDL (41).

E. Oxysterols in LDL Modified by Oxidation

It has been proposed that oxidative modifications of LDL increase its atherogenicity and that the resulting oxidized LDL, and not native LDL, plays a pivotal role in the development of foam cells of the fatty acid streak and its progression to more mature forms of the lesion. This subject has been reviewed by Steinberg et al. (1041, 1042, 1044) and others (156, 252, 372). The formation of oxidized LDL has been reported after incubation of LDL with a variety of cell types including cultured endothelial cells, monocytes, macrophages, and smooth muscle cells. These cells constitute the major cell types in the arterial wall. The bulk of the foam cells in fatty streaks is believed to be derived from circulating monocytes taken up beneath the endothelium and, to a lesser extent, from smooth muscle cells. The oxidation of LDL by incubation with cells results in marked changes which, as summarized by Steinberg et al. (1044), include an increased lyssolecithin content; a decreased content of polyunsaturated fatty acids associated with an abundance of products of the peroxidation of these acids; a fragmentation of
apoprotein B100 and decreased levels of lysine, histidine, and proline; an increased negative charge; and an increased density. In addition, as noted below, the oxidized LDL has been claimed to have an increased content of oxysterols. LDL modified by oxidation also exhibits a number of changes in biological activity, including an increased rate of uptake through the acetyl LDL receptor or the “scavenger” receptor leading to the formation of foam cells; a reduced rate of uptake into cells via the LDL receptor; increased chemotactic activity for circulating monocytes, but not neutrophils; a marked cytotoxicity; an increased secretion of apoE from macrophages; and an increased message level for apoE. Similar changes in the properties of LDL have also been reported after oxidation of LDL with copper sulfate. The oxidized LDL from cell incubations and from chemical oxidation appear to be commonly considered as equivalent. The oxidized LDL preparations obtained by incubation of LDL with Cu²⁺ or with cells are very extensively changed and represent complex chemical mixtures. In almost all cases, the oxidized LDL preparations in the culture media (or after incubation with Cu²⁺) were analyzed or utilized directly. In only a few cases has centrifugation been used to isolate the modified LDL before analysis or utilization in biological experiments. Some workers believe a less modified form of LDL to be potentially of more physiological importance. This form(s) is termed minimally modified LDL (MM-LDL) and is defined operationally (and differing from the forms of oxidized LDL noted above) by its retention of efficient uptake by the LDL receptor and its lack of uptake by the scavenger receptor (68, 833). MM-LDL has been prepared by brief oxidation of LDL with Fe²⁺ or by prolonged storage at 4°C (68). Relatively little information is available concerning the chemical composition of MM-LDL.

The oxidative changes in the modified LDL and in MM-LDL are associated with significant changes in the levels of oxysterols. In reviewing the general area of oxidized LDL, Steinberg et al. (1044) briefly noted that “LDL cholesterol is also oxidized during LDL modification, which could enhance its cytotoxicity and atherogenicity.” However, despite this suggestion and the very high biological potencies of oxysterols and the suggestions of the possible role of oxidation products of Chol in the pathogenesis of atherosclerosis (676, 794), relatively little attention has been directed toward studies of the oxysterols in modified LDL.

Two early studies (452, 1221) concerned the formation of oxysterols during the chemical oxidation of LDL with Cu²⁺. In one study (452), LDL was incubated with CuSO₄ (2.5 μM) for 5, 8, and 24 h at 37°C. It was concluded that “oxidation of LDL produced a series of oxidative products of cholesterol as revealed by gas-liquid chromatography. The major oxidative product was 7-keto-cholesterol.” One component was “tentatively identified” as 22-OH-Chol on the basis of its GC behavior (although the formation of this sterol by chemical oxidation of Chol is unlikely). Substantial amounts of other components, ascribed to oxidation products of Chol, were observed but were not identified. The GC data were interpreted as showing very high levels of oxygenated sterol in the chemically oxidized LDL with, in one experiment, values of 335, 243, and 117 μg/mg LDL protein for Chol, 7-keto-Chol, and “remaining sterols,” respectively. However, assignments of structure based solely on comparison with the retention times of a limited number of authentic standard samples (and not including several probable oxidation products of Chol) using a single GC condition permitting only incomplete resolution of two markedly different sterols (i.e., Chol and 7-keto-Chol) can only be taken as, at best, suggestive. The same group (452) also reported on “the oxysterol content of LDL that had been modified in a cell-oxidation system using human monocyte-derived macrophages.” In two experiments, it was claimed that “20% and 32% of total lipoprotein sterol was converted to the oxysterol, 7-ketocholesterol, after the macrophage oxidation of LDL.” Other than the provision of the incubation conditions, no other experimental details were given. Moreover, the interpretation of these experiments is extremely difficult for the reasons noted above. However, these results, if correct with respect to the identification of the 7-keto-Chol, would indicate very substantial levels of the oxygenated sterol in modified LDL. The macrophages were incubated with 100 mg/ml LDL. With the assumption of normal levels of Chol in the LDL, this would yield 7-keto-Chol at a level of ~160 μM (or ~640 μg/mg of LDL protein). Such an extraordinarily high level of the oxysterol could, by itself, provide a ready explanation for the widely observed cytotoxicity of oxidized LDL. Because extensive degradation of 7-keto-Chol occurs under the alkaline saponification conditions (30 min at 70°C) employed by the authors, the true level of the 7-keto-Chol could be very substantially higher. In another early study (1221), LDL prelabeled with [³H]Chol linoleate was incubated with CuSO₄ (5 μM) for 5 or 20 h at 37°C. TLC of the lipids recovered from the LDL after 5 h indicated decreases (relative to control LDL) with time in the amount of ³H with the mobility of Chol linoleate and the formation of labeled polar material. The results of enzymatic analyses of control LDL and of oxidized LDL (5 and 20 h) were interpreted as showing decreases in both free Chol and total Chol, which were much greater in the latter component, suggesting much more marked decrease in the Chol esters. Capillary GC analysis of the polar material, obtained after TLC and hydrolysis, showed six components more polar than Chol. Two were said to have the same mobility as standards of 5,6-epoxy-Chol (stereoisomer of epoxide not specified) and 7-OH-Chol (stereoisomer of OH at C-7 not specified). The GC system was stated to be incapable of the resolution of the 7-OH-
Chol and 25-OH-Chol. One peak was said to have the chromatographic mobility of 7-keto-Chol. The peak with the GC behavior of “7-OH-Chol” and 25-OH-Chol was said to yield “a fragmentation identical to 7-OH-Chol.” The authors stated that the predominant sterol in oxidized LDL was 7-keto-Chol. Quantitative data on oxysterol concentrations in the treated LDL were not presented. Thus both of these early studies suggested the formation of very substantial amounts of oxysterol upon oxidation of LDL with Cu²⁺, and both suggest 7-keto-Chol as a major product. A number of other subsequent studies have indicated the presence of substantial amounts of 7-keto-Chol in LDL after oxidation with Cu²⁺ (66, 134, 181, 201, 272, 410, 516, 609, 612, 673, 1092).

Maor and Aviram (612) observed that oxidation of LDL, previously labeled with [³H]Chol linoleate, in the presence of CuSO₄ (10 μM) for 24 h at 37°C, led to the formation of labeled 7-keto-Chol (both free and esterified) as the major oxysterol detected. Identification of the 7-keto-Chol was limited to TLC and HPLC. The level of the labeled ³H in the oxidized LDL ascribed to fatty acid esters of 7-keto-Chol was less than that ascribed to the free 7-keto-Chol. Hughes et al. (410) reported on the levels of free oxysterols in LDL that had been subjected to oxidation with cupric sulfate (5 μM) for 24 h at 37°C. Reported levels were as follows (in μM): 5β,6β-epoxy-Chol, 2.0; 5α,6α-epoxy-Chol, 0.5; 7-keto-Chol, 10.8; 7α-OH-Chol, 1.5; and 7β-OH-Chol, 2.5. The levels of oxysterols present as esters were not studied. Characterization of the various oxysterols was made on the basis of GC-MS (retention times and partial MS data with comparisons with authentic standards).

Malavasi et al. (609) reported studies of the oxidation of human LDL by CuSO₄ (20 μM; 37°C). Material with the chromatographic (TLC) behavior of the 7-hydroperoxides of Chol was detected in the oxidized LDL but not in native LDL. This material was reduced with NaNBH₄ to give equal amounts of 7α-OH-Chol and 7β-OH-Chol, thus indicating the presence of both the 7α- and 7β-hydroperoxides of Chol in the oxidized LDL. Although quantitative data were not presented, it was stated that the concentration of the 7-hydroperoxide decreased with time of oxidation, which was accompanied by increases in the levels of 7α-OH-Chol, 7β-OH-Chol, cholesta-3,5-dien-7-one, and 7-keto-Chol. The only detectable (by TLC and GC-MS) sterol in native LDL was free and esterified Chol, and it was only after oxidation with CuSO₄ that oxysterols were observed. The limits of detection were not presented. The same laboratory (166) also reported the presence of 5-hydroperoxy-5α-cholest-6-en-3β-ol in human LDL subjected to oxidation with CuSO₄ (20 μM) for 8 h at 37°C. Evidence for structure was based on its chromatographic behavior upon reverse-phase HPLC with particle beam-electron impact MS and GC-MS of the TMS derivative of the product of the reduction of the putative 5-hydroperoxide with sodium borohydride. Subsequently, Chisholm et al. (201) reported the presence of 7β-hydroperoxycholest-5-en-3β-ol in human LDL subjected to oxidation with cupric sulfate (2–6 μM). Native or oxidized LDL was lyophilized and then extracted three times with acetone using brief sonication. The extracts were subjected to reverse-phase HPLC. A polar fraction was then subjected to normal-phase HPLC. A major polar fraction obtained therefrom was identified as 7β-hydroperoxycholest-5-en-3β-ol on the basis of its ¹H- and ¹³C-NMR spectra and sodium borohydride reduction to give a product whose TMS derivative corresponded to that of authentic 7β-OH-Chol by retention time on GC and MS by positive ion chemical ionization. No comment was made regarding the previous results of Malavasi et al. (609), indicating the presence of both the 7α- and 7β-hydroperoxides of Chol in LDL oxidized by Cu²⁺. Brown et al. (135) noted the formation of the 7α- and 7β-hydroperoxides of Chol upon oxidation of human LDL with cupric chloride (20 μM) at 37°C. The β-isomer was the major isomer formed. These results were obtained from the oxidized LDL subjected to saponification (at low temperature to suppress decomposition of the hydroperoxides) and thus represent total sterol and not unesterified sterol as in other publications. The 7-hydroperoxides were the major initial oxysterols detected upon Cu²⁺ oxidation of LDL; at later times, 7-keto-Chol and, to lesser extents, 7α-OH-Chol and 7β-OH-Chol were the major oxysterols detected. Structure assignments were based solely on HPLC.

Colles et al. (211) noted that oxidation of LDL in the presence of cupric sulfate led to the formation of material with the expected TLC behavior of 7α-OH-Chol, 7β-OH-Chol, 7-keto-Chol, a 5,6-epoxy derivative of Chol, 7α-hydroperoxy-Chol, and 7β-hydroperoxy-Chol. The latter sterol was the major oxidation product under the conditions studied. It is important to note that evidence for structure assignments was limited solely to TLC. Dzeletovic et al. (272) studied the oxidation of LDL, catalyzed by Cu²⁺ or soybean lipoxygenase, as a function of time. Oxidation of LDL with soybean lipoxygenase was carried out in the presence of added linoleic acid at 37°C at pH 7.4. Oxysterols were studied by GC-MS using their previously described methodology (273). In the Cu²⁺ oxidation (5 μM CuSO₄ at pH 7.4 for 0.5 to 24 h) of LDL, the level of linoleic acid (relative to stearic acid) decreased very rapidly and substantially and fairly closely corresponded to an increase in absorbance at 234 nm (due to the formation of conjugated dienes). The electrophoretic mobility of the LDL appeared to be unchanged at 0.5 h, slightly increased at 1 h, and progressively increased at each time point studied until 24 h. The free Chol levels appeared to decrease slightly. However, the levels of esterified Chol dropped markedly (detectable at 2 h and maximal, i.e., ~50% drop at 8 h). 7-Oxgenated sterols (sum of 7α-OH-Chol, 7β-OH-Chol, and 7-keto-Chol) increased perceptibly by 1 h and in-
increased to a maximum of \( \sim 65 \, \mu g/ml \) by 8 h. 5-Oxygenated sterols (sum of the \( \Delta^5 \)-6,6a-epoxide, the \( \Delta^5 \)-6,6\( \beta \)-epoxide, and \( \Delta^5 \)-6,6\( \beta \)-diOH-Chol) increased perceptibly by 2 h and reached a maximum (\( \sim 20 \, \mu g/ml \)) at 12 h. “There was a negligible formation” of 24-OH-Chol and 26-OH-Chol. The major oxysterols formed were 7-oxygenated sterols. 7-Keto-Chol constituted 60–70% of the 7-oxygenated sterols. 7a-OH-Chol and 7b-OH-Chol were formed in approximately equal amounts. The 5-oxygenated sterols “constituted 20–30% of the total amount of oxysterols determined.” The formation of Chol 7-hydroperoxides was followed by TLC. The hydroperoxide spot “was barely detectable” at 1 h “and the intensity increased up to 8 h and thereafter decreased (12 h) and was absent at 24 h.” Oxidation of LDL with lipoxigenase also gave a mixture of mostly 7-oxygenated and 5-oxygenated sterols. With the enzymatic oxidation, it appeared that more 7-oxygenated sterols (relative to 5-oxygenated) were formed when compared with the case of the Cu\( ^{2+} \) oxidations. Esterified Chol was oxidized to a considerably larger extent than free Chol. Portions of the incubation products from the oxidation of LDL with Cu\( ^{2+} \) and lipoxigenase were subjected to TLC, and the bands corresponding to the Chol hydroperoxides were eluted and reduced with sodium borohydride. The major product (>90%) was 7b-OH-Chol (as studied by GC-MS). The other products were 7a-OH-Chol and an unidentified cholestenediol. Chol 7a-hydroperoxide, prepared by chemical synthesis, was carried through the analytical procedures and was reported to be quantitatively converted to 7-keto-Chol and 7a-OH-Chol (3:1 ratio).

Breuer et al. (124) reported on the oxysterols present in LDL subjected to oxidation with CuSO\(_4\) (5 \( \mu M \)) at pH 7.4 at 37°C for 0.5 to 24 h. Substantial formation of 7-keto-Chol, 7b-OH-Chol, and 7a-OH-Chol was reported. Other oxysterols formed in significant amounts were 5b,6b-epoxy-Chol and (in lesser amounts) 5a,6a-epoxy-Chol. Oxysterols formed in very low amounts were 5a,6b-diOH-Chol and 5a,6a-diOH-Chol (in approximately the same amounts, i.e., \( \sim 1.2 \, \mu M \) by 12 h) and 4b-OH-Chol and 4a-OH-Chol (in approximately the same amounts, i.e., \( \sim 0.5 \, \mu M \) by 4 h). Brown et al. (134) studied the oxysterols present in LDL after oxidation in the presence of cupric chloride (20 \( \mu M \)) at 37°C for 1 to 48 h. Methodology involved capillary GC of the sterols (as TMS ethers), with analyses with and without saponification (20% KOH in methanol, room temperature for 3 h in the presence of ethyl ether). Identifications were based solely on GC retention times. Any cholest-3,5-dien-7-one detected was ascribed to base-catalyzed decomposition of 7-keto-Chol in the saponification step. It should be noted that the separation of cholest-3,5-dien-7-one and the TMS derivative of 7b-OH-Chol was very slight under the conditions employed, a situation that complicates the quantitation of either of these compounds. The major oxysterol formed in the Cu\( ^{2+} \) oxidation of LDL was reported to be 7-keto-Chol. Other oxysterols detected were 7b-OH-Chol, 7a-OH-Chol, 5b,6b-epoxy-Chol, 5a,6a-epoxy-Chol (lower levels than the 5b,6b-epoxide), and a sterol believed to be cholest-4-ene-3,6b-diol (designated as 6b-hydroxycholesterol). These oxysterols were reported to be present both as free sterols and as fatty acid esters. The proportion of the oxysterol that was esterified was reported to be high for 7-keto-Chol, 7b-OH-Chol, and 7a-OH-Chol. In the case of the 5,6-epoxides, the amounts in the free and esterified states were roughly comparable. Sterols not detected in the oxidized LDL were 22-OH-Chol, 25-OH-Chol, 26-OH-Chol, and cholest-4-en-3-one. Mori et al. (673) studied the formation of oxysterols after incubation of human LDL with CuCl\(_2\) (8 \( \mu M \); 30°C) for varying periods of time (1–20 h). By 2 h, clear increases in 7b-OH-Chol and 7-keto-Chol were observed and were very substantial by 4 h. The 5a,6a-epoxide was increased to a lesser extent (increases at 2, 4, and 20 h). The authors reported formation of “6b-OH-Chol” and 4b-OH-Chol. The levels of the latter two sterols were very much less than those of the other sterols. Not measured were the 5b,6b-epoxide (too close to 7b-OH-Chol) and 7a-OH-Chol (too close to Chol). Methodology involved GC-MS of TMS derivatives. The designation “6b-OH-Chol” was unfortunate, since correspondence with one of the authors indicated the compound in question was considered to be cholest-4-ene-3,6b-diol on the basis of similarity of the MS of the bis-TMS derivative with that from a MS from the NBS data library. Patel et al. (780) reported detailed studies of the formation of oxygenated sterols upon oxidation (8.5 h at 37°C) of human LDL with Cu\( ^{2+} \) (20 \( \mu M \)), myoglobin (5 \( \mu M \)) and 3-morpholinosydnonimine-N-ethylcarbamide (1 mM). The latter compound decomposes to form nitric oxide (NO) and superoxide, which have been suggested to form peroxynitrite. Substantial levels of the following oxysterols were formed under each of the three oxidation conditions: 7a-OH-Chol, 7b-OH-Chol, 7-keto-Chol, 5a,6a-epoxy-Chol, 5b,6b-epoxy-Chol, and 5a,6b-diOH-Chol.

Information on oxysterols in MM-LDL is very limited. In one study (68), MM-LDL (prepared by prolonged incubation of LDL at 4°C) was analyzed for oxysterols by capillary GC. Despite the fact that autoxidation of Chol is expected to give a spectrum of oxidation products, data were reported only for nonesterified 5a,6a-epoxy-Chol (and not for the 5b,6b-epoxide, 7-keto-Chol, 7a- and 7b- OH-Chol, 25-OH-Chol). Apparently, only the nonesterified sterols were examined despite the fact that a very substantial fraction of many of the oxysterols in LDL is in ester form (see above). Levels of the epoxide were reported to be \( \sim 1 \) and \( \sim 5.5 \, \mu g/mg \) of Chol for “fresh” and MM-LDL, respectively. The reported findings suggest that substantial autoxidation of Chol occurred during the isolation and/or analysis of the oxysterols of the “fresh” LDL. Moreover, if the identification of the 5a,6a-epoxide in
MM-LDL is correct, then it would be unexpected for this oxysterol to be present alone. Carpenter et al. (159) reported the formation of 7β-OH-Chol upon incubation of a Chol linoleate-BSA complex with human monocytes for 24 h at 37°C. Only the extracellular medium was studied. The formation of the 7β-OH-Chol by the monocytes was reported to be markedly suppressed by BHT, probucol, and α-tocopherol and partially inhibited by EDTA. Bhadra et al. (74) studied the oxygenated sterols recovered after incubation of LDL with human endothelial cells. Analyses of the cells plus medium after 48 h of incubation showed only an unresolved mixture of the 5α,6α- and 5β,6β-epoxides of Chol. It was reported that the 5α,6α-epoxide, after purification by TLC, was identified by capillary GC analysis and GC-MS. The published mass spectra of the isolated material and of an authentic sample of the 5α,6α-epoxide were not identical. Brown et al. (135) reported the formation of 7β-Keto-Chol, 7α- and 7β-OH-Chol, and the 7α- and 7β-hydroperoxides of Chol upon incubation of human LDL with mouse peritoneal macrophages. Structure assignments were based solely on HPLC.

Hodis et al. (390) studied the oxysterol composition of an electronegatively charged LDL (LDL2−) from hypercholesterolemic cynomolgous monkeys. LDL2− has been reported to be similar to LDL modified by in vitro chemical oxidation. Extraordinarily high levels of oxysterols were reported in LDL2−. The oxysterols included 7α-OH-Chol, 7β-OH-Chol, 7-keto-Chol, cholesta-3,5-dien-7-one, 5α,6α-epoxy-Chol, 5β,6β-epoxy-Chol, and 5α,6β-dioxy-Chol, and with a total oxysterol level of ~300 μM. The same laboratory (951) also reported on the mean levels of a number of oxysterols in LDL2− obtained from 11 normocholesterolemic human subjects. Mean levels of the oxysterols (expressed as a percentage of Chol) were as follows: 7α-OH-Chol, 0.46; 7β-OH-Chol, 0.55; 7-keto-Chol, 1.94; 5α,6α-epoxy-Chol, 0.66; 5β,6β-epoxy-Chol, 0.69; 5α,6β-dioxy-Chol, 0.19; and 25-OH-Chol, 0.17, with a value for total oxysterols of 4.66%. The mean percentage of LDL2− of total LDL was reported as 4.5%.

In consideration of the possible actions of oxygenated sterols in oxidized LDL, it is important to consider the potency of the concerned oxysterols in the regulation of sterol biosynthesis and the levels of HMG-CoA reductase activity. As noted above, the major oxygenated sterols found in these LDL preparations include 7-keto-Chol, 7β-OH-Chol, and 7α-OH-Chol, and the 5β,6β-epoxide of Chol, accompanied by lower levels of the 5α,6α-epoxide derivative of Chol and even lower levels of 25-OH-Chol and 5α,6β-dioxy-Chol, 4β-OH-Chol, and 4α-OH-Chol. With the exception of 25-OH-Chol, these sterols are not among those oxysterols that show high potency in lowering HMG-CoA reductase activity. Finally, some general considerations are in order with regard to the levels of oxysterols in LDL samples modified by oxidation. Because of the very significant interest in oxidized LDL as a potentially important species in the pathogenesis of atherosclerosis, a very large number of recent and current investigations involve studies of the effects of oxidized LDL on various cells in culture. In most cases, the LDL samples used are minimally characterized, typically by measurement of TBARS and/or electrophoretic mobility. The studies outlined above indicate that very substantial levels of oxygenated sterols exist in LDL samples subjected to chemical or cellular oxidation. In view of the significant biological actions of various oxygenated sterols on cultured cells (as reviewed herein) and the reported high levels of the oxygenated sterols, serious attention should be directed toward the determination of the chemical nature and levels of the oxysterols present in the various oxidized LDL samples used in the investigations. The oxidized LDL samples clearly represent mixtures of a variety of biologically active species, of which the oxysterols appear to be potentially important. It would appear that, to understand the effects of oxidized LDL on various processes in cells and to allow for comparisons of results obtained in different investigations, definition of the chemical nature and levels of the oxysterols, as well as other components, present in the oxidized LDL samples is in order.

F. Oxysterols in Other Lipoprotein Fractions

In 1983, Brooks et al. (132) reported the presence of four oxysterols in VLDL, LDL, and HDL of serum from one subject with type II hypercholesterolemia: 5α,6α-epoxy-Chol, 5β,6β-epoxy-Chol, 7-keto-Chol, and 26-OH-Chol. The oxysterols were studied by GC-MS of the TMS ethers. The reported values of oxysterols (in ng/mg Chol) would correspond to the following approximate concentrations (in μM): 5α,6α-epoxy-Chol, VLDL (0.06), LDL (1.23), HDL (0.33); 5β,6β-epoxy-Chol, VLDL (0.06), LDL (1.17), HDL (0.25); 7-keto-Chol, VLDL (0.02), LDL (0.84), HDL (0.08); and 26-OH-Chol, VLDL (0.01), LDL (0.51), HDL (0.25). Thus, for each of the oxysterols studied, the majority was in LDL. Addis et al. (3) reported the distribution of six oxysterols (5α,6α-epoxy-Chol, 5β,6β-epoxy-Chol, 5α,6β-dioxy-Chol, 7-keto-Chol, 7β-OH-Chol, and 25-OH-Chol) in lipoprotein fractions (VLDL, LDL, and HDL) from six human subjects in the fasting state. High levels of the “total oxysterols” were reported in VLDL (2.4 μM), LDL (10.4 μM), and HDL (4.1 μM). Very high levels of several oxysterols were reported in the lipoprotein fractions. For example, the sum of the reported oxysterol levels in the 3 lipoprotein fractions were as follows: 5β,6β-epoxy-Chol (7.5 μM), 5α,6α-epoxy-Chol (6.3 μM), and 7-keto-Chol (2.3 μM). These levels far exceed the levels of the same oxysterols in unfractionated plasma from normal human subjects in studies with serious attention to suppression of autodigestion of Chol during sample processing (41, 521).
The methodology employed was based on capillary GC analyses of the oxysterols. The conditions used in sample processing (i.e., the use of ethyl ether for extraction, lack of added antioxidants, and the lack of procedures to exclude oxygen other than flushing with nitrogen), the lack of an internal control to detect and quantitate autoxidation of Chol, and the incomplete separation of various oxysterols on GC analysis do not lead to confidence in the results presented.

Dyer et al. (271) studied the levels of 7-keto-Chol in lipoprotein fractions prepared from serum samples of 10 “healthy male volunteers.” Mean values of 7-keto-Chol were as follows: VLDL, 0.66 ± 0.49 mM; LDL, 0.58 ± 0.38 mM; and HDL, 1.17 ± 0.55 mM, with a sum for all fractions of 4.38 mM. It appears that there is an error in the units which should, in fact, be micromolar rather than millimolar. The methodology was based on capillary GC analyses of the TMS derivatives of the oxysterols (after lipid extraction, saponification, and extraction of NSL). EDTA, BHT, and phenylmethylsulfonyl fluoride were added to serum before processing. “Serum was stored at −20°C until separated by ultracentrifugation (within 10 days).” Apart from the initial inclusion of antioxidant and flushing with nitrogen at various stages, no rigorous exclusion of oxygen was made during the processing of the serum samples. Regarding the possibility for artifactual generation of 7-keto-Chol by autoxidation of Chol during the ultracentrifugation steps, the authors stated “there was no difference in concentration of 7-keto-Chol in samples left to stand at 20°C for 4 days (equivalent to the time and conditions of ultracentrifugation) and processed immediately.” The authors attempted to determine the artifactual generation of 7-keto-Chol from Chol due to “the extraction” of the LDL samples. [3,4,13C]Chol (1 mg) was added to eight duplicate LDL samples (amount of LDL or LDL Chol was not specified). “In four samples this standard was added before, and in four samples, after lipid extraction.” The TMS derivatives of the samples were then analyzed by GC-MS. When the [3,4,13C]Chol standard was added “after the extraction,” the ratio of “m/z 472.7 to m/z 474.7” for the TMS derivative of 7-keto-Chol was reported as 0.027, indicating that almost all (~97.3%) of the 7-keto-Chol contained two atoms of 13C. If the reporting of the ratio is correct, this implies almost quantitative oxidation of the [13C]Chol to [7-keto-13C]Chol “after the extraction” of the 7-keto-Chol. When the internal standard was added before “extraction” of the LDL, the reported mean ratio of m/z 472.7 to 474.7 was 0.028. The authors claimed no artifactual generation of the 7-keto-Chol because the ratio of m/z 472.7 to 474.7 did not decrease. However, it would be technically difficult to measure a decrease in this ratio. In any event, artifactual generation of 7-keto-Chol at one or more steps in the processing of the samples appears likely because the sum of the reported 7-keto-Chol levels in the lipoprotein fractions (4.38 μM) (see above) far exceeds the mean level of 7-keto-Chol in unfractionated plasma samples from normal human subjects as reported in other studies of higher credibility, e.g., 0.055 μM (273) and 0.030 μM (41).

Babiker and Diczfalusy (41) recently reported on the distribution of a number of oxysterols (7α-OH-Chol, 7β-OH-Chol, 7-keto-Chol, 5α,6α-epoxy-Chol, 5β,6β-epoxy-Chol, 5α,6β-dioH-Chol, 24-OH-Chol, 25-OH-Chol, 26-OH-Chol) in different plasma lipoprotein fractions, i.e., VLDL, LDL, HDL, and lipoprotein-free plasma derived from seven normal subjects in the fasting state. For the major oxysterols detected, i.e., 26-OH-Chol, 5β,6β-epoxy-Chol, 24-OH-Chol, and 7α-OH-Chol, most of the oxysterol was found in LDL and HDL. For all oxysterols studied, very low levels were observed in VLDL. The levels of 5α,6α-epoxy-Chol, 5β,6β-epoxy-Chol, 5α,6β-dioH-Chol, 7-keto-Chol, 7β-OH-Chol, and 25-OH-Chol in the lipoprotein fractions in this study are very markedly lower than those reported by Addis et al. (3), and they are very much lower than the values reported by Dyer et al. (271) for 7-keto-Chol in lipoprotein fractions. Moreover, Babiker and Diczfalusy (41) considered that only their data on 24-OH-Chol, 26-OH-Chol, and 5α,6β-dioH-Chol were valid. In the case of the other oxysterols, the sum of the oxysterol in the various lipoprotein fractions exceeded that of the measured value for the oxysterol in total plasma. The increase (from +20% for 7α-OH-Chol to +256% for 5α,6α-epoxy-Chol) was suspected to have been due to artifactual generation of the oxysterol from Chol during the isolation of the lipoprotein fractions. For the distribution of 26-OH-Chol, the authors reported mean levels as follows (in μM): VLDL, 0.007; LDL, 0.154; and HDL, 0.187. For 24-OH-Chol, mean levels reported were as follows (in μM): VLDL, 0.007; LDL, 0.082; and HDL, 0.077. For 5α,6β-dioH-Chol, reported values were as follows (in μM): VLDL, 0.002; LDL, 0.019; and HDL, 0.007.

There have been several published studies describing the distribution of oxysterols in plasma lipoprotein fractions after oral administration of oxysterols to animals. Peng et al. (796) reported the presence of material with the TLC mobility of free oxysterols in VLDL and LDL (and to a lesser extent in HDL) of serum of rabbits at 24 h after the intragastric administration of a [4-14C]Chol sample that had been subjected to autoxidation (5 wk at 60°C in air). Peng et al. (796) reported that, at 24 h after the oral administration of [1H]25-OH-Chol (location of label not specified) to monkeys, most of the 3H was located in LDL (55%) and VLDL (35%), whereas only a small percentage (10%) was associated with HDL. The chemical nature of the 3H in the lipoprotein fractions was not studied. In more extensive studies with another oxysterol (747), it was shown that, at 24 h after the oral administration of [2,4,3H]3β-hydroxy-5α-cholest-8(14)-en-15-one to baboons, almost all of the 15-ketosterol in plasma was in the form of esters that were located in LDL and HDL, with
very little associated with VLDL or intermediate-density lipoprotein. In addition, other studies with the 15-keto-sterol (912) demonstrated the major importance of chylomicrons in the transport and early metabolism of the 15-keto-sterol after its oral administration. The distribution of $^3$H and $^{14}$C in rat intestinal lymph was studied after the intragastric administration of [2,4-$^3$H]3β-hydroxy-5α-cholesten-8(14)-en-15-one and [4-$^{14}$C]Chol in triolein. Of the lymph collected between 6 and 8 h, 75% of the $^3$H and 61% of the $^{14}$C was associated with chylomicrons. In four experiments, 9.4% of the $^3$H was in the form of the free 15-keto-sterol, and 89.6% was recovered in the form of fatty acid esters of the 15-keto-sterol, of which the major fatty acid ester (89%) was the oleate ester. It is noteworthy that little or no conversion of the administered 15-[$^3$H]ketosterol to Chol of the lymph chylomicrons was observed. Thus, after intragastric administration of this oxygenated sterol, most was incorporated into fatty acid esters that were assembled into chylomicron particles. Therefore, the oxygenated sterol would enter the general circulation not as the free sterol but as its fatty acid esters that are associated with a lipoprotein particle that is known to undergo rapid clearance from the blood and rather specific uptake in organs. These combined findings have very important implications as to the initial events occurring after the intestinal absorption of the oxysterol. It is likely, but not certain, that other oxygenated sterols are also handled similarly after their oral administration. The results suggest that chylomicrons of plasma could be expected to be enriched in oxysterol at early times after its administration.

Emanuel et al. (277) reported on the presence of oxysterols in chylomicrons of plasma at early times after the administration to human subjects of a test meal of scrambled eggs prepared from dried egg powder containing oxysterols (reported to contain 5β,6β-epoxy-Chol, 50 ppm; 5α,6α-epoxy-Chol, 90 ppm; 7β-CHol, 60 ppm; and 7-keto-Chol, 30 ppm). Increases in “total Chol oxidation products” in chylomicrons were claimed after administration of the test meal to five female subjects. The authors stated that “…in all subjects and at all time intervals studied cholesterol oxidation products (COPS) concentrations in total plasma were 5- to 10-fold higher than in chylomicrons alone. This suggested either an absorption mechanism distinct from chylomicrons or a rapid net transfer of COPS from chylomicrons to other lipoprotein and/or plasma fractions.” Separate graphical data were presented on plasma and chylomicrons. In one case, a graph of individual oxysterols was presented. Each of 7-keto-Chol (the highest ~3.50 μM), 7β-CHol (~1.49 μM), and the individual 5α,6α- and 5β,6β-epoxides of Chol (each at ~0.63 μM) showed peaks at ~3.5 h. No data were presented on other oxysterols. Confirmation of the structures of Chol oxides in chylomicrons was mentioned; however, no data were presented. In this study, chylomicrons were subjected to Folch extraction, and the extracts were apparently saponified to give NSL (conditions not given) that were silylated and analyzed by capillary GC. Chromatograms of plasma sterols showed poor resolution.

G. Oxysterols in Tissues

Knowledge of the levels of oxysterols in tissues is very limited. Moreover, none of the studies described below utilized procedures to detect and quantitate artifactually generation of oxysterols during sample processing and analysis. Nonetheless, very interesting results have been obtained in a number of studies.

In 1973, Smith and Pandya (1007) reported the presence of 7-keto-Chol in samples of various organs obtained from autopsies of human subjects. 25-OH-Chol was also detected in each of the same organs with the exception of kidney. The authors noted that “both 7-keto-Chol and 25-OH-Chol may be artifacts of autoxidation during sample preparation.”

A number of oxysterols have been reported in livers of mice fed a diet “essentially free of Chol” (889). These included (in order of decreasing levels and in μg/g liver) the following unesterified sterols: 7-keto-Chol (0.27), (24S)-24-OH-Chol (0.22), 7β-OH-Chol (0.10), (25R)-26-OH-Chol (0.09), 7α-OH-Chol (0.03), and 25-OH-Chol (0.003). In two other experiments, the levels of unesterified oxysterols were reported as follows: 7-keto-Chol (0.07 and 0.13), (24S)-24-OH-Chol (0.19 and 0.17), 7β-OH-Chol (0.16 and 0.21), (25R)-26-OH-Chol (0.04 and not detected), and 7α-OH-Chol (0.02 and 0.07). Each of the same sterols was also found as esters, which, with one exception (7-keto-Chol), were reported to occur at substantially higher levels than the unesterified oxysterols. In one experiment with animals fed the control diet, the ester levels (μg/g liver) were as follows (in order of decreasing levels): 7α-OH-Chol (5.71), 7β-OH-Chol (4.87), (24S)-24-OH-Chol (0.51), (25R)-26-OH-Chol (0.31), 7-keto-Chol (0.12), and 25-OH-Chol (0.04). These results indicate that, with the exception of 7-keto-Chol, the bulk of the oxysterols were found in esterified form. The high levels of 7β-OH-Chol are worthy of note and raise the possibility that this material arose from autoxidation of Chol and also raise the possibility that a portion or all of the 7-keto-Chol and 7α-OH-Chol may have been formed similarly. However, it is important to note that this origin is very unlikely for the cases of the 26- and 24-hydroxysterols, generally not considered to be significant autoxidation products of Chol. Moreover, both of these sterols were reported to be single diastereomers on HPLC. Information on the levels of the 5α,6α- and 5β,6β-epoxy derivatives of Chol, both known autoxidation products of Chol, were not provided. Additionally, the levels of esterified 7-keto-Chol should be
viewed cautiously due to the marked lability of this sterol under the saponification conditions employed. Mice fed Chol (recrystallized; 3% in diet) for one night showed higher levels of (24S)-24-OH-Chol (most of which was in esterified form). The levels of 7α-OH-Chol and 7β-OH-Chol were considerably lower in the Chol-fed animals (Table 3). It is important to note that the levels of the individual oxysterols were determined on pooled liver samples and, as a consequence, the lack of estimation of the variation between animals complicates the interpretation of these experiments. However, the results of an additional similar experiment also showed a substantially higher level for (24S)-24-OH-Chol (also mostly in ester form) in Chol-fed animals relative to animals fed the control diet.

A study by Lund et al. (593) also concerned the levels of oxysterols in mouse liver. This study differed in design and methodology from that of Saucier et al. (899). Male mice (C57BL/6J; 11–14 wk old) were fed a “cholesterol-free” diet containing peanut oil (10%) with and without Chol (2%) for 4 days. The levels of individual oxysterols (free plus esterified) were determined after saponification (0.5 M KOH at 55°C for 45 min) of a homogenized preparation of liver followed by extraction with CHCl₃ and methanol (2:1). GC-MS analyses of the TMS derivatives of the oxysterols, employing deuterated internal standards, were made on three control and three Chol-fed mice. The results of these analyses indicated significant levels of a number of oxysterols in mouse liver. In a number of cases, the observed levels of the individual oxysterols (Table 3) differed from those reported by Saucier et al. (899). Most notably, the very high levels of 7α-OH-Chol and 7β-OH-Chol observed by Saucier et al. (899) in the livers of mice fed a Chol-free diet were not observed in the study of Lund et al. (593). However, the finding of levels comparable to or in excess of the levels of 7α-OH-Chol for 7β-OH-Chol, 7-keto-Chol, and the 5β,6β-epoxide of Chol again raises concern about the artifactual origin of some of the oxysterols by autoxidation of Chol during sample processing and/or analysis. Especially noteworthy in this regard is the fact that the oxysterol present at the highest level in mouse liver (either from control or Chol-fed animals) was the 5β,6β-epoxide of Chol. The results of the two studies also differed with regard to 24-OH-Chol, with considerably lower levels reported in the study by Lund et al. (593). In the study of Lund et al. (593), mice fed Chol (2%) for 4 days showed higher levels of most of the individual oxysterols. The increase in the level of 24-OH-Chol (+395%) reported by Saucier et al. (899) upon Chol feeding was considerably less (+88%) in the study by Lund et al. (593). In the latter study the levels of 26-OH-Chol were slightly higher (+28%) in the livers of the Chol-fed mice. The increases observed by Lund et al. (593) in 24-OH-Chol and 26-OH-Chol upon Chol feeding were less than those observed for 7α-OH-Chol (+940%) and 7β-OH-Chol (+385%).

Johnson et al. (456) reported studies of the levels of oxysterols in rat liver. Male Sprague-Dawley rats were maintained on a commercial diet. The animals were fasted for 6 h (5–11 A.M.). At 11 A.M., the rats were fed either a powdered sterol-free rat chow diet for 60 min or the same diet containing 5% purified Chol. The purified Chol was reported to “be free of contaminating sterols” by GC-MS. Rats were housed in pairs. Although the amount of food consumed in the 1-h feeding was measured, no

### Table 3. Oxysterols in mouse and rat liver

<table>
<thead>
<tr>
<th>Oxysterol</th>
<th>Mouse</th>
<th>Rat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control*</td>
<td>Chol fed*</td>
</tr>
<tr>
<td>7α-OH-Chol</td>
<td>5.74</td>
<td>0.69</td>
</tr>
<tr>
<td>7β-OH-Chol</td>
<td>4.97</td>
<td>0.72</td>
</tr>
<tr>
<td>(24S)-24-OH-Chol</td>
<td>0.73</td>
<td>3.61</td>
</tr>
<tr>
<td>(25R)-26-OH-Chol</td>
<td>0.40</td>
<td>0.30</td>
</tr>
<tr>
<td>7-Keto-Chol</td>
<td>0.39</td>
<td>0.54</td>
</tr>
<tr>
<td>25-OH-Chol</td>
<td>0.043</td>
<td>0.09</td>
</tr>
<tr>
<td>5α,6α-Epoxy-Chol</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>5β,6β-Epoxy-Chol</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>5α,6β-diolOH-Chol</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>26-Hydroxycholesterol</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Cholest-4-ene-3β,26-diol</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Cholest-4-ene-3β,6-diol</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Total of above</td>
<td>12.27</td>
<td>5.95</td>
</tr>
</tbody>
</table>

ND, not determined. a Adapted from Saucier et al. (899); sum of free plus esterified oxysterols. Diet employed was “essentially free of cholesterol” with or without added Chol (3%) for 1 night. † Adapted from Lund et al. (593). Definition of expression of variation (SD or SE) was not given. Diet employed was a Chol-free diet supplemented with peanut oil (10%) with or without added Chol (2%) for 4 days. ‡ Configurations at C-24 and C-25 for the 24-OH-Chol and 26-OH-Chol, respectively, were not determined in this study. § Adapted from Johnson et al. (456). Diet was sterol-free rat food with or without 5% Chol for 60 min.
data were presented. The livers of “pairs of rats (typically 6 to 8 pairs)” were frozen in liquid nitrogen and then combined (~70 g) and saponified with a mixture of 50% KOH and 95% ethanol “at reflux temperature for 3 h in the dark in an atmosphere of nitrogen.” “A small amount (~0.001%) of BHT was added as antioxidant.” The NSL were extracted and dissolved in toluene, and the solution was stored in the dark at 4°C for an unspecified length of time before reverse-phase flash chromatography on silica gel. Fractions containing early eluting polar sterols (eluted before Chol) were pooled and “concentrated under vacuum.” The column was then eluted with a mixture of CHCl₃ and methanol (2:1) and then with methanol. The material eluted with these two solvents was combined with the Chol-containing fractions, and the mixture was “concentrated under vacuum.” The polar sterol mixture and the Chol-containing fraction were then each “dissolved in toluene” (100 µl) and stored for an unstated period of time before GC-MS studies. GC-MS was carried out on TMS ether derivatives. A number of oxysterols were reported to be present in pooled samples of livers from the Chol-fed rats and from control rats (Table 3). These included 7α-OH-Chol, 7β-OH-Chol, 25-OH-Chol, 26-OH-Chol, and sterols listed as 26-hydroxycholesterol, cholest-4-ene-3β,26-diol, and cholest-4-ene-3β,6-diol. No authentic standards corresponding to the latter three sterols were mentioned, nor were any comments made in the text about the occurrence of these sterols in liver or the basis for the structure assignments. No retention time or MS data were provided for any of the sterols except for 25-OH-Chol. The authors noted that “no evidence was obtained to support the presence of lanosterol-24,25-epoxy-Chol, 24-OH-Chol, or 5α-7-dehydrocholesterol in human liver. No special procedures were described to suppress or detect autoxidation. The HPLC methodology described did not include mention of the mobility of any other oxysterols except 25-OH-Chol. Under the conditions employed, 25-OH-Chol, (24S)-24,25-epoxy-Chol, and Chol were reported to have retention times of 10.5, 13.5, and 39.3 min, respectively. The MS fragmentation of the isolated (24S)-24,25-epoxy-Chol was not presented. However, it was reported that “the same fragmentation pattern” as authentic (24S)-24,25-epoxy-Chol. The levels of the 24-epoxy-Chol present in liver samples from two human subjects were reported to be 0.40 and 0.13% of the amount of Chol present. In another study, Taylor et al. (1102) reported the presence of (24S)-24,25-epoxylanosterol in human liver (0.062 and 0.031% of total amount of Chol in samples from the same two human subjects). If one assumes a level of Chol in the human liver of ~2 mg/g, then levels of 0.40 and 0.13% of (24S)-24,25-epoxy-Chol in Chol would correspond to ~8.0 and ~2.6 mg/g, respectively. It should be noted that these levels appear to be very high, especially in comparison with the levels of individual oxysterols reported by Lund et al. (593) to be present in the livers of Chol-fed or control mice. Furthermore, as noted above, Johnson et al. (456) reported inability to detect the presence of 24,25-epoxy-Chol or 24,25-epoxylanosterol in liver from Chol-fed or control rats. The limits of detection for these sterols were not stated; however, the limit of detection of 25-OH-Chol in this study appeared to be ~0.005 mg/g.

Hasan and Kushwaha (376) reported on the levels of 26-OH-Chol in the livers of baboons on a high-fat (40% of calories), high-Chol (1.7 mg/kcal) diet. Results were presented for animals reported to be “low responders” (n = 6) and “high responders” (n = 6) to dietary Chol. The duration of administration of the experimental diet was not provided. Levels of 26-OH-Chol in the livers of baboons on a chow diet were not provided. No study was made of the levels of oxysterols in the diet fed to the baboons. The authors reported very high levels of 26-OH-Chol in liver: low responders, 86 ± 32 μg/g; and high responders, 26 ± 13 μg/g. These values are very considerably in excess of those reported for 26-OH-Chol in livers of rodents on Chol-deficient diets (456, 593, 899) and of rodents fed high levels of Chol (456, 593, 899). As noted previously in the section on oxysterols in plasma (see sect. v.A), the methodologies employed in these studies for the identification and quantitation of 26-OH-Chol were less than ideal. Identification of 26-OH-Chol was limited to comparison of its retention time with that of an authentic sample on reverse-phased HPLC. The retention time of only one other oxysterol standard (7-keto-Chol) in this HPLC system was presented. Equally serious was the unfortunate choice of 7-keto-Chol as an internal standard for quantitation of the 26-OH-Chol. The potential presence of 7-keto-Chol in liver and/or its artifactual generation during sample processing was not considered. In addition, the base-labile 7-keto-Chol was added to a strongly alkaline solution (after saponification) before extraction of the NSL.

Staprans et al. (1036) reported on the levels of selected oxysterols in livers of NZW rabbits (n = 13) after feeding of a Chol-containing diet (0.33%) for 12 wk. The source and purity of the added Chol were not provided; however, it was reported that the diet (prepared weekly
by adding the sterol to the diet with evaporation of the solvent under nitrogen) contained “no detectable oxidized Chol during the feeding period.” The oxysterols (presumably total oxysterol since mention of a saponification step was included) were estimated by GC. The reported oxysterol levels (as estimated from the graphical presentation; expressed as μg/mg liver) were as follows: 7α-OH-Chol, ~100; 7β-OH-Chol, ~160; 5β,6β-epoxy-Chol, ~110; 5α,6α-epoxy-Chol, ~100; and 7-keto-Chol, ~130. It should be noted that these values are very much higher than those reported for the same oxysterols in mouse liver after short-term feeding of a diet very high (2%) in Chol (593). The authors ascribed the presence of the oxysterols in liver to their endogenous production. However, no data were presented that excluded their artificial generation from Chol during sample processing. The levels of some of the oxysterols (7β-OH-Chol, 7-keto-Chol, and 5β,6β-epoxy-Chol) in liver were reported to be significantly higher in animals fed the same diet in which ~5% of the Chol had been oxidized.

Osada et al. (740) reported on the levels of oxysterols in liver from 4-wk-old male Sprague-Dawley rats fed one of three diets: a basal Chol-free diet, the basal diet containing added Chol (0.5%), or the basal diet supplemented with Chol (0.5%) and a mixture of oxidized Chol species (0.5%). The percentage composition of the oxidized Chol mixture was reported as follows: Chol (8.2), 7α-OH-Chol (5.5), 7β-OH-Chol (5.5), 7-keto-Chol (22.0), 5α,6α-epoxy-Chol (2.9), 5β,6β-epoxy-Chol (9.0), 25-OH-Chol (3.8), and unknown oxidized sterols (23.5). Because dietary administration of the diet supplemented with Chol plus the oxidized Chol mixture suppressed food consumption, the animals receiving the basal diet or that supplemented with Chol were given food only in the amount consumed by the animals receiving the diet supplemented with both Chol and the oxidized Chol mixture. In the rats fed the Chol-free and Chol diets, the only oxysterol detected was 7β-OH-Chol, which was present at levels of 60 ± 5 and 151 ± 49 μg/g, respectively. Chol levels in liver in the two groups were reported to be 7.4 ± 0.8 and 56.6 ± 0.6 mg/g, respectively. The value for the Chol-free group appears to be considerably higher than that commonly found in rat liver (~2–3 mg/g). In the livers of animals fed the diet containing Chol plus oxidized Chol, the following oxysterols were observed (in μg/g): 7β-OH-Chol, 432 ± 33; 7-keto-Chol, 48 ± 6; 5α,6α-epoxy-Chol, 60 ± 12; 5β,6β-epoxy-Chol, 106 ± 33; and 5α,6β-diOH-Chol, 98 ± 22.

Tamasawa et al. (1091) reported on the levels of unesterified 7-keto sterol derivatives of Chol in liver microsomes from male Wistar rats maintained on a chow diet. The levels in liver microsomes from rats fed a chow diet and those fed a chow diet supplemented with 7-keto-Chol (0.1% in diet) for 6 days were as follows (in μg/g): for animals fed chow diet, 7α-OH-Chol, 8.7 ± 1.0; 7β-OH-Chol, 14.5 ± 4.4; and 7-keto-Chol, 51.6 ± 5.9; for animals fed the chow diet plus 7-keto-Chol, 7α-OH-Chol, 7.9 ± 0.8; 7β-OH-Chol, 438 ± 47; and 7-keto-Chol, 449 ± 37. Sterols were determined by GC-MF of their TMS derivatives. No special precautions were noted to suppress or quantitate autoxidation of Chol during sample processing. Breuer et al. (125) reported on the levels of selected oxysterols in liver microsomes after intravenous administration of 7-keto-Chol in a commercial fat emulsion (2 mg/ml; 0.5 mg/kg; 1 ml/h over 48 h) to female Sprague-Dawley rats maintained on a fat-free commercial pelleted diet. The levels of oxysterols were determined by GC-MS methodology (121). The following levels (ng/mg protein) were observed in the control animals: 7α-OH-Chol, 4 ± 3; 7β-OH-Chol, <1; 7-keto-Chol, 11 ± 4; 5α,6α-epoxy-Chol, 1 ± 1; 5α,6β-diOH-Chol, 3 ± 1; 24-OH-Chol, <1; 25-OH-Chol, 2 ± 1; and 26-OH-Chol, <1. The levels observed in the rats receiving the 7-keto-Chol were: 7α-OH-Chol, 14 ± 6; 7β-OH-Chol, 1,600 ± 350; 7-keto-Chol, 2,200 ± 410; 5α,6α-epoxy-Chol, <1; 5β,6β-epoxy-Chol, 1 ± 0; 24-OH-Chol, 12 ± 7; 25-OH-Chol, 2 ± 1, and 26-OH-Chol, <1. It is noteworthy that, in both studies (125, 1091), administration of 7-keto-Chol resulted in very high levels not only of 7-keto-Chol in microsomes but also of 7β-OH-Chol.

Ryzlak et al. (879) reported on the levels of unesterified 7-keto-Chol, cholesta-3,5-dien-7-one, and cholesta-4,6-dien-3-one in liver from normal subjects and patients with alcoholic fatty livers and cirrhosis. In control subjects, the levels of the three sterols were as follows (in μg/g): 7-keto-Chol, 37 ± 22; cholesta-3,5-dien-7-one, 0.2 ± 0.1; and cholesta-4,6-dien-3-one, 0.3 ± 0.3. In patients with fatty liver, the levels were reported as (in μg/g): 7-keto-Chol, 6.8 ± 3.5; cholesta-3,5-dien-7-one, 13 ± 3; and cholesta-4,6-dien-3-one, 2.3 ± 0.9. None of the three steroids was detected in the cirrhotic liver group. Adachi et al. (2) reported the presence of cholesta-3,5-dien-7-one [1.35 ± 1.34 (SD) μg/100 mg Chol] in erythrocyte membranes from alcoholic patients. The authors noted that this sterol “was not detected in significant amounts in control subjects.”

24-OH-Chol has been reported to occur in rat (575), bovine (251, 302, 821), equine (302), and human brain (596, 941, 1008). Smith et al. (1008) reported on the levels of 24-OH-Chol in different regions of human brain. The mean values for the concentration (expressed per g dry wt) of the 24-hydroxysterol in cortex, subcortical white matter, midbrain, and pons were similar (~58–67 μg/g dry tissue), whereas a lower mean value (~24 μg/g dry tissue) was reported for cerebellum. The levels of 24-OH-Chol, expressed as a percentage of total sterols, were reported to be similar (0.05–0.08%) for all brain regions studied. The 24-OH-Chol in human cerebral cortex, human cerebellum, and bovine cerebral cortex was reported (251) to be concentrated in a microsomal fraction with none detected in nuclear and mitochondrial fractions. Prasad et al. (821) reported on the occurrence of 24-OH-
Chol in bovine brain as a sulfate ester. The nature of the sulfate ester (mono- or disulfate or the position of the ester) was not established. The concentration of the 24-OH-Chol as the sulfate ester in bovine brain was estimated to be ~84 μg/kg. Zhang et al. (1223) reported the presence of 24-OH-Chol in rat brain microsomes at levels between 0.49 and 0.66 nmol/mg protein. Identification was based on GC-MS of its TMS derivative. The configuration of the hydroxyl function at C-24 was not studied. Prasad et al. (821) also reported the occurrence of 24-OH-Chol in bovine adrenals. Identification was based on GC and GC-MS of its bis-TMS derivative (no spectral data were presented). The configuration at C-24 was not established. The 24-OH-Chol was present as the free sterol (2–3 μg/kg) and as fatty acid esters (10–12 μg/kg). None of the 24-OH-Chol present in bovine adrenal was detected as the sulfate ester. Lütjohann et al. (596) reported on the levels of 24-OH-Chol in various organs of one human subject (and on the levels of the sterol in cerebellum and cerebrum of two subjects). The levels of 24-OH-Chol (free plus fatty acid esters; μg/g) were as follows: heart, 0.11; spleen, 0.03; liver, 0.07; adrenals, 3.40; kidney, 0.07; thymus, 0.11; duodenum, 0.07; muscles, 0.03; lung, 0.07; testes, 0.04; fat tissue, 0.01; skin, 0.13; bone marrow, 0.16; tendons, 0.08; cerebellum, 3.8–4.8; and cerebrum, 8.6–15.1. Data on other oxysterols were not presented. However, it was stated that the samples from brain contained other oxysterols in addition to 24-OH-Chol. It was stated that “the concentration of 27-hydroxycholesterol varied between 5 and 30% of that of 24-hydroxycholesterol, whereas the concentration of 25-hydroxycholesterol was always <3% of that of 24-hydroxycholesterol.” In very early work, Fieser et al. (302) reported the presence of very low levels of 24-OH-Chol in wool fat. Characterization was limited to melting point and mixed melting point.

Vatassery et al. (1163) reported on the levels of certain oxysterols present in synaptosomes from rat brain after incubation for 4 h at 37°C in a medium containing NaCl (135 mM), KCl (5 mM), MgCl₂ (1 mM), CaCl₂ (1 mM), sodium phosphate (1 mM), glucose (10 mM), and HEPES (10 mM) at pH 7.4 in the absence or presence of added Fe²⁺ (100 μM) and ascorbate (100 μM). In the absence of the oxidants, only 7β-OH-Chol was detected (0.58 ± 0.01 nmol/mg protein). After incubation in the presence of Fe²⁺ and ascorbate for 4 h, 7α-OH-Chol, 7β-OH-Chol, 7-keto-Chol, and 5α,6α-epoxy-Chol were reported to be present at levels of 3.4 ± 0.03, 5.1 ± 0.3, 6.8 ± 1.3, and 1.6 ± 0.2 nmol/mg protein, respectively. Samples were saponified at 60°C for 30 min in the presence of added BHT and ascorbic acid. TMS ethers were analyzed by capillary GC. Standards available for this study included 7α-OH-Chol, 19-OH-Chol, 7β-OH-Chol, 5α,6α-epoxy-Chol, 22-keto-Chol, and 7-keto-Chol, the TMS ether derivatives of which were nicely resolved in the capillary GC system used. In an extension of this work, Vatassery et al. (1164) noted that the oxidants did not lead to the in vitro formation of 24-OH-Chol, a sterol reported by others (see above) to be present at significant levels in brain. The formation of the oxygenated derivatives of Chol upon incubation of synaptosomes was dependent on the concentrations of the oxidants (Fe²⁺ and ascorbate) and time of incubation (1164).

Dixon et al. (257) reported the occurrence of (22R)-22-OH-Chol and (20α,22R)-dihydro-7β-Chol as free sterols (by their isolation method) from bovine adrenal glands. The levels of the two sterols were 1.5 and 2.15 mg/kg, respectively. Neither of the sterols could be detected in adrenal as their sulfate esters. Reich et al. (841) reported the indication of the presence of 7β-OH-Chol in sheep thymus and claimed that this sterol was responsible for an intense yellow-green primary fluorescence in thymus tissue. Evidence supporting these claims was, at best, weak.

Emmons et al. (278) demonstrated the presence of 3β-hydroxy-5α-cholestan-8(14)-ene-15-one in rat skin and hair. Investigation of this matter was prompted by the well-documented occurrence of relatively high levels of sterol precursors of Chol in skin. After the addition of a doubly labeled internal standard of the 15-ketosteroid, i.e., [3α-3H][2,2,4,4-²H₆]β-hydroxy-5α-cholestan-8(14)-ene-15-one, the NSL were successively subjected to reverse-phase MPLC, TLC, reverse-phase HPLC, and, after formation of the TMS derivative, to GC-MS. The results of five independent analyses of portions of rat skin showed a mean value of 84.5 ± 4.1 ng/g skin. Analyses of samples of rat hair from 10 rats showed a mean value of 143 ± 19 ng/g hair. Most (~72%) of the 15-ketosterol in hair was esterified.

Adachi et al. (1) reported the presence of cholesta-3,5-dien-7-one (4.8 ± 0.2 μg/mg fat) in the opacified cornea of fish. In contrast, the Δ⁵,7-ketosterol was not observed in control corneas. These findings suggest the presence of 7-keto-Chol in the opacified cornea, since authentic 7-keto-Chol was decomposed by the procedures employed to give the Δ⁵,7-ketosterol. Girao et al. (338) reported the presence of several oxysterols in human cataracts. GC analyses of the TMS derivatives of unesterified oxysterols obtained from homogenate preparations of the nuclear portion of totally opacified cataracts (n = 54) were reported to contain the following oxysterols (expressed as a molar percentage of Chol): 7β-OH-Chol, 0.73 ± 0.07; 5α,6α-epoxy-Chol, 0.09 ± 0.02; 20α-OH-Chol, 0.06 ± 0.01; 25-OH-Chol, 0.01 ± 0.002; and 7-keto-Chol, 0.42 ± 0.03. In contrast, nuclear preparations from clear lenses (n = 48) showed only low levels of 7β-OH-Chol (0.04 ± 0.02 molar%). Assignments of structure were based solely on GC data (with incomplete separations of a number of the oxysterols). It was suggested that the oxysterols might be responsible for the membrane damage associated with cataract formation. It was also suggested that the formation of the oxysterols might be related to reported high levels of hydrogen peroxide in the
aqueous humor and lens of patients with cataracts (1026, 1027). The oxysterol levels in the cataract preparations were quite high, i.e., total of ~1.3% of Chol for the five oxysterols studied.

Two interesting studies (520, 1108) have concerned oxysterols present in membranes of erythrocytes from normal subjects and patients with sickle cell disease. Both groups reported significant levels of 19-OH-Chol in sickle cell membranes and its absence in membrane preparations from normal subjects. Kucuk et al. (520) reported the presence of four oxysterols in membranes from sickle cells from three subjects: 5α,5α-epoxy-Chol, 5α,6β-dioOH-Chol, 7-keto-Chol, and 19-OH-Chol at mean levels of 1.10, 1.20, 0.90, and 0.60 μg/ml red blood cells. Lower mean levels of the first three oxysterols were observed in membranes from four normal subjects, i.e., 0.29, 0.02, and 0.23 μg/ml red blood cells, respectively. Other oxysterols reported to have been identified in the sickle cell membranes but for which no quantitative data were reported included 7α-OH-Chol, 7β-OH-Chol, 20α-OH-Chol, and 25-OH-Chol. Evidence for the identification of the various oxysterols was not presented, although GC and GC-MS studies were apparently made. Teng and Smith (1108) also observed higher levels of oxysterols in membranes from sickle cells than in those from normal subjects. Oxysterols in erythrocyte membranes from four “healthy subjects” were reported as “not detected”; however, in one subject, a low level of 7-keto-Chol (30 μg/ml whole blood) was noted. The lower limits of detection of the levels for the various oxysterols were not presented. It should be noted that the “low level” of 7-keto-Chol in the healthy subject was in fact higher than that observed in 4 of 13 patients and higher than 2 of 3 values reported for another patient. Teng and Smith (1108) presented data on the levels of seven oxysterols, i.e., 7α-OH-Chol, 7β-OH-Chol, 7-keto-Chol, 20α-OH-Chol, 25-OH-Chol, 26-OH-Chol, and 19-OH-Chol. Three oxysterols reported to be present in sickle cell membranes by Kucuk et al. (520), i.e., 5α,6α-epoxy-Chol, 5β,6β-epoxy-Chol, and 5α,6β-dioOH-Chol, were not detectable by the methodology employed by Teng and Smith (1108). Levels of the oxysterols in the sickle cell membranes varied considerably in the different patients and in the same patients at different times. The levels of 7α-OH-Chol were from undetectable to 19 μg/dl whole blood, and those for 7β-OH-Chol were from undetectable to 18.2 μg/dl. 7-Keto-Chol was detected in each patient (from 1.0 to 75.0 μg/dl). 20-OH-Chol levels varied from undetectable to 20.2 μg/dl, and 25-OH-Chol varied from undetectable to 25.2 μg/dl. 26-OH-Chol was detected in only 3 of 13 patients at levels varying from trace to 9.0 μg/dl. 19-OH-Chol was detected in 4 of 13 patients at levels from trace to 30.6 μg/dl whole blood. Methodology for analyses of the oxysterols involved preliminary TLC (to remove Chol) followed by treatment of the oxysterols with Chol oxidase with subsequent HPLC analyses of the resulting Δ^4-3-ketosterols (which provided the basis of identification and quantitation). The authors noted that “the variable presence and levels of oxysterols may reflect the periodic crisis nature of the disease in which variable nonenzymic oxidation processes occur.” It should be noted that both studies involved the isolation of membranes by lysis of the erythrocytes and despite extensive washing, hemoglobin retention in the membrane preparations was noted to be a problem (both with normal and sickle cell preparations) (520) and could therefore facilitate autoxidation of Chol. Nonetheless, both groups indicated clear differences in the oxysterol levels in membranes from erythrocytes from normal subjects and sickle cell patients. The reported occurrence of 19-OH-Chol in the sickle cell preparations is particularly intriguing and should provide a stimulus to further studies regarding its identification and potential pathophysiological significance.

**H. Oxysterols in Meconium**

26-OH-Chol, 22-OH-Chol, 24-OH-Chol, and 7α-OH-Chol have been reported to be present in human meconium, in which they are found predominantly as sulfate esters (283, 368, 547). The levels of the oxysterols in meconium were noteworthy. For example, the levels of 26-OH-Chol and 22-OH-Chol were, in one study (547), reported as 4 – 8 and 3.8 – 6.4 μg/g meconium, respectively. In another study (283), 22-OH-Chol, 24-OH-Chol, and 26-OH-Chol were reported to be present as sulfate esters in human meconium. In addition, another OH-Chol was tentatively assigned the structure 23-OH-Chol on the basis of mass spectral data on the TMS derivative (in the absence of comparisons with an authentic standard). The 22-OH-Chol was assigned the 22S configuration on the basis of GC retention time data. The 22S assignment differs from that ascribed to the 22-OH-Chol isolated from adrenal gland.

**I. Oxysterols in Cerebrospinal Fluid**

Lütjohann et al. (596) reported the presence of 24-OH-Chol in cerebrospinal fluid from human subjects. Other side-chain oxygenated sterols were not detected. The levels of the 24-hydroxysterol in cerebrospinal fluid were reported to be ~10% those found in plasma. The mean ratio of 24-OH-Chol to Chol in cerebrospinal fluid was 0.2% of Chol for the five oxysterols studied.
ural and processed foods is a matter of considerable potential importance in public health. The influences of modes and lengths of storage as well as methods of processing (especially in those cases in which the drying or preparation of food materials involved processing at elevated temperatures in the presence of oxygen) on the generation of various oxysterols by autoxidation of Chol and other naturally occurring sterols are subjects of obvious practical importance. Food products of special importance include those such as dried egg powders (very extensively used in a large number of commercial products), milk powders (extensively used in infant formulas and other commercial foods), dried or stored fish and cheese products, stored butter preparations, and a variety of foods subjected to deep frying (especially with repeated use of the heated various fats).

A number of studies have attempted to determine the levels of oxysterols in various commercial food products and related materials. These have included various powdered preparations of whole eggs or egg yolks (307, 308, 357, 359, 537, 538, 811, 892, 1154), fresh egg yolks (720, 811), various powdered milk preparations including those for consumption by infants (12, 721, 864, 892), fresh fried potatoes (269, 270, 773), potato chips (269, 270), beef tallow from deep-fat fryers (50), butter oil (12), Indian ghee (432, 834), freeze-dried meat products (1154), raw brain concentrate (773), processed marine foods including dried sardine and squid (736), other commercial fish preparations (727), cheeses and commercial cheese spread (906), and commercial meat products including salami, pork links, and liverwurst (906). Methodologies employed varied considerably in sophistication, with separation and quantitation based on approaches ranging from simple TLC to GC and GC-MS. Substantial levels of oxysterols in the food products have been reported. In most preparations, the major oxysterols were 7-oxygenated sterols (7β-Oh-Chol, 7α-Oh-Chol, and 7-keto-Chol) and 5,6-oxygenated sterols (5α,6α-epoxy-Chol, 5β,6β-epoxy-Chol, and 5α,6β-dioH-Chol), accompanied, in lesser amounts in some studies, by materials reported as 25-OH-Chol (308, 720, 721, 727, 864, 892, 1154), 20α-Oh-Chol (432, 720, 721, 906), 3β-hydroxy-5α-cholestan-6-one (12), 3β,5α-dihydroxycholestan-6-one (906), 19-OH-Chol (906), and “25-hydroxycholesterol epoxide” (432). Evidence for structural assignments were frequently limited to chromatographic behavior on GC or, in two cases, to TLC mobility alone (432, 834). Another major deficiency in almost all of the studies has been a lack of serious attention to the problem of artifactual generation of oxysterols from Chol during processing and analyses of the various samples. The importance of this matter has been indicated above in considerations of the levels of oxysterols in plasma. One study (864) demonstrated the necessity of the detection of artifactual generation of oxysterols during their analysis in food products.

In 1995, Rose-Sallin et al. (864) presented an important paper on the analysis of oxysterols in milk powders. The special feature of this work was the use of an internal standard of [25,26,26,27,27,27-2H7]-Chol to monitor artifactual formation of oxysterols during sample processing and analysis. The methodology used involved addition of the deuterated Chol to the sample, mild saponification (1 N KOH in methanol overnight at room temperature), and extraction of the nonsaponifiable lipids with “deperoxidized diethyl ether.” The pooled ether extracts were washed once with 0.5 N aqueous KOH and three times with 0.47 N sodium sulfate, and dried over anhydrous sodium sulfate. The sample was subjected to solid-phase extraction with elution of Chol and nonpolar materials with a mixture of hexane and ethyl acetate followed by elution of the oxysterols with acetone. The sterols were analyzed (as TMS derivatives) by capillary GC-MS (30 m DB-1 column), and 19-OH-Chol (as its TMS derivative) was used as an internal standard for GC. In this study, 20α-OH-Chol was not estimated due to the fact that with the silylation conditions used, 20α-OH-Chol was not quantitatively silylated. The 5,6-epoxides of Chol were not quantitated because the 5α,6α-epoxide “was not accurately purified using our method.” With the use of the deuterated Chol internal standard, data were presented on the artifactual generation of oxysterols from Chol during 20 analyses of one sample of milk powder containing 1 mg Chol/g milk powder. The results indicated that, under the conditions studied, at least 2% of the Chol in the sample underwent autoxidation. Specific oxysterol levels, formed artifactualy, were as follows (in ng/g milk powder): 7α-OH-Chol, <10; 7β-OH-Chol, 80 ± 30; 7-keto-Chol, 1,490 ± 380; 5α,6β-dioH-Chol, 10 ± 5; and 25-OH-Chol, 340 ± 40 (total of above, 1,920). With the use of this methodology (with corrections for artifactualy generated oxysterols), fresh milk powders and infant milk powders were found to contain very much lower levels of oxysterols than those reported previously by others (892). In a subsequent report (255) from the same laboratory on the artifactual generation of five different oxysterols from Chol, the extent of artifactual generation varied with different oxysterols (substantial for 7-keto-Chol, 7β-OH-Chol, and 7α-OH-Chol, and negligible for 25-OH-Chol and 5α,6β-dioH-Chol) and with different methods of sample processing. It is important to note that, in 1992, Wasilchik et al. (1185) developed very detailed conditions for detecting artifactual generation of oxysterols using a [3H4]Chol as an internal standard. However, to the knowledge of this reviewer, there have been no subsequent accounts of applications of this methodology.

Jacobson (432) reported the presence of high levels of oxygenated derivatives of Chol in Indian ghee and suggested that the ingestion of these compounds in ghee might be responsible for the high incidence of atherosclerosis in immigrant Indian populations. Ghee is prepared...
from butter under conditions in which oxidation might be anticipated. Fresh butter was reported to contain Chol at a level of 2,500 µg/g and “no detectable oxides.” Ghee was reported to contain Chol at a level of 2,100 µg/g and oxides of Chol at a level of 12.3% of sterols. Reported levels (µg/g Chol) of the oxysterols were as follows: 7α-OH-Chol, 72.9 ± 37; 7β-OH-Chol, 67.1 ± 28; a sterol designated as “25-OH-Chol epoxide,” 58.0 ± 23; 20α-OH-Chol, 60.7 ± 40; and “cholestanetriol,” trace. Identification and quantitation of the oxysterols were based on simple TLC. Rama Prasad and Subramanian (834) subsequently claimed the presence of a “5,6-epoxide” of Chol and 5α,6β-diol-3-one in home-made ghee but not in two samples of commercial ghee. Workup of the samples was similar to that of Jacobson (432), and “identification” of the sterols was limited to behavior on TLC. No quantitation data were presented. Neither study employed controls for autoxidation of Chol during sample processing, and the claimed “identifications” of the sterols were not based on rigorous study.

Substantial levels of oxysterols in commercial egg powders (especially those prepared with spray drying with high heating) (307, 357, 359, 558) and in materials heated with various fats (especially those rich in unsaturated fatty acids) (50, 269, 270, 563, 727, 736) have been reported.

V. METABOLISM OF OXYSTEROLS

Knowledge of the metabolism of oxysterols is essential to an understanding of the actions of oxysterols in various biological systems. As illustrated by the case of 3β-hydroxy-5α-cholesta-8(14)-en-15-one (described below), marked differences in the metabolism of this oxysterol were observed with different cultured mammalian cells (e.g., CHO-K1 cells vs. Hep G2 cells). Also, significant differences between metabolism in the cultured cells and that observed in intact animals were noted. The metabolism of the 15-ketosterol resulted in a complex array of products of varying potency (relative to the parent 15-ketosterol) for different biological actions. Moreover, an understanding of the metabolism of the 15-ketosterol provided the basis for the rational design and construction of analogs with increased in vivo potency.

A. Metabolism of Oxysterols in In Vitro Preparations

1. 7-Oxygenated sterols

The conversion of 7α-OH-Chol to 7α-hydroxycholesterol-4-en-3-one is catalyzed by rat liver microsomes incubated in the presence of NAD (70, 416), and aspects of the mechanism of the transformation were studied by Björkhem (80). Skrede et al. (993) reported the conversion of 7α-hydroxycholesterol-4-en-3-one, an intermediate in the conversion of 7α-OH-Chol to bile acids, to cholesta-4,6-dien-3-one upon incubation with rat liver microsomes. The product was characterized by its chromatographic behavior and by GC-MS of its methoxime derivative. Significant nonenzymatic conversion was also noted under the conditions studied. It was proposed that the dehydroxylation of the 7α-hydroxysterol may be an important reaction leading to the high levels of cholestanol observed in tissues of patients with CTX. Boberg et al. (101) reported the conversion of (22R)-cholesterol-5-ene-3β,7β,22-triol to (22R)-22-hydroxycholesta-4,6-dien-3-one upon incubation with rat liver microsomes in the presence of NAD. The product was characterized by MS of the methoxime-TMS derivative. The same product was obtained after incubation of (22R)-cholesterol-5-ene-3β,7α,22-triol. The overall conversions were proposed to occur via initial NAD-dependent formation of a Δ1,3-keto-7-hydroxy analog followed by formation of the Δ1,6,3-ketosterol.
tritol-26-aldehyde to the 3α,7α,12α-tritol-26-oic acid (with no conversion to the corresponding tetrol). Incubation of the 3α,7α,12α,26-tetrol with the combination of the two gel filtration fractions in the presence of NAD led to the formation of the 26-acid with no detectable accumulation of the 26-aldehyde. The two gel filtration fractions were shown to correspond to ethanol-NAD oxidoreductase and acetaldehyde-NAD oxidoreductase, respectively. In 1970, Okuda and Takigawa (731) reported the purification of rat liver 5β-cholestan-3α,7α,12α,26-tetrol:NAD oxidoreductase. The correspondence of the enzyme to alcohol dehydrogenase was strongly indicated by ratios of the two enzyme activities through the purification and by the results of studies involving inactivation of the enzyme by heating or treatment with p-chloromercuribenzoate. No evidence of the presence of multiple forms of the enzyme was observed upon electrophoresis. Subsequently, Björkhem et al. (92) studied various known isoenzymes of horse liver alcohol dehydrogenase (LADH) with regard to their action on 5β-cholestan-3α,7α,12α,26-tetrol. The isozyme LADH_{SS} was found to catalyze the conversion of the tetrol to the corresponding 26-acid (although no details of the characterization of the product were presented). The LADH_{SS} was reported to show “more than 50 times higher o-steroid dehydrogenase activity than LADH_{EE}.” Thus this isoenzyme from horse liver apparently catalyzed the formation of the carboxylic acid without the need of an additional enzyme, the aldehyde dehydrogenase, as in the case of the cytosolic enzymes from mouse liver as suggested by the work of Okuda and Takigawa (730). In addition to cytosolic localization, enzymatic conversion of the 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid has been demonstrated in rabbit liver mitochondria in the presence of added NAD (229). No conversion of the tetrol to the 26-acid was detected with a subcellular preparation enriched with peroxisomes. The mitochondrial conversion was inhibited by 4-heptylpyrazol, a known inhibitor of cytosolic alcohol dehydrogenase, but was not affected by disulfiram, a known inhibitor of cytosolic acetaldehyde dehydrogenase. It should also be noted that in addition to the presence of enzymes in the cytosol and mitochondria for the catalysis of the conversion of the 26-hydroxy steroids to the corresponding C-26 acids, it has been reported that peroxisomes are capable of converting 26-OH-Chol to 3β-hydroxycholest-5-enoic acid (515).

Dahlbäck and Holmberg (230) reported that incubation of 5β-cholestan-3α,7α,12α-triol with highly purified rabbit liver mitochondrial P-450_{5α} in the presence of ferredoxin, ferredoxin reductase, and NADPH gave two products, 5β-cholestan-3α,7α,12α,26-tetrol and 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid. The latter material was identified as follows. The TMS derivative of the methyl ester had the same retention time and MS as those of an authentic sample. Further incubation of 5β-cholestan-3α,7α,12α,26-tetrol with the cytochrome P-450_{5α} system gave one major product, the corresponding C-26 acid. NADPH could not be replaced by NAD or NADP. Very recently, Holmberg-Betsholtz et al. (393) reported the identification of 3α,7α,12α-trihydroxy-5β-cholestan-26-al upon incubation of either 5β-cholestan-3α,7α,12α,26-tetrol or 5β-cholestan-3α,7α,12α-triol with highly purified rabbit liver mitochondrial cytochrome P-450_{5α} in the presence of ferredoxin, ferredoxin reductase, and NADPH. When incubation mixtures were treated with hydroxylamine hydrochloride or methoxylamine hydrochloride, the “properties of the compound were changed as anticipated for an oxime or methoxime derivative of an aldehyde.” Treatment of an “incubation extract followed by TLC of the reduced sample resulted in the disappearance of the unknown metabolite, but not of the 27-carboxylic acid or the 27-hydroxysteroid.” When the “incubation extract was treated with deuterium-labeled sodium borohydride, combined GC-MS analysis revealed that the disappearance of the unknown compound peak was coupled with the incorporation of one atom of deuterium in the 27-hydroxysteroid.” A synthetic sample of 3α,7α,12α-trihydroxy-5β-cholestan-26-al was prepared in 6.8% yield by reduction of the triacetate of the methyl ester of 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid from alligator bile with diisobutylaluminum hydride. No characterization was provided except for the presentation of the MS for its tris-TMS derivative. The isolated C-27 aldehyde was reported to have, as its tris-TMS derivative, the same retention time and MS as those of the synthetic sample. It was reported that “the identity of the aldehyde was further confirmed by conversion into the corresponding methoxime trimethylsilyl derivative, which gave the expected mass spectrum, identical to that of the synthetic compound.” Kinetic studies of the formation of the C-26 aldehyde and the C-26 carboxylate from the 3α,7α,12α,26-tetrol were compatible with a precursor-product relationship between the aldehyde and the acid. In a subsequent study with the purified rabbit liver mitochondrial enzyme (394), further evidence was presented indicating the catalysis of the conversion of (in order of catalytic efficiency) 5β-cholestan-3α,7α,12α,27-tetrol, 5β-cholestan-3α,7α,27-triol, and 5β-cholestan-3α,7α,27-diol to the corresponding carboxylic acids. Ketoconazole inhibited the 26-hydroxylation of 5β-cholestan-3α,7α,12α-triol and the conversion of the triol to the corresponding carboxylic acid. Interestingly, whereas carbon monoxide (CO:O_{2}, 98:2 vs. N_{2}:O_{2}, 98:2) inhibited the 26-hydroxylation of 5β-cholestan-3α,7α,12α-triol, it had no inhibitory effect on the conversion of the triol to the carboxylic acid.

Pikuleva et al. (803) reported that the purified recombinant human cyp26, which was expressed in E. coli, catalyzed the conversion of 5β-cholestan-3α,7α,12α-triol into 5β-cholestan-3α,7α,12α,27-tetrol and the corresponding carboxylic acid and the conversion of Chol to...
26-OH-Chol. In a subsequent study (804) with the same recombinant enzyme, the catalysis of the conversion not only of Chol to 26-OH-Chol but also to the corresponding aldehyde and acid (as judged by HPLC) was reported. Incubation of the 26-hydroxysterol with the enzyme yielded the aldehyde and acid (as judged by HPLC and, in the case of the aldehyde, by MS of its TMS derivative). Incubation of 3β-hydroxycholest-5-en-26-oic acid with the enzyme yielded two products that were not identified but that were believed, on the basis of the MS of their TMS derivatives, to represent hydroxylated derivatives (with the hydroxy function on the sterol nucleus).

Establishment of the quantitative in vivo importance in animals of the two main reaction types (i.e., oxidase vs. dehydrogenase) in the conversion of 26-hydroxysteroids to the corresponding acids is clearly a difficult matter. However, the use of a strain of the deer mouse with a genetic lack of alcohol dehydrogenase indicated no changes in bile acid composition or pool sizes (989). If this finding in mice extends to other species, it would indicate the potential importance of the oxidase pathway.

Microsomes of liver (96, 619, 976, 1118, 1119) and brain (1222) have been shown to catalyze the 7α-hydroxylation of 26-OH-Chol. Norlin and Wikvall (717) reported the purification of a P-450 species from pig liver microsomes (distinct from cyp7a) that catalyzed the 7α-hydroxylation of 26-OH-Chol and 25-OH-Chol (as well as of dehydroepiandrosterone and pregnenolone). Chol and testosterone did not serve as substrates for the purified enzyme.

3. 25-Oxygenated sterols

Alsema et al. (7) reported the conversion of 25-OH-Chol to pregnenolone by mitochondria of bovine adrenal cortex under standard incubation conditions (pH 7.40). In incubations carried out at pH 7.80, the conversions of 25-OH-Chol to a 20,22,25-trihydroxycholesterol and of (20S)-20-OH-Chol to a 20,25,26-trihydroxycholesterol were detected. Assignments of structure were based on GC-MS studies of their TMS derivatives (in the absence of authentic standards). Morisaki et al. (678) studied the substrate specificity of highly purified adrenocortical cytochrome P-450

...
4. 20- and 22-Oxygenated sterols

It is well established that (22R)-22-OH-Chol and (20R,22R)-20,22-dihydroxy-Chol can be converted to pregnenolone in steroid hormone-forming organs and that the enzyme catalyzing these conversions and their formation from Chol, Chol-side-chain cleavage enzyme (P-450sc), is localized in mitochondria (7, 148 and references cited therein). (20S)-20-OH-Chol can also act as a substrate for pregnenolone formation from labeled Chol in JEG-3 choriocarcinoma cells, an effect that was ascribed to an inhibition of the P-450sc. A protein, steroidogenic acute regulatory protein, appears to be important in the regulation of steroid hormone formation and appears to do so by affecting the transport of Chol into mitochondria, providing access to the side-chain cleavage enzyme (490 and references cited therein, Ref. 572).

5. 15-Oxygenated sterols

3β-Hydroxy-5α-cholesterol-8(14)-en-15-one, itself a very potent inhibitor of sterol synthesis in cultured mammalian cells, has been shown to be convertible to Chol upon incubation with the 10,000-g supernatant fraction of rat liver homogenate preparations (666, 667). The conversion to Chol occurred in preparations of livers from both male and female rats; however, the conversion was substantially higher in liver preparations from male rats (666). Under anaerobic conditions, the conversion of the 15-ketosterol to 5α-cholesta-8,14-dien-3β-ol and 5α-cholesta-7-en-3β-ol was demonstrated (667). Under either aerobic or anaerobic conditions, upon incubation with rat liver microsomal plus cystolic fractions, the conversion of the Δ8,14-15-ketosterol to material corresponding to fatty acid esters of the 15-ketosterol was demonstrated. Earlier studies established that 5α-cholesta-8(14)-ene-3β,15α-diol and 5α-cholesta-8(14)-ene-3β,15β-diol, each labeled with 3H, undergo efficient conversion to Chol upon incubation with the 10,000-g supernatant fraction of rat liver homogenate preparations under aerobic conditions (411, 412). Under anaerobic conditions, 5α-cholesta-7-en-3β-ol was demonstrated to be the major product. Other labeled products identified were 5α-cholesta-8,14-dien-3β-ol, 5α-cholesta-8-en-3β-ol, and 5α-cholesta-8(14)-en-3β-ol. Incubation of each of the labeled diols with washed rat liver microsomes gave 5α-cholesta-8,14-dien-3β-ol as the major product. Incubation of labeled 5α-cholesta-8(14)-ene-3β,15β-diol with the 10,000-g supernatant fraction in the presence of 100 µM AY-9944 (trans-1,4-bis-[2-chlorobenzylaminomethyl]cyclohexane dihydrochloride) gave 5α-cholesta-8,14-dien-3β-ol as the sole product. Prior studies, reviewed previously (910, 918), established the convertibility to Chol (and the sequence of enzymatic reactions) for the following sterols: 5α-cholesta-8,14-dien-3β-ol, 5α-cholesta-8-en-3β-ol, 5α-cholesta-7-en-3β-ol, and cholesta-5,7-dien-3β-ol. The combination of results permitted the formulation of a pathway for the overall conversion of the Δ8,14-15-ketosterol to Chol (667).

Pascal et al. (778) studied the metabolism of two 15-oxygenated C27 sterols with the unnatural cis-C-D ring junction, i.e., 5α,14β-cholesterol-7-ene-3β,15α-diol and 5α,14β-cholesterol-7-ene-3β,15β-diol. Only the 15α-hydroxy compound was convertible to Chol upon incubation with rat liver homogenate preparations. Other products of the 3β,15α-diol were 5α-cholesta-8,14-dien-3β-ol, cholesta-5,7-dien-3β-ol, 5α-cholesta-8-en-3β-ol, and 5α-cholesta-7-en-3β-ol. A scheme was presented to account for the overall conversion to Chol and the other sterol precursors of Chol.

14α-Methyl-5α-cholesterol-7-ene-3β,15β-diol was shown to be convertible to Chol, albeit in low yield, in rat liver homogenate preparations (618, 1030, 1031), whereas no conversion of its 15α-hydroxy epimer to Chol was observed. Gibbons and co-workers (330, 332, 333) reported on the metabolism of 16-3H]-lanost-8-ene-3β,15α-diol and 16-3H]-lanost-8-ene-3β,15β-diol in rat liver homogenate preparations. The nature of products was studied by radio-TLC in various systems. Slight conversion of each of the sterols to Chol was observed that appeared to be higher for the 15α-hydroxysterol. Substantial conversion to polar material was observed. Control experiments indicated that the polar material did not arise from autoxidation. The results of GC-MS studies suggested that a substantial portion of the polar material was represented by a lanostenetriol. The formation of this polar metabolite was reported to be suppressed by carbon monoxide. The structure of the putative lanostenetriol was not established. A substantial fraction of the 3H was recovered in nonpolar material that appeared to correspond to fatty acid ester(s) of the incubated substrate.

6. 32-Oxygenated sterols

Gibbons et al. (332) reported the conversion of lanost-8-ene-3β,32-diol and 3β-hydroxylanost-8-ene-32-al to Chol and 5α-cholesta-7-en-3β-ol upon incubation with the 10,000-g supernatant fraction of a rat liver homogenate. Sekigawa et al. (947) reported on the metabolism of 32-hydroxylated 24,25-dihydrolanosterols by partially purified cytochrome P-450p from rat liver microsomes in the presence of cytochrome P-450 reductase of rat liver and NADPH under aerobic conditions. Under the conditions studied, lanost-8-ene-3β,32-diol and 24,25-dihydrolanosterol were converted to 4,4-dimethyl-5α-cholesta-8,14-dien-3β-ol, which was characterized by GC-MS of its TMS derivative and by reverse-phase HPLC (with ultraviolet detection at 248 nm). Under the same conditions, lanost-7-ene-3β,32-diol and lanost-6-ene-3β,32-diol were reported to give no detectable metabolites. This finding
with the lanost-7-ene-3β,32-diol is in apparent conflict with the previous reports of the conversion of 14α-hydroxymethyl-5α-cholesterol-7-en-3β-ol (777) and lanost-7-ene-3β,32-diol (910) to Chol upon incubation with rat liver microsomes or 10,000-g supernatant fractions of rat liver homogenates. However, the demonstration of the catalysis, by rat liver microsomes, of the conversion of 14α-hydroxymethyl-5α-cholesterol-7-en-3β-ol to 14α-hydroxymethyl-5α-cholesterol-8-en-3β-ol and of 14α-methyl-5α-cholesterol-7-en-3β-ol to 14α-methyl-5α-cholesterol-8-en-3β-ol (776) provides an explanation of the overall conversion of 24,25-dihydroxy-5α-cholesterol-7-en-3β-ol to Chol in incubations containing microsomes.

Sono et al. (1016) reported the metabolism of lanost-8-ene-3β,32-diol and 3β-hydroxylanost-8-en-32-al to 3β-hydroxy-4,4-dimethyl-5α-cholesta-8,14-dien-3β-ol by a highly purified preparation of cytochrome P-450 from pig liver microsomes in the presence of NADPH and purified NADPH cytochrome P-450 reductase of pig liver microsomes.

B. Metabolism of Oxysterols in Cultured Cells

1. 7-Oxygenated sterols

Krut et al. (519) reported that, upon incubation of a mixture of 14C-labeled 7α-hydroxy-, 7β-hydroxy-, and 7-ketocholesterol with fetal bovine pulmonary artery endothelial cells for 24 h, marked formation of more polar material(s) in the culture medium was detected by TLC. The chemical nature of this polar material(s) was not established.

2. 24-Hydroxysterols

Because 24-OH-Chol is a major oxygenated sterol in brain, the metabolism of this sterol by cultured brain cells is of particular interest. Zhang et al. (1223) studied the metabolism of unlabeled (24R)-24-OH-Chol in cultured rat astrocytes, Schwann cells, and neurons. Only one metabolite was detected which, on the basis of GC-MS studies of its TMS derivative, was reported as a 24,25-dihydroxycholesterol-4-en-3-one. No formation of 7α,24-dihydroxycholesterol-4-en-3-one was detected despite intensive efforts toward this end. Thus the metabolism of the 24-OH-Chol differed from that of the 26- and 25-hydroxycholesterols for which significant formation of 7α-hydroxylated metabolites was observed.

3. 25-Hydroxysterols

Falke et al. (291) reported that incubation of 25-OH-Chol, 20α-OH-Chol, and (22R)-22-OH-Chol with isolated rat adrenal cells caused a dose-dependent increase in the production of corticosterone (as measured by fluorimetric assay). Toaff et al. (1116) studied the metabolism of 25-OH-Chol by rat luteal mitochondria and dispersed luteal cells. The incorporation of [6-3H]25-OH-Chol into labeled pregnenolone and progesterone (as judged by TLC) by mitochondria of rat ovaries (obtained at 7 or 8 days after human chorionic gonadotropin administration) was reported. 25-OH-Chol stimulated mitochondrial steroid formation in a dose-dependent fashion. 25-OH-Chol also stimulated progesterin secretion from rat luteal cells (as determined by an immunoassay). Silavin et al. (984) reported that cytocholasin B, but not cytocholasin D, resulted in decreased side-chain cleavage of [26,27-3H]25-OH-Chol as assayed by recovery of labeled water-soluble products (not characterized) in hamster ovarian granulosa cells. Cytocholasin B had no effect on the uptake of the labeled 25-OH-Chol into the granulosa cells.

Taylor and Kandutsch (1100) reported the conversion of labeled 25-OH-Chol to a slightly less polar component and to more polar components (by radio-TLC) upon extended (60 h) incubation with mouse L929 cells. The less polar component was characterized as pregnenolone on the basis of its chromatographic behavior (TLC and reverse-phase HPLC) and cocrystallization experiments. Evidence was presented that indicated that one of the more polar components corresponded to a 20,22-dihydroxy derivative of the 25-OH-Chol on the basis of its chromatographic behavior (TLC), MS data, and the TLC behavior of the steroidal component obtained upon periodate oxidation of the polar material. Only partial MS data were presented, and a comparison with spectral data for synthetic cholest-5-ene-3β,20,22,25-tetrol was not presented. The formation of labeled pregnenolone was not detected in the L cells upon incubation with labeled mevalonate or labeled Chol in the presence or absence of added 8-bromoadenosine 3′,5′-cyclic monophosphate (which stimulates the side-chain cleavage reaction in endocrine cells). It was suggested that the reported conversion of 25-OH-Chol to pregnenolone in the L cells might represent a side-chain cleavage pathway “accessible only to oxysterols.” Relatively little metabolism (~11%) of the 25-OH-Chol to material with the TLC behavior of its oleate ester was observed upon extended (51 h) incubation with the L cells incubated in serum-free medium. It was reported that no further metabolism of labeled pregnenolone was detected upon its incubation with the L cells. Taylor and Kandutsch (1100) reported that incubation of the labeled 25-OH-Chol with medium (in the absence of cells) yielded ~10% autoxidized steroid solvents despite the addition of α-tocopherol. Major metabolism of the 25-OH-Chol to polar materials (or to material with the mobility of pregnenolone) analogous to that observed with the L cells was not observed with Chinese hamster lung cells or with CHO cells. This lack of metabolism of the 25-OH-Chol was correlated with the absence of a loss of suppressive action of the 25-OH-Chol on HMG-CoA reductase activity in the lung cells. In the lung cells, clear
lowering of HMG-CoA reductase activity was observed at an early time point (3 or 4 h); however, thereafter the values for the control and treated cells did not differ markedly. The authors suggested the possibility that the differences in reductase activity with time in the L cells and the lung cells incubated with 25-OH-Chol were related to the capacity of L cells, but not lung cells to inactivate the 25-OH-Chol by metabolism. In a subsequent study with human fibroblasts, Zhang et al. (1225) were unable to detect the formation of pregnenolone or 20,22-dihydroxysterols upon incubation of 25-OH-Chol with human fibroblasts. However, as noted previously, substantial formation of 7\(\alpha\),25-dihydroxycholest-4-en-3-one and lesser amounts of the 7\(\alpha\)-hydroxy derivative of 25-OH-Chol were observed.

The formation of pregnenolone from 25-OH-Chol has also been detected in cultured swine granulosa cells (1166) and in cultured bovine luteal cells (354) using immunoassay procedures. Babischkin et al. (42) reported the conversion of 25-OH-Chol to progesterone (as judged by immunoassay) by human placental syncytiotrophoblasts. Incubation of the cells with estradiol (0.1 \(\mu\)M) was reported to stimulate the conversion of 25-OH-Chol to progesterone. 25-OH-Chol has also been shown to serve as an efficient precursor of testosterone (as determined with an immunoassay procedure) in Leydig cells of mouse and rat testes (829). In the rat cells, relative testosterone production (relative to cAMP-stimulated conversion of Chol to testosterone) from 25-OH-Chol and 20\(\alpha\)-OH-Chol were essentially the same and significantly higher than from Chol. (22R)-22-OH-Chol was superior to the 25- and 20\(\alpha\)-hydroxysterols in this respect. In the cells from mice, the metabolism of the three hydroxysterols to testosterone was less than that observed in the rat cells. In the mouse cells, the formation of testosterone from 25-OH-Chol was lower than that from Chol in the cAMP-stimulated cells. Lichtstein et al. (570) reported the conversion, by primary rat adrenal cells, of \([1,2-3\text{H}]\)25-OH-Chol, but not \([26,27-3\text{H}]\)25-OH-Chol, into a labeled component with the chromatographic behavior of digitalis-like material. It was suggested that side-chain cleavage was the initial stage in the conversion of 25-OH-Chol to the digitalis-like material, the chemical nature of which was not established.

Axelson and Larsson (33) studied the metabolism of \([26,27-3\text{H}]\)25-OH-Chol in human fibroblasts. Substantial conversion to a polar metabolite, indicated to be 7\(\alpha\),25-dihydroxycholest-4-en-3-one, was observed in normal but not in transformed fibroblasts. Low levels of conversion to material with the properties of fatty acid esters of 25-OH-Chol were observed in both normal and transformed fibroblasts. The 7\(\alpha\),25-dihydroxy-\(\Delta^{4}\)-3-ketoster showed high activity in lowering reductase activity in both normal and transformed human fibroblasts. Zhang et al. (1224) reported the conversion of 25-OH-Chol to cholest-5-ene-3\(\beta\),7\(\alpha\),25-triol and 7\(\alpha\),25-dihydroxycholest-4-en-3-one by human fibroblasts. However, 7\(\alpha\)-hydroxylation of 25-OH-Chol was not observed with a virus-transformed fibroblast cell line, a colon carcinoma cell line, or with a breast cancer cell line. These findings, coupled with those of Axelson and Larsson (33), clearly indicate that 7\(\alpha\)-hydroxylation of 25-OH-Chol does not occur in all cell types. The paper by Zhang et al. (1224) needs some clarification since it describes the formation of labeled 7\(\alpha\),25-dihydroxycholest-4-en-3-one from \([3\alpha,\text{H}]\)25-OH-Chol. Communication with one of the authors indicated an omission in this paper of the fact that formation of the \(\Delta^{4}\)-3-ketosterol was based on release of \(\text{H}^{3}\) to the medium in a form ascribed to water. In studies of the metabolism of 25-OH-Chol in a virus-transformed fibroblast cell line, no conversion to 7\(\alpha\)-hydroxylated products was observed. However, a more polar metabolite of 25-OH-Chol was detected for which, on the basis of GC-MS studies of its TMS derivative, a tentative structure of cholest-5-ene-3\(\beta\),4\(\delta\),25-triol was suggested.

Zhang et al. (1223) studied the metabolism of \([3\alpha,\text{H}]\)25-OH-Chol and \([26,27,\text{H}]\)25-OH-Chol in cultures of rat astrocytes, Schwann cells, and neurons. In contrast to the results of incubations with 26-OH-Chol, essentially no conversion of the 25-OH-Chol to acids was observed. Also in contrast to the metabolism of 26-OH-Chol, 25,26-dihydroxysterols were observed. Also, in contrast to results of others with other cell types (see above), no significant conversion of the labeled 25-OH-Chol to pregnenolone, progesterone or 7\(\alpha\)-hydroxypregnenolone was observed. The major metabolites of 25-OH-Chol observed were cholest-5-ene-3\(\beta\),7\(\alpha\),25-triol and 7\(\alpha\),25-dihydroxycholest-4-en-3-one. Zhang et al. (1226) also observed the formation of 7\(\alpha\)-hydroxylated metabolites of 25-OH-Chol upon incubation of the labeled 25-hydroxysterol with mouse thymus tissue or with isolated mouse thymus epithelial cells. Corresponding metabolites were not observed upon incubation with isolated thymocytes. It is important to note that 7\(\beta\)-hydroxylated metabolites of 25-OH-Chol were not detected in this study. Upon incubation of 7\(\alpha\),25-diOH-Chol with isolated thymus epithelial cells, conversion of the 7\(\alpha\),25-dihydroxysterol to the corresponding 7\(\alpha\),25-dihydroxy-\(\Delta^{4}\)-3-ketosterol was observed. Interestingly, interleukin-1B stimulated the 7\(\alpha\)-hydroxylation of 25-OH-Chol in the epithelial cells. Kilsdonk et al. (484) mentioned that they had observed the metabolism of 25-OH-Chol to more polar compounds in hepatoma cells. No additional information was provided.

4. 26-Oxygenated sterols

Zhang et al. (1225) reported on studies of the metabolism of 26-OH-Chol and 25-OH-Chol by human fibroblasts. Formation of the 7\(\alpha\)-hydroxy derivatives of these sterols was reported along with indications of their subsequent metabolism to 7\(\alpha\)-hydroxy-\(\Delta^{4}\)-3-ketosterols. Evi-
idence indicating that the formation of the 7α-hydroxysterols was not due to autoxidation involved lack of detection of the corresponding 7β-hydroxysterols or 7-keto-steroids in the cells and the reported lack of formation of the 7α-hydroxysterols when the 26-OH-Chol and 25-OH-Chol were incubated in control experiments with fibroblasts treated with ethanol for 2 h before use. Axelson et al. (34) reported metabolism of 26-OH-Chol, 7α,26-dihydroxycholest-5-en-3-one, and 7α,26-dihydroxycholest-4-en-3-one to the corresponding 26-oic acids in human fibroblasts. In addition, 7α,26-dihydroxycholest-4-en-3-one and 7α-hydroxy-3-oxo-cholest-4-en-26-oic acid were recovered as metabolites of the 7α,26-diol-Chol. The 26-oic acids differed in their effects on HMG-CoA reductase activity in human fibroblasts, with the 3β-hydroxycholest-5-en-26-oic acid and 3-oxocholest-4-en-26-oic acid showing high potency and 3β,7α-dihydroxycholest-5-en-26-oic acid showing little or no activity.

The conversion of 26-OH-Chol to the corresponding acid can be catalyzed by either the 26-hydroxylase or by a dehydrogenase-dependent mechanism. Babiker et al. (40) reported efficient conversion of 26-OH-[3α-3H]Chol to cholest-5-en-26-oic acid (as judged by radio-HPLC) in human alveolar pulmonary macrophages. Cyclosporin (20 μM) caused a very marked inhibition of the formation of the 26-carboxylic acid. In contrast, 4-methylpyrazole, an inhibitor of alcohol dehydrogenase, was reported to have no effect on the conversion of the labeled 26-alcohol to the corresponding acid. Zhang et al. (1223) studied the metabolism of unlabeled 26-OH-Chol and 26-OH-[3α-3H]Chol in cultures of rat astrocytes, Schwann cells, and neurons. After incubation of the 3H-labeled substrate with rat astrocytes for 24 h, a substantial amount of the 3H (47% of total) in the culture medium was recovered in a water-soluble volatile form (presumably water) consistent with the formation of a 3-keto metabolite. Other 3H-labeled metabolites included cholest-5-ene-3β,7α,26-triol, cholest-5-ene-3β,25,26-triol, cholest-5-ene-3β,7α,25,26-tetrol, and an acidic fraction (3.7% of total). GC-MS studies of the TMS derivatives of the acids formed from unlabeled 26-OH-Chol indicated that the acidic fraction was composed of 3β-hydroxycholest-5-en-26-oic acid, 3β,7α-dihydroxycholest-5-en-26-oic acid, and 7α-hydroxy-3-oxocholest-4-en-26-oic acid. Another metabolite detected by GC-MS in the culture medium after incubation of the 26-OH-Chol with the rat astrocytes was 7α,26-dihydroxycholest-4-en-3-one, which presumably accounted for the loss of the 3α-labeled hydrogen of the substrate. Analyses of the cells after incubation with the labeled 26-OH-Chol were reported to show 7α-hydroxylated products as the major metabolites. Metabolism of 26-OH-Chol similar to that observed in astrocytes was also found in Schwann cells. Products reported were 7α,26-diol-Chol, 7α,26-dihydroxycholest-4-en-3-one, 3β-hydroxycholest-5-en-26-oic acid, 3β,7α-dihydroxycholest-5-en-26-oic acid, and 7α-hydroxy-3-oxocholest-4-en-26-oic acid. 25-Hydroxylation appeared to be much lower in the Schwann cells than in the astrocytes. The metabolism of 26-OH-Chol to 7α-hydroxylated or 25-hydroxylated products by neurons appeared to be considerably less than that observed with astrocytes or Schwann cells. Zhang et al. (1226) reported the formation of 7α-hydroxylated metabolites of 26-OH-Chol upon incubation of the labeled 26-hydroxycholesterol with mouse thymus tissue. Babiker et al. (40) reported inability to detect products of 7α-hydroxylation of 26-OH-Chol or 3β-hydroxycholest-5-en-26-oic acid in human macrophage or endothelial cells. Experimental details were not presented. Sauter et al. (901) studied the metabolism of 7α-OH-Chol and 26-OH-Chol to bile acids by cultured human hepatocytes. Addition of either sterol resulted in increased production of chenodeoxycholic acid and cholic acid. Contrary to earlier ideas, the authors postulated that the relative formation of chenodeoxycholic acid and cholic acid is regulated by metabolism distal to the initial hydroxylation of Chol to yield either 7α-OH-Chol or 26-OH-Chol.

5. 5,6-Epoxysterols and cholestan-3β,5α,6β-triol

Sevanian and Peterson (954) reported variable conversion of labeled 5α,6α-epoxy-Chol to 5α,6β-diol-Chol in V79 Chinese hamster lung fibroblasts. The isotope variability in the conversion was not identified. Sevanian et al. (950) reported on the uptake and metabolism of 3H-labeled 5α,6α-epoxy-Chol and 5β,6β-epoxy-Chol by rabbit aortic endothelial cells. Conversion of each of the 5,6-epoxides (10 μM) to 5α,6β-diol-Chol was reported (as judged by radio-TLC). The extents of uptake of each of the epoxides were similar as was the extent (∼17%) of conversion of each of the epoxides to the triol. Approximately 40% of the total [3H]5α,6β-diol-Chol formed was recovered from the medium; at 24 h, the triol in the medium was ∼1 μM (or ∼10%) of the initial level of the epoxide in the medium. A portion of the [3H]triole in the cells and in the medium appeared to be in the form of fatty acid esters. Sevanian et al. (950) studied the uptake of [1,2,3H]5α,6β-diol-Chol, at various concentrations (from 1 to 5 μM), by the rabbit aortic endothelial cells. At each concentration, near-maximal uptake into confluent cells was observed at the earliest time point studied (4 h), and thereafter, little or no further uptake was observed (at 8 and 24 h) as judged by the amount of 3H in the cells at the various time points. Cao et al. (154) studied the uptake and metabolism of [1,2,6,7-3H]5β,6β-epoxy-Chol (9.8 μM) by a murine macrophage cell line (J774A.1). Rapid and substantial uptake of 3H was observed, along with substantial conversion of the [3H]epoxysterol to 5α,6β-diol-Chol (as judged by radio-TLC). Addition of LDL was reported to markedly suppress cellular uptake of the labeled epoxysterol and its conversion to the 5α,6β-diol-Chol. Hrelia et al. (404) reported that upon incubation of unlabeled 5α,6α-epoxy-Chol (1, 10, and 100 μM) in ethanol with primary cultures of rat cardiomyocytes, de-
tectable levels of the epoxide and of 5α,6β-diOH-Chol were observed (GC of TMS derivatives).

6. 24,25-Epoxysterols

Taylor et al. (1102) studied the metabolism of the 24S- and 24R-isomers of [2-3H]24,25-epoxylanosterol in mouse L cells and in Chinese hamster lung fibroblast (Dede) cells. In the mouse L cells, the 24S- and 24R-isomers were converted to (24S)-24,25-epoxy-Chol and (24R)-24,25-epoxy-Chol, respectively. Different results were obtained with the Dede cells. In these cells, the 24S-isomer of 24,25-epoxylanosterol was converted to (24S)-24,25-epoxy-Chol, whereas the 24R-isomer of 24,25-epoxylanosterol was converted to (24R)-24-OH-Chol (plus other radioactive components which were not studied further). Characterization of the various products was limited to their behavior on HPLC. Panini et al. (758) studied the metabolism of the 24S-isomer of 24,25-epoxylanosterol by cultured intestinal epithelial (IEC-6) cells. 5β-H-labeled (24S)-24,25-epoxylanosterol was prepared by incubation of IEC-6 cells with [5βH]acetate in the presence of U-18666A (to cause the accumulation of labeled 2,3:22,23-diepoxy-squalene) followed by further incubation of the cells with fresh medium containing ketoconazole to inhibit the metabolism of the 24,25-epoxylanosterol. Incubation of the IEC-6 cells with the biosynthetically labeled (24S)-24,25-[5βH]epoxylanosterol resulted in the formation of (24S)-24,25-epoxy-Chol, as judged by reverse-phase HPLC and lithium aluminum hydride reduction of the product to material with the mobility of 25-OH-Chol on reverse-phase HPLC. Ketoconazole (30 μM) caused a substantial inhibition of the conversion of the 24,25-epoxylanosterol to 24,25-epoxy-Chol. The conversion of 14C-labeled 24,25-epoxylanosterol, formed biosynthetically upon incubation of CHO-K1 cells with [14C]acetate in the presence of ketoconazole (15 μM), to material with the chromatographic mobility (reverse-phase HPLC) of 24,25-epoxy-Chol in cultured rat hepatocytes and in CHO-K1 cells has been reported (757). Ketoconazole (20 μM) inhibited the conversion in both cell types (757). Full characterizations of the labeled substrate and product were not presented.

Taylor et al. (1102) studied the metabolism of the 24S- and 24R-isomers of [23-3H]24,25-epoxy-Chol in mouse L cells and in Chinese hamster lung fibroblast (Dede) cells. In the mouse L cells, little metabolism of the labeled 24S-isomer was observed. Reverse-phase HPLC analysis of the cellular sterols recovered after incubation of the labeled 24R-isomer indicated that, whereas most of the recovered 3H had the chromatographic mobility of the incubated substrate, at least two additional, more polar components were reported without further characterization. With the Dede cells, little metabolism of the labeled 24S-isomer was observed. In contrast, reverse-phase HPLC of the cellular sterols recovered after incubation of the labeled 24R-isomer indicated significant conversion to material with the chromatographic mobility of a standard sample of 24-OH-Chol and to at least two more polar materials that were not further characterized. Triparanol, at a concentration of 2 μM, was reported to inhibit (60%) the conversion by Dede cells of the labeled 24R-isomer of 24,25-epoxy-Chol to material with the mobility of 24-OH-Chol on reverse-phase HPLC.

7. 15-Oxygenated sterols

Gibbons et al. (334) studied the metabolism of lanost-8-ene-3β,15β-diol, lanost-8-ene-3β,15α-diol, lanost-7-ene-3β,15β-diol, and lanost-7-ene-3β,15α-diol in cultured rat hepatocytes and in Hep G2 cells. The major metabolite in these cells (apart from formation of its 15-ketosterol) was 3β,26-dihydroxy-5α-cholest-8(14)-ene-15-one, which was found as a 10.5:1 mixture of the 25R- and 25S-isomers along with small amounts of its
3α-hydroxy-derivative 3α,26-dihydroxy-5α-cholest-8(14)-en-15-one. The major acidic metabolites of the 15-ketosterol in the Hep G2 cells were 3β-hydroxy-15-oxo-5α-cholest-8(14)-en-26-oic acid and 3β-hydroxy-15-oxo-5α-cholestan-8(14)-en-24-oic acid. Also formed were polar conjugated forms of the metabolites of the 15-ketosterol. No indication of metabolites resulting from reduction of the 15-ketone function were observed, a finding in accord with the lack of formation of Chol from the 15-ketosterol in these cells.

8. 32-Oxygenated sterols

Gibbons et al. (334) reported on the metabolism of lanost-8-ene-3β,32-diol, lanost-7-ene-3β,32-diol, 3β-hydroxylanostan-8-en-32-al, and 3β-hydroxylanost-7-en-32-al (all labeled with ➤3H at C-2) with mouse L cells or primary cultures of fetal mouse liver cells. In L cells (5-h incubation), the major metabolism of each of the sterols was to nonpolar materials (on TLC), which were not identified (but which possibly represent fatty acid esters). Less extensive conversion of the oxygenated sterols to material with the TLC mobility of C27 monohydroxysterols was also observed. Little or no conversion of the sterols to more polar metabolites was observed in the L cells. The major metabolism of the four C32 oxygenated C30 sterols in fetal mouse liver cells (12-h incubation) was also to nonpolar materials (on TLC), which may represent fatty acid esters. Less extensive conversion to material with the TLC mobility of C27 monohydroxysterols was observed. Substantial conversion of lanost-8-ene-3β,32-diol and lanost-7-ene-3β,32-diol into unidentified polar material was reported.

C. Metabolism of Oxysterols in Intact Animals and Human Subjects

1. 7-Oxygenated sterols

Krut et al. (519) reported on the absorption of 7-oxygenated Chol derivatives in the rat. A mixture of ➤14C-labeled 7α-OH-Chol (60.2%), 7β-OH-Chol (17.1%), 7-keto-Chol (22.7%), and [1,2-3H]Chol in a mixture of ethanol (100 µl) and Intralipid (900 µl) was administered by gavage to male Sprague-Dawley rats. Feces were collected daily, homogenized in water, extracted "by the method of Bligh and Dyer," and saponified, and the sterols were recovered and counted. It should be noted that the amount of sterol mass administered was not specified. However, the specific activity of the [4-14C]Chol, from which they were prepared, was reported as 51.3 mCi/mmol, and the specific activity of the [1,2-3H]Chol was given as 50.3 mCi/mmol. Because the saponification conditions were not given, the extent of base-induced decomposition of the labeled 7-keto-Chol cannot be predicted. The solvent used for extraction of the NSL was not given, and the completeness of the extraction of the oxysterols from the feces cannot be predicted. The authors reported cumulative recovery of ➤3H and ➤14C in NSL of feces over 4 days as 25.0 ± 1.1 and 8.5 ± 0.3%, respectively. From this, they stated that this "corresponds to absorption of 75% for Chol and 91.5% for oxysterols."

Björkhem et al. (89) studied the metabolism of [1,2-3H]7-keto-Chol after its administration to rats and guinea pigs with bile cannulation. After intraperitoneal injection in a suspension with albumin to the bile fistula rats, 10–20% of the administered ➤3H was excreted in bile within 48 h. After strong alkaline hydrolysis of the bile, the resulting acids showed a very complex distribution upon chromatographic analyses (a situation which may have been the consequence of the lability of keto-bile acids to strong basic hydrolysis conditions). After intraperitoneal administration of the [1,2-3H]7-keto-Chol to bile duct-cannulated guinea pigs, 5–10% of the injected ➤3H was recovered in bile in the first 24 h. After intraperitoneal injection of [1,2-3H]7β-OH-Chol into bile duct-cannulated rats, 10–25% of the administered ➤3H was recovered in bile within 48 h. After strong alkaline hydrolysis of the bile, the resulting acids showed a very complex distribution of ➤3H. Sarma et al. (895) studied the distribution of ➤14C at 24 h after the intragastric and intravenous administration of [4-14C]7-keto-Chol to female rabbits in an emulsion containing sodium glycocholate and saline. The ➤14C-labeled 7-keto-Chol (250 mg) and sodium glycocholate (400 mg) in saline was provided to each of two rabbits by intragastric administration. Data were presented on the ➤14C-labeled material in lipid extracts of plasma, bile, feces plus intestinal contents, stomach contents, and selected organs or tissues (liver, spleen, adrenals, ovaries, and aortas). The highest concentrations of ➤14C were observed in stomach contents, intestinal contents plus feces, and bile. The lowest levels of ➤14C were observed in aorta. The distribution of ➤14C in each of the samples was analyzed by TLC, which showed ➤14C-labeled material with a variety of mobilities. Noteworthy were the relatively high percentages of material with very high polarity, especially in liver, plasma, and ovaries. The identification of the labeled materials was not presented, nor were data permitting evaluation of the total recovery of the administered ➤14C. Erickson et al. (284) reported that, after perfusion of livers of male Sprague-Dawley rats for 2 h with [3H]7-keto-Chol (either labeled at C-1 and C-2 or "uniformly labeled," >96% of the ➤3H in liver was recovered in lipids. On the basis of TLC, it was estimated that ~70 and ~20% of the ➤3H corresponded chromatographically to 7-keto-Chol and 7β-OH-Chol, respectively. A smaller percentage of ➤3H (~11%) corresponded chromatographically to 7α-OH-Chol. Approximately 3% of the ➤3H corresponded in its TLC behavior to Chol. Although the [3H]7-keto-Chol used in these experiments was reported to contain a low level of labeled Chol, the authors interpreted their findings as
indicating conversion of the 7-keto-Chol to Chol. The authors suggested that the apparent conversion of the 7-keto-Chol to Chol involved the intermediate formation of 7β-OH-Chol and speculated that the latter sterol underwent enzymatic dehydration to give 7-dehydrocholesterol. This matter was not studied further, nor was the [3H]Chol characterized except for TLC. Erickson et al. (284) also examined the distribution of 3H in bile after perfusion of livers of rats with [3H]7-keto-Chol for 2 h. At 1 h of perfusion, 24 ± 6% of the 3H in bile was recovered in the aqueous phase after extraction with CHCl₃ and methanol (2:1). After 2 h of perfusion, the percentage of 3H in bile recovered in the aqueous phase increased to 40 ± 5%. TLC analysis of the lipid extract of bile was reported to show the following percentage distribution: Chol, 23%; 7-keto-Chol, 19%; 7α- and 7β-OH-Chol, 5%; and other unidentified compounds, 54%. TLC analysis of the polar materials in the aqueous phase from the extraction of bile showed the following percentage distribution: taurocholate, 15%; tau-rochenodeoxycholate, 21%; glycocholate, 12%; and unidentifid, 52%. Also described was an experiment with one rabbit in which labeled 7-keto-Chol (25 mg) and sodium glycocholate (50 mg) in a suspension in saline (5 ml) was injected into an ear vein. TLC analyses of the 14C-labeled lipids recovered from the various organs and other samples indicated distribution of 14C into materials with different mobilities. The identification of the labeled materials was not presented, nor were data permitting evaluation of total recovery of the administered 14C.

Lindstedt (577) reported the conversion of [3H]7α-OH-Chol to cholic acid and chenodeoxycholic acid (characterized by reverse-phase chromatography and cocystallization studies) after its intraperitoneal administration to bile duct-cannulated rats. Quantitative data were not presented. Anderson et al. (10) studied the fate of [4-14C]7α-OH-Chol in human subjects with partial biliary diversion after its intravenous administration in 25% human serum albumin containing a small amount of ethanol. Conversion of the labeled 7α-OH-Chol to cholic acid and chenodeoxycholic acid was reported. The acids were characterized by radio-GC and cocystallization experiments.

Norii et al. (716) studied the distribution of 14C in the acidic fraction of bile of bile duct-cannulated rats after the intraperitoneal injection of [4-14C]7β-OH-Chol. Almost all (∼94%) of the administered 14C was recovered in bile within 120 h. The bulk of the 14C was recovered in the four possible stereoisomers of 3,7-dihydroxycholest-5-enoic acid (i.e., the 3β,7α-, 3β,7β-, 3α,7α-, and 3α,7β-dihydoxy-C24 acids) and 3β-hydroxy-7-ketocholest-5-en-24-oic acid. Characterizations of the labeled products were based on reverse-phase column chromatography and cocystallization experiments. Ogura and Yamasaki (725) reported on the fate of [4-14C]7α-OH-Chol and its 3β-stearoyl ester after intraperitoneal administration to male Wistar rats with bile duct cannulation. The free diol and its 3β-stearate ester were converted mainly to cholic acid and chenodeoxycholic acid, which were characterized by reverse-phase column chromatography and cocystallization experiments.

Vlahcevic et al. (1174) conducted extensive studies of the metabolism of a number of labeled 7α-hydroxycholesteryl in human subjects with bile duct cannulation. Specific compounds investigated were 7α-OH-Chol, 7α-hydroxycholesterol-4-en-3-one, 5β-cholestan-3α,7α,26-triol, and 5β-cholestan-3α,7α,12α,26-tetrol. In each case, high percentages of the administered radioactivity were recovered in bile. Also, very little (<5%) of the labeled material in bile was recovered in the CHCl₃ phase upon Folch extraction. Each of the compounds was metabolized to cholic acid and chenodeoxycholic acid (characterized by chromatography and cocystallization experiments) except for the cases of 5β-cholestan-3α,7α,12α-triol and 5β-cholestan-3α,7α,26-tetrol, for which little or no conversion (<3%) to chenodeoxycholic acid was observed. Swell et al. (1077) injected several 7α-hydroxycholesteryl into human patients with essentially complete biliary diversion. 7α-Hydroxycholesteryl studied were 7α-OH-Chol, 7α-hydroxycholesterol-4-en-3-one, 5β-cholestan-3α,7α,26-triol, and 7α,26-dihydroxycholesterol-4-en-3-one. All of these were efficiently converted to primary bile acids (cholic acid and chenodeoxycholic acid, with a preponderance of labeled chenodeoxycholic acid from 7α,26-dihydroxycholesteryl-4-en-3-one).

Conflicting results have been presented regarding one aspect relative to the in vivo metabolism of 7α-OH-Chol. Skrede et al. (991) reported results that were interpreted as indicating the formation of 5α-cholestanol from 7α-OH-Chol in rats and humans. They further suggested that a pathway from Chol to 7α-OH-Chol to cholesta-4,6-dien-3-one to cholesterol-4-en-3-one to 5α-cholestan-3-one and finally to cholestanol to account for the overall conversion. Moreover, they suggested that this pathway may be quantitatively more important in humans with CTX and might account, in part, for the elevated levels of cholestanol in blood and tissues of subjects with CTX. The experiments on which these suggestions were made were based on decreases in the ratios of 3H and 14C in cholestanol recovered from tissues of rats and of feces of rats and a human subject after administration of a mixture of [4-14C]Chol and [7α-3H]Chol. In addition, after intravenous administration of a mixture of [7β-3H]7α-OH-Chol and [4,14C]7α-hydroxycholesterol-4-en-3-one to one patient with CTX, the formation of [3H]cholestanol in plasma, urine, and feces was reported. However, there was no formation of [14C]cholestanol, a finding attributed by the authors to the low amount of 14C administered (0.84 μCi). The samples of [7α-3H]Chol used in this study were reported to have been prepared according to a procedure published by Corey and Gregoriou (216). The latter paper describes two methods for the preparation of [7α-
It is noteworthy that both methods gave varying amounts of cholest-6-en-3β-ol as a by-product. The potential presence of the 3H-labeled Δ5-sterol is of particular relevance to studies of the formation of cholesterol, since reduction of Δ5-steroidal compounds occurs as a discrete step in the overall in vivo dehydroxylation of 7α-hydroxylated bile acids. Relatively little information was provided by Skrede et al. (991) relative to the identity and purity of the putative [7α-3H]Chol. It was stated that the sterols were purified by HPLC before preparation of the mixture to be injected. Oxidation of a mixture of the [3H]Chol and [4-14C]Chol to 7-keto-Chol acetate was reported to result in loss of >99% of the 3H, a finding interpreted as indicating that essentially all of the 3H in the [3H]Chol was at C-7. It should be noted that a similar result could be obtained even if the [3H]Chol sample contained substantial amounts of [3H]cholest-6-en-3β-ol (with the reasonable assumption that the Δ5-sterol would not give the Δ5,7-keto system upon oxidation under the conditions employed). The putative [7α-3H]Chol was also mixed with [4-14C]Chol and incubated with rat liver microsomes. Analysis of the resulting labeled 7α-OH-Chol showed 3H/14C ratios consistent with 81 and 92% of the 3H in two preparations of the [3H]Chol as being in the 7α-configuration. The remaining 19% and 8 were ascribed to 3H in the 7β-configuration. In contrast to the results of Skrede et al. (991), indicating conversion of 7α-OH-Chol to cholesterol, Salen et al. (889) reported that intravenous administration of a mixture of [1,2-3H]cholest-4-en-3-one and [4-14C]7α-OH-Chol to one CTX patient resulted in the appearance of significant amounts of [3H]cholesterol (but not [3H]Chol) in plasma at 2, 7, and 11 days after injection. No conversion of the [14C]7α-OH-Chol to choles-3ol was detected. However, the [14C]-labeled sterol served as an efficient precursor of cholesterol in bile. Little characterization data were given for substrates and products.

Krut et al. (519) reported very rapid clearance of 7α-OH-Chol and 7β-OH-Chol after intravenous administration of a mixture of [26,26,26,27,27,27-2H6]Chol, [26,26,26,27,27,27-2H6]7α-OH-Chol, and [26,26,26,27,27,27-2H6]7β-OH-Chol in “Intralipid” into male Sprague-Dawley rats. Blood sampling was carried out at various times from 5 to 180 min, followed by GC-MS analyses of the sterols of plasma. The remaining fraction of each of the injected sterols in plasma was estimated. Although the indicated more rapid clearance of the two oxygenated sterols appears justified, the location of the label (C-26 and C-27) in the injected substrates is less than ideal in view of possible isotope effects in the formation of side-chain metabolites and the probable formation of the 7-hydroxylated sterols from the δ5-Chol after its intravenous administration. That 7α-OH-Chol is cleared more rapidly than Chol from plasma was indicated by studies of the fate of [4-14C]7α-OH-Chol and [1,2-3H]Chol at 5 min after their intravenous administration in Intralipid to three rats.

HPLC analysis of the 14C sterols present in plasma and liver indicated that most (81%) of the 14C in plasma and liver (∼90%) at this time point had a mobility different from that of 7α-OH-Chol, suggesting the presence of a metabolite(s), the structure of which was not determined. Under the conditions studied, 78% of the administered 14C and 10% of the administered 3H were estimated to have been recovered in the combination of plasma, liver, red blood cells, muscle, fat, lung, and spleen at 5 min after their intravenous injection. Bile was not sampled. In these experiments, the effect of the administration of the sterols in Intralipid was not investigated. Intralipid is a “phospholipid emulsion containing 10 g of triglyceride per 100 ml (Intralipid, Kabi Pharmaceutical) in the ratio of 1.9.”

2. 5,6-Epoxysterols

In one study (304), impure unlabeled 5,6-epoxycholestan-3β-ol was fed to Sprague-Dawley rats at levels of 0.5 and 1.5% for 90 days. TLC analyses of lipids of serum, liver, epididymal fat pads, spleen, and kidney were reported to show no detectable amounts of the epoxysterol in any of the tissues. GC analyses were reported to show that ~50% of the administered sterol was recovered in fecal lipids. Another study (305) involved administration of 1.0–1.2 g of a 10% solution of unlabeled 5α,6α-epoxy-Chol in monolein to Sprague-Dawley rats. Information on the identity and purity of the administered epoxysterol was not presented. The “entire GI tract” was removed, homogenized, extracted, saponified, and studied by GLC and MS. The results of this study suggested conversion to 5α,6β-diolOH-Chol.

Bowden et al. (111) reported on the fate of [4-14C]5α,6α-epoxy-Chol upon intragastric administration in corn oil to two B6C3F1 mice. Approximately 92% of the recovered 14C was found in feces at 18 h after its administration. Small amounts (1.3–2.4% of the recovered 14C) were found in urine and each of the other individual organs studied. Characterization of the chemical nature of the 14C in the various organs was not made. The same workers also studied the distribution of 14C after application of the labeled epoxysterol in acetone to skin of mice. At 18 h after the application of the epoxysterol, most (~65%) of the recovered 14C was found in feces. Most of the 14C in feces was reported to have the chromatographic mobility (TLC and GC) of the administered epoxysterol. Substantial fractions of the total 14C in skin, liver, stomach and duodenum (and their contents), small intestine, large intestine, and feces were recovered in a water-soluble fraction after extraction with CHCl3-methanol (2:1). The nonextractable fraction of the contents of small intestine appeared to be present as polar conjugates as...
indicated by release of $^{14}$C into organic solvent extractable material after treatment with a commercial enzyme preparation with $\beta$-glucuronidase and sulfatase activity. The chemical nature of the $^{14}$C in feces or in the various organs was not established. Apparently, most of the $^{14}$C in the organs studied was more polar than the epoxysterol on TLC.

Bascoul et al. (49) reported efficient absorption of labeled $[^4,^{14}$C]$\alpha$,6$\alpha$-epoxy-Chol after its intragastric administration to male Wistar rats. Little information was provided on the identity and purity of the labeled sterol that was administered to the animals in a sonicated mixture of ethanol and physiological saline. The chemical nature of the labeled sterols in blood and feces was not established, although it was stated that, at the termination of the experiment (7 days), "a large part of the radioactivity was found in the liver and in the fecal matter in the form of acid compounds accompanied by a small population of neutral compounds." Raicht and Cohen (831) reported that administration of $\alpha$,6$\alpha$-epoxy-Chol to male Fischer CD344 rats at an average dose of 40 mg/day (177 mg/kg body wt) in a liquid diet for 17 days resulted in a marked increase in fecal acidic steroids (12.2 ± 4.1 vs. 2.8 ± 1.0 mg/day for controls) but had no effect on fecal neutral sterols. The same authors studied the fecal bile acids after administration of $[^4,^{14}$C]$\alpha$,6$\alpha$-epoxy-Chol to rats. Analyses of the bile acids by GC of the labeled fractions obtained by TLC indicated the presence of lithocholic acid and hyodeoxycholic acid (although it should be noted that the GC mass analysis does not establish the form of acid compounds accompanied by a small population of neutral compounds). Raicht and Cohen (831) provided evidence for the occurrence of liver and in the fecal matter in the form of acid compounds accompanied by a small population of neutral compounds. The chemical nature of the labeled sterols in blood and feces was not established, although it was stated that, at the termination of the experiment (7 days), "a large part of the radioactivity was found in the liver and in the fecal matter in the form of acid compounds accompanied by a small population of neutral compounds." Raicht and Cohen (831) reported that administration of $\alpha$,6$\alpha$-epoxy-Chol to male Fischer CD344 rats at an average dose of 40 mg/day (177 mg/kg body wt) in a liquid diet for 17 days resulted in a marked increase in fecal acidic steroids (12.2 ± 4.1 vs. 2.8 ± 1.0 mg/day for controls) but had no effect on fecal neutral sterols. The same authors studied the fecal bile acids after administration of $[^4,^{14}$C]$\alpha$,6$\alpha$-epoxy-Chol to rats. Analyses of the bile acids by GC of the labeled fractions obtained by TLC indicated the presence of lithocholic acid and hyodeoxycholic acid (although it should be noted that the GC mass analysis does not establish the nature of the labeled material). Information on the nature and purity of the unlabeled and labeled samples of the epoxysterol was not provided. Maerker et al. (602) studied the reactions of $\alpha$,6$\alpha$-epoxy-Chol and 5$\beta$,6$\beta$-epoxy-Chol with simulated gastric juice (pH 1.2) at 37°C. The epoxides were rapidly converted to chlorohydrins, i.e., the $\alpha$,6$\alpha$-epoxide gave 6$\beta$-chloro-3$\beta$,5$\alpha$-diol and the 5$\beta$,6$\beta$-epoxide gave 5$\alpha$-chloro-3$\beta$,6$\beta$-diol. In the presence of the simulated gastric juice, both chlorohydrins underwent facile conversion to $\alpha$,6$\alpha$-dihydroxy-Chol. Upon incubation of the 6$\beta$-chloro-5$\alpha$-cholestan-3$\beta$,5$\delta$-diol with phosphate buffer at pH 7.44 at 37°C, conversion of the chlorohydrin to the $\alpha$,6$\alpha$-epoxide was observed.

3. Cholestane-3$\beta$,5$\alpha$,6$\beta$-triol

Roscoe et al. (860) studied the distribution of $^{14}$C at 24 h after the intragastric administration of $[^4,^{14}$C]$\alpha$,6$\beta$-dihydroxy-Chol (25 mg; ~147 mg/kg) in an emulsion of Tween 80 and water to each of three rats. Of the recovered $^{14}$C, most (84%) was found in feces plus intestinal contents, and 16 and 0.1% were found in the body and urine, respectively. Of the $^{14}$C recovered in the body, most was associated with intestine (32%), carcass (20%), blood (28%), and liver (6%), whereas low levels (~1.4% each) were associated with kidneys, adrenals, testes, spleen, brain, epididymal fat pads, lung, and heart. Of the $^{14}$C recovered in feces and intestinal contents, ~70% was found in neutral sterols, and ~30% was recovered as acids. TLC of the neutral sterol fraction showed several components, none of which was characterized. TLC of the fecal bile acid fraction showed two major labeled components. Roscoe and Fahrenbach (859) studied the fate of $[^4,^{14}$C]$\alpha$,6$\beta$-dihydroxy-Chol after its oral administration (in an emulsion with 10% Tween 80) to male rats. Analysis of fecal lipids led to the isolation of two labeled components for which evidence was presented suggesting the structures 3$\beta$,5$\alpha$-dihydroxycholestan-6-one and a mixture of fatty acid esters of $\alpha$,6$\beta$-dihydroxy-Chol.

4. 24-Oxygenated sterols

Lin and Smith (576) studied the metabolism of $[24$-H$]$(24R)-24-OH-Chol, its C-24 epimer, and $[^4,^{14}$C]Chol by intracerebral injection of an ethanol solution of the sterol(s) (0.5–1 $\mu$Ci) into immature male rats. After periods of 0.5 h to 30 days, pairs of rats were killed, and the excised whole brains were homogenized and separated into subcellular fractions by discontinuous gradient centrifugation. Sterol material was isolated by solvent extraction, separated from other brain lipids by preparative TLC, and analyzed by scintillation counting. The relative uptake and longevity of $[24$-H$]$(24R)-24-OH-Chol and $[^4,^{14}$C]Chol differed somewhat among the various subcellular fractions.

Saucier et al. (900) studied the metabolism of $[3,24$-H$]$(24R)-24-OH-Chol and $[3,24$-H$]$(24S)-24-OH-Chol after intragastric administration to mice. Details of the synthesis and characterization of the labeled sterols were not provided. The limited description provided indicated oxidation of (24RS)-24-OH-Chol with pyridinium chlorochromate to give a crude 3,24-diketone (with the assumption of retention of the double bond at the $\Delta^5$ position; the only characterization reported was an absorption at 1,720 cm$^{-1}$ in the infrared spectrum) followed by reduction of the diketone with labeled sodium borohydride. The 24R- and 24S-isomers were then reported to have been separated by chiral column HPLC. Other than the provision of retention times for the two labeled isomers, no additional characterization was reported. The individual sterols (2.2 mg) were administered in a mixture (0.2 ml) of olive oil (87.5%) and ethanol (12.5%). The levels of the 24-hydroxy-sterol in the free and esterified states in liver were studied by HPLC methodology at various times (from 1 to 8 h) after the administration of the labeled sterols. A very serious limitation of the study was that only one mouse per time point was studied. Ignoring this very real limitation, the levels of total 24-OH-Chol (free plus esters) in liver appeared to be highest at 3 h for both the 24S- and 24R-isomers. After administration of either of the labeled isomers of 24-OH-Chol, most of the 24-OH-Chol was found.
in the ester fraction. It was not noted whether these were monoesters or diesters. In the case of the 24S-isomer, the levels of the 24-OH-Chol found in the free sterol fraction were low and did not appear to change over the period from 1 to 8 h. This finding differed from that made with the 24R-isomer, in which case a more significant fraction of the sterol was found in the free state. With both the 24S- and 25R-isomers, substantial additional amounts of $^3$H in the CHCl$_3$ phase obtained after Bligh and Dyer extraction of liver were recovered in unidentified components of higher polarity that were reported to be found in the free and esterified states. Significant amounts of $^3$H were found in the water phase after the Bligh and Dyer extraction of liver after the administration of either of the isomers of the 24-OH-Chol. This was very much more pronounced in the case of the 24S-isomer. In this case, the highest level of $^3$H in liver was observed at 6 h. With each of the isomers, very little $^3$H was recovered in acidic compounds. In consideration of the latter finding, location of the label in the administered sterols (i.e., at 3 and 24) must be noted. Thus, in the likely formation of a C-24 acid or a ketone at C-24, all of the labeled hydrogen would be lost from this position. The chemical nature of the polar materials formed from the two isomers of labeled 24-OH-Chol was not studied. In the same animals, the levels of $^3$H in NSL were studied, but the chemical nature of the $^3$H was not investigated. After administration of the $[3^3H]$(24S)-24-OH-Chol, the levels of $^3$H in blood were similar at each time point studied from 1 to 8 h. In contrast, the levels of $^3$H in the NSL of blood after intragastric administration of the labeled (24R)-24-OH-Chol appeared to be higher at 2, 3, and 4 h than at 1 and 6 h. Again, only one animal per time point was employed. Saucier et al. (900) also reported the formation of water-soluble $^3$H-labeled material upon incubation of either isomer of the 3,24-$^3$H-labeled material that was not studied further. Erickson et al. (285) studied the distribution of $^3$H after perfusion of livers of rats with [26,27-$^3$H]25-OH-Chol. TLC analyses of subcellular fractions of liver obtained after 30 or 120 min of perfusion showed that a substantial fraction of the recovered $^3$H had the TLC behavior of 25-OH-Chol. $^3$H was also detected in bile but was not studied further. Kandutsch et al. (472) reported on the distribution of $^3$H in blood, liver, and segments of the intestine after intragastric administration of [26,27,3$^3$H]25-OH-Chol (source and purity not specified) into Sprague-Dawley rats (150–200 g; sex not specified). Lipids were extracted according to Folch. The level of total $^3$H in aorta was studied at 1, 2, 5, 7, and 13 days. The maximum level was observed at 5 days, which was much higher than those on days 1 and 13. The distribution of $^3$H in the total lipid extract from brain and liver was studied by TLC with a limited number of standards. In both tissues, on day 1 $^3$H was distributed widely across the TLC plate. However, on day 5, most of the $^3$H showed a very polar behavior on TLC. The chemical nature of the labeled materials was not studied.

5. 25-Oxygenated sterols

Swell et al. (1079) studied the metabolism of [2$^3$H]25-OH-Chol, prepared by Wilzbach labeling, in two human patients with bile duct cannulation and one normal subject by aspiration of duodenal contents. The labeled 25-hydroxysterol, after intravenous administration in 25% human albumin containing 0.3% ethanol, did not serve as an efficient precursor of primary bile acids (reported conversions of 9.7–18.9% to cholic acid and chenodeoxycholic acid). Almost all (>98%) of the radioactivity in bile was reported to be in the aqueous-methanol phase after Folch extraction. Although small amounts of the administered $^3$H were recovered in bile in the form of cholic acid and chenodeoxycholic acid, most of the $^3$H in bile (22 and 31% of the administered $^3$H) was found in polar material that was not studied further. Erickson et al. (285) studied the distribution of $^3$H after intraperitoneal injection of a suspension of [2$^3$H]25-OH-Chol (location of label and purity not presented) into male mice. Interpretation of the results is limited by the use of only one animal per time point and lack of determination of the chemical nature of the recovered $^3$H-labeled material. Nonetheless, the results indicate rapid appearance of $^3$H in blood and liver, perhaps more rapidly than for [2$^3$H]Chol. Formas et al. (310) reported on the distribution of $^3$H after intraperitoneal injection of a suspension of [2$^3$H]25-OH-Chol (location of label and purity not presented) into Sprague-Dawley rats (150–200 g; sex not specified). Lipids were extracted according to Folch. The level of total $^3$H in aorta was studied at 1, 2, 5, 7, and 13 days. The maximum level was observed at 5 days, which was much higher than those on days 1 and 13. The distribution of $^3$H in the total lipid extract from brain and liver was studied by TLC with a limited number of standards. In both tissues, on day 1 $^3$H was distributed widely across the TLC plate. However, on day 5, most of the $^3$H showed a very polar behavior on TLC. The chemical nature of the labeled materials was not studied.

6. 26-Oxygenated sterols

In a very early and important study by Frederickson and Ono (314), biosynthetic (mouse liver mitochondria) $[4,14^1C]26$-OH-Chol or $[4,14^1C]25$-OH-Chol (~0.1 mg in each
case) were administered by intravenous injection (in a suspension in Tween 20 in saline) to bile duct-cannulated rats. $^{14}$C derived from the 26- and 25-hydroxysterols was very rapidly and substantially excreted in bile. Approximately 97 and 80% of the administered label of $[^{4,14}$C]$25$-OH-Chol and $[^{4,14}$C]$26$-OH-Chol, respectively, were excreted in bile in 5 h, whereas under the same conditions, only $\sim$1–2% of the label of $[^{4,14}$C]$Chol$ was recovered in bile. Most (>90%) of the $^{14}$C (derived from the labeled 26- and 25-hydroxysterols) in bile was recovered in polar acid products that were not identified. On the basis of cocrystallization studies with unlabeled cholic acid, essentially none of the $^{14}$C-labeled acids (formed from either labeled 26-OH-Chol or 26-OH-Chol) corresponded to cholic acid. Danielsson (234) studied the metabolism of $[^{4,14}$C]$26$-OH-Chol after its intraperitoneal injection into a bile duct-cannulated rat. During the first 18 h after the injection, a total of 64% of the administered $^{14}$C was recovered in bile. Most of the $^{14}$C had the chromatographic behavior of cholic acid and chenodeoxycholic acid. Wachtel et al. (1175) reported on the distribution of $^{3}$H in bile after the intravenous infusion of $[^{3}$H]$26$-OH-Chol in hamsters. The $[^{3}$H]$26$-OH-Chol, labeled in the Clemmensen reduction of kryptogenin with presumed localization of the $^{3}$H at carbon atoms 16 and 22 (1175), was administered by intravenous injection. Analysis of bile indicated significant conversion to chenoxycholic acid and cholic acid that were characterized by TLC and cocrystallization experiments. Cholic acid was also reported as a biliary metabolite after intravenous administration of the labeled 26-OH-Chol to rats (1175).

Anderson et al. (10) investigated the metabolism of $[^{16,22,3}$H]$26$-OH-Chol in human subjects with partial biliary diversion after its intravenous administration in 25% human serum albumin containing a small amount of ethanol. Conversion to cholic acid and chenodeoxycholic acid was reported. The acids were characterized by radio-GC and cocrystallization experiments. In comparison with the results obtained with simultaneously administered $[^{4,14}$C]$7a$-OH-Chol, a greater proportion of the 26-OH-Chol was converted to chenoxycholic acid. Swell et al. (1077) reported the conversion of $[^{2,3}$H]$26$-OH-Chol to cholic acid and chenodeoxycholic acid after its intravenous administration to human patients with essentially complete biliary diversion. The conversion of the 26-OH-Chol to the two primary bile acids was less than that observed for several $7a$-hydroxysterols. Ayaki et al. (37) studied the metabolism of $[^{16,22,3}$H]$26$-OH-Chol and also of $[^{16,22,3}$H]$3b$-hydroxycholesterol-5-enoic acid after their intravenous administration (0.75–0.90 mmol; vehicle not stated) to bile duct-cannulated male rabbits. Bile was collected “for up to 4 h during which time most of the administered tritium was recovered.” After administration of the labeled 26-OH-Chol, most (84 and 80% in two experiments) of the $^{3}$H in bile was reported to be in the form of cholic acid. However, the data presented indicated that, in each experiment, 12% of the $^{3}$H in bile was recovered as monohydroxy bile acids and 12% was recovered as chenodeoxycholic acid. The labeled cholic acid was characterized by chromatographic and cocrystallization experiments. Although cholic acid was the major $^{3}$H-labeled bile acid formed from the labeled 26-OH-Chol, cholic acid was a minor metabolite (3 and 8% of the labeled material in bile) in two experiments after the administration of $[^{16,22,3}$H]$3b$-hydroxycholesterol-5-en-26-oic acid. In this case, the remainder of the $^{3}$H corresponded to unidentified nonpolar material (34 and 35%), monohydroxy bile acids (39 and 46%), and chenodeoxycholic acid (17 and 13%). The labeled cholic acid and chenodeoxycholic acid, formed from the $\Delta^{5}$-C$_{27}$ acid, was characterized by chromatographic and cocrystallization experiments.

7. $15$-Oxygenated sterols

$3b$-Hydroxy-$5\alpha$-cholesta-8(14)-en-15-one has been shown to be convertible to Chol in rats (115, 116, 912, 914) and baboons (747, 912, 920). The 15-ketosterol is readily absorbed upon intragastric or intraduodenal administration to rats (115, 116, 912) and upon oral administration to baboons (747, 920). Studies in lymph duct-cannulated rats (912) demonstrated that most of the 15-ketosterol is absorbed via lymphatics and that most of the compound in intestinal lymph is found as its fatty acid esters (predominantly the oleate ester) that are associated with chylomicrons. After intragastric administration of the $[^{2,4,3}$H]$15$-ketosterol to rats, most of the $^{3}$H was found in tissues and blood as its fatty acid esters and as Chol and its fatty acid esters (115, 130) with no unusual concentration of $^{3}$H in any organs. Upon oral administration of $[^{2,4,3}$H]$15$-ketosterol and $[^{4,14}$C]$Chol$ to baboons (747, 920), very rapid absorption of the 15-ketosterol was indicated by high levels of $^{3}$H in blood (relative to $^{14}$C) at an early time (4 h) after its administration. Almost all of the 15-ketosterol in plasma was found as its fatty acid esters at all time points studied (from 4 to 24 h). Substantial conversion of the $[^{3}$H]$15$-ketosterol to Chol and Chol esters of plasma was observed. At 4 h, most of the $^{3}$H in plasma was found as esters of the 15-ketosterol; at 24 h, most of the $^{3}$H in plasma was in the form of Chol and Chol esters. Studies of the metabolism of $[^{2,4,3}$H]$15$-ketosterol after its intravenous administration, along with $[^{4,14}$C]$Chol$ to a baboon, demonstrated very rapid disappearance of the $[^{3}$H]$15$-ketosterol from plasma, which was associated with rapid appearance of its fatty acid esters in plasma (917). Decreases in the levels of the free and esterified 15-ketosterol in plasma were associated with increasing levels of $[^{3}$H]$Chol$ and its fatty acid esters. Studies of the metabolism of the $[^{4,14}$C]$15$-ketosterol after its intravenous administration to bile duct-cannulated rats demonstrated very rapid and substantial conversion of the 15-
ketosterol to polar biliary metabolites (914). Approximately 86% of the administered $^{14}$C was recovered in bile in the first 38 h. Of the total amount of $^{14}$C recovered in bile in 38 h, $\sim 50\%$ was excreted in the first 70 min after its administration, and $\sim 90\%$ was excreted within 8 h. Of the $^{14}$C not recovered in bile or other excreta, most ($\sim 79\%$) was in the form of Chol and Chol esters of tissues and blood. After intravenous administration of doubly labeled chylomicrons, obtained from rats with lymph duct cannulation after the intraduodenal administration of the [2,4-$^{3}$H]-15-ketosterol and [4-$^{14}$C]Chol, very rapid and selective uptake of $^{3}$H and $^{14}$C by liver was observed (912). In the administered doubly labeled chylomicrons, most of the $[^{3}$H]15-ketosterol and the [$^{14}$C]Chol were found as their fatty acid esters. The rates of disappearance of $^{3}$H and $^{14}$C from plasma were very similar, as were the rates of uptake of $^{3}$H and $^{14}$C by liver. However, the rate of disappearance of $^{3}$H from liver was much faster than that for $^{14}$C. At early time points (from 3 to 30 min), almost all of the $^{3}$H in liver was in the form of the unesterified 15-ketosterol (indicating hydrolysis of the 15-ketosteryl esters after their uptake by liver). By 120 min after administration of the doubly labeled chylomicrons, most of the $^{3}$H in liver was in the form of Chol and its fatty acid esters. After the intravenous administration of the doubly labeled chylomicrons to bile duct-cannulated rats, very rapid and substantial excretion of $^{3}$H was observed in bile in the form of polar metabolites of the 15-ketosterol. Approximately 80% of the administered $^{3}$H was excreted in bile, with $\sim 56\%$ excreted in the first 80 min. Of the $^{3}$H not excreted in bile at 49 h, most was found in blood and tissues, and almost all of this was in the form of Chol and Chol esters. Very little $^{14}$C was recovered in the 15-ketosterol (or its esters) or in Chol (or its esters) in bile after intravenous administration of the [4-$^{14}$C]15-ketosterol to bile duct-cannulated rats (914). Almost all of the $^{14}$C in bile was recovered as polar material. Further studies of the chemical nature of the labeled polar metabolites in bile indicated considerable complexity (unpublished data). Neutral labeled compounds in bile included $3\beta,26$-dihydroxy-$5\alpha$-cholesta-8(14)-ene-15-one (and its $3\alpha$-hydroxy isomer), $5\alpha$-cholesta-8(14)-ene-3$\beta$,15$\beta$,26-triol (and its $3\alpha$-hydroxy isomer), $5\alpha$-cholesta-8,14-diene-3$\beta$,26-diol (and its $3\alpha$-hydroxy isomer), and 26-OH-Chol. Acidic compounds included $3\beta,26$-dihydroxy-5-oxy-$5\alpha$-cholesta-8(14)-ene-26-oic acid (and its $3\alpha$-hydroxy isomer), $3\beta$-hydroxy-5-oxy-$5\alpha$-cholesta-8(14)-ene-24-oic acid, and bile acids characteristic of the rat (presumably formed from Chol and including chenodeoxycholic acid, $\alpha$-muricholic acid, and $\beta$-muricholic acid). A substantial fraction of a number of the above was present as conjugates (with glucuronic acid, taurine, and sulfate). A simplified summary of the metabolism of the 15-ketosterol, as derived from in vivo studies with rats and studies with rat liver mitochondria, rat liver homogenate preparations, and Hep G2 cells, is shown in Figure 7.

The importance of understanding the metabolism of the 15-ketosterol in the various systems derives in part from the demonstration that a number of these metabolites show high potency in certain biological actions. For example, the (25R)-26-hydroxy, 25-hydroxy, and 24-hydroxy analogs of the 15-ketosterol have been shown to have high potency (approximately the same as the parent 15-ketosterol) in lowering HMG-CoA reductase activity in CHO-K1 cells (485, 916, 1071, 1072) as well as (25R)-5$\alpha$-cholesta-8(14)-ene-3$\beta$,15$\beta$,26-triol (1073). Acidic metabolites, i.e., $3\beta$-hydroxy-5-oxy-$5\alpha$-cholesta-8(14)-en-26-oic acid (982) and $3\beta$-hydroxy-15-oxy-$5\alpha$-cholesta-8(14)-en-24-oic acid (382), lowered reductase activity in the CHO-K1 cells, albeit with a potency considerably less than that of the parent 15-ketosterol. In addition, understanding the me-
metabolism of the 15-ketosterol provided the basis for the rational design of analogs of the 15-ketosterol in which its major metabolism was blocked (1074–1076), leading to increased potency or other favorable actions in in vivo studies in animals (328, 1074).

The high incorporation of orally administered 15-ketosterol into fatty acid esters of chylomicrons of intestinal lymph also has important potential implications with regard to its actions, metabolism, and safety in the intact animal. The combined findings noted above indicate that, after its oral administration, the 15-ketosterol enters the general circulation not as the free sterol but as its fatty acid esters that are associated with chylomicrons, a triglyceride-rich lipoprotein particle for which considerable information exists regarding its fate. It should be noted that traditional pharmacological studies of the metabolism of a drug involve determination of its distribution and fate after its intravenous administration. In the case of the 15-ketosterol, and presumably a number of other oxygenated sterols, such studies should in fact entail the use not of the free sterol in a conventional pharmaceutical vehicle but of the compound in the form in which it enters the circulation after its oral administration, i.e., as its esters which are incorporated into chylomicrons. In the case of the 15-ketosterol, the major in vivo metabolism after its intravenous administration in a pharmaceutical vehicle in the rat corresponded closely to that observed when the compound was administered largely as its fatty acid esters in chylomicrons (912). Chylomicrons themselves are very rapidly metabolized in vivo with very rapid clearance from blood either as such or as chylomicron remnants, with major uptake by liver. The selective delivery of the 15-oxygenated sterol (as its fatty acid esters) to liver via chylomicrons (or chylomicron remnants) also has significant implications with regard to its actions and safety.

After oral administration of the 15-ketosterol, two organs will be exposed to high levels of the 15-ketosterol, i.e., small intestine and liver, both of which are recognized as important in the regulation of newly synthesized Chol, which contributes to Chol in blood plasma. However, it has been shown that in certain animal species, most notably rabbits and marmosets, significant uptake of chylomicrons and chylomicron remnants occurs not only in liver but also in bone marrow (413–415). Perisinusoidal macrophages appear to be intimately involved in the uptake of chylomicrons in the bone marrow of rabbits (413). The uptake of chylomicrons by bone marrow in rabbits was shown to be dependent on the dosage of injected chylomicrons. With high amounts of chylomicrons, relatively high amounts of uptake by bone marrow were observed (413). Lipoprotein lipase appears to be involved in the overall uptake of chylomicrons by liver but not by bone marrow in the rabbit (415). Administration of a monoclonal antibody against lipoprotein lipase to rabbits has been reported to inhibit the uptake of chylomicrons by liver but not by bone marrow (415). In contrast to rabbits and marmosets, the clearance of chylomicrons from plasma was much faster in rats, guinea pigs, and dogs, and the amounts of uptake of the chylomicrons by bone marrow were low in the latter animals (414). Comparable information for humans is not available. It is notable that morphological studies of bone marrow of rats (998) and baboons (unpublished) were found to be normal after oral administration of the 15-ketosterol.

8. Mixtures of oxygenated sterols

Peng et al. (793) reported on studies relative to the absorption of oxygenated derivatives of Chol in rabbits. [4,14C]Chol (50 mCi/mm mol) was maintained at 60°C in air for 5 wk. The resulting mixture was analyzed by TLC. The bulk of the 14C (88%) had the mobility of Chol. Other labeled material corresponded to the general mobility of oxygen sterols (25-OH-Chol, 7α-OH-Chol, 7β-OH-Chol, 7-keto-Chol, and 5α,6β-di-OH-Chol); however, the identity of these polar labeled materials was not established. The mixture of the labeled sterols (as a suspension in gelatin) was administered to rabbits by gastric lavage. Analyses of 14C in serum at 4, 24, and 48 h were reported to show no differences in the distribution on TLC analysis from that observed in the sample before administration. This finding was interpreted as indicating no differences in the rates of absorption of the oxysterols from that of Chol. At 24 h after dosing, materials with the TLC mobilities of chylomicrons (or chylomicron remnants) also had significant implications with regard to its actions and safety in the intact animal. The combined findings noted above indicate that, after its oral administration, the 15-ketosterol enters the general circulation not as the free sterol but as its fatty acid esters that are associated with chylomicrons, a triglyceride-rich lipoprotein particle for which considerable information exists regarding its fate. It should be noted that traditional pharmacological studies of the metabolism of a drug involve determination of its distribution and fate after its intravenous administration. In the case of the 15-ketosterol, and presumably a number of other oxygenated sterols, such studies should in fact entail the use not of the free sterol in a conventional pharmaceutical vehicle but of the compound in the form in which it enters the circulation after its oral administration, i.e., as its esters which are incorporated into chylomicrons. In the case of the 15-ketosterol, the major in vivo metabolism after its intravenous administration in a pharmaceutical vehicle in the rat corresponded closely to that observed when the compound was administered largely as its fatty acid esters in chylomicrons (912). Chylomicrons themselves are very rapidly metabolized in vivo with very rapid clearance from blood either as such or as chylomicron remnants, with major uptake by liver. The selective delivery of the 15-oxygenated sterol (as its fatty acid esters) to liver via chylomicrons (or chylomicron remnants) also has significant implications with regard to its actions and safety.

After oral administration of the 15-ketosterol, two organs will be exposed to high levels of the 15-ketosterol, i.e., small intestine and liver, both of which are recognized as important in the regulation of newly synthesized Chol, which contributes to Chol in blood plasma. However, it has been shown that in certain animal species, most notably rabbits and marmosets, significant uptake of chylomicrons and chylomicron remnants occurs not only in liver but also in bone marrow (413–415). Perisinusoidal macrophages appear to be intimately involved in the uptake of chylomicrons in the bone marrow of rabbits (413). The uptake of chylomicrons by bone marrow in rabbits was shown to be dependent on the dosage of injected chylomicrons. With high amounts of chylomicrons, relatively high amounts of uptake by bone marrow were observed (413). Lipoprotein lipase appears to be involved in the overall uptake of chylomicrons by liver but not by bone marrow in the rabbit (415). Administration of a monoclonal antibody against lipoprotein lipase to rabbits has been reported to inhibit the uptake of chylomicrons by liver but not by bone marrow (415). In contrast to rabbits and marmosets, the clearance of chylomicrons from plasma was much faster in rats, guinea pigs, and dogs, and the amounts of uptake of the chylomicrons by bone marrow were low in the latter animals (414). Comparable information for humans is not available. It is notable that morphological studies of bone marrow of rats (998) and baboons (unpublished) were found to be normal after oral administration of the 15-ketosterol.
heated in air at 150°C for 12 h. The resulting mixture was subjected to chromatography on a silicic acid column. A polar fraction was collected that contained the following (expressed as % by weight): Chol, 12.0; 7α-OH-Chol, 8.8; 7β-OH-Chol, 8.2; 5α,6α-epoxy-Chol, 6.0; 5β,6β-epoxy-Chol, 7.1; 5α,6β-diOH-Chol, 4.7; 7-keto-Chol, 30.2; and unknowns (composed of >20 components), 23.0. The oxidized sterole mixture (25 mg) was administered in an emulsion (3 ml) containing sodium taurocholate (200 mg), fatty acid-free albumin (50 mg), and triolein (200 mg) via a tube into the stomach of rats with a lymph duct cannula. The lymph was collected at intervals over a 24-h period. Control rats received purified Chol (25 mg) in the same vehicle. The oxidized sterole mixture was reported to cause a decrease in lymph flow and, at early times, a decrease in the amount of oleic acid appearing in lymph. Analysis of the oxysterols in lymph was reported to show differences in the absorption of the various sterols, with 7β-OH-Chol being the best absorbed. Oxysterols reported to have been less well absorbed were 7α-OH-Chol, 5α,6α-epoxy-Chol, and 5β,6β-epoxy-Chol. 7-Keto-Chol and 5α,6β-diOH-Chol were reported to be the least absorbed. In each case, most (≥50%) of the oxysterol was reported to be in chylomicrons. The experimental design, i.e., lack of labeled oxysterols and the use of the oxysterol mixture, restricts the interpretation of this experimentation.

Vine et al. (1171) reported studies relative to the absorption of oxygenated derivatives of Chol in lymph duct-cannulated rats. A mixture of Chol and unlabeled oxygenated derivatives of Chol (50 mg) and triolein (600 mg) was infused into the duodenum as a bolus. The oxysterol mixture was prepared by heating purified Chol at 135°C for 10 h and was reported to contain the following sterols: Chol, 69.3%; 7β-OH-Chol, 7.0%; 5α,6α-epoxy-Chol, 4.8%; 5β,6β-epoxy-Chol, 4.8%; 7-keto-Chol, 8.6%; a sterol designated as “6β-hydroxycholesterol,” 1.0%; choleste-4-en-3-one, 0.4%; cholesta-4,6-dien-3-one, 1.5%; cholestane-3,6-dione, 1.0%; and unknowns, 1.6%. In contrast to the results of Osada et al. (735), administration of the oxysterol mixture was reported to have no effect on the intestinal lymph flow. Three oxysterols, i.e., 7β-OH-Chol, 5α,6α-epoxy-Chol, and 7-keto-Chol, were reported to have been recovered in significant amounts in intestinal lymph collected over 12 h, with estimated values of percentage absorption of 7.9, 5.5, and 5.8%, respectively. The peak values of absorption of the three sterols appeared to vary, with maximum values for the 7β-hydroxy-, 7-keto-, and 5α,6α-epoxy-sterols at 3, 4, and 5 h, respectively. 5β,6β-Epoxy-Chol was not detected in intestinal lymph, although present at a level of 4.8% of total sterols administered. The authors suggested that the 5β,6β-epoxysterol was either degraded in intestinal epithelial cells or not incorporated into chylomicrons.

D. Formation of Fatty Acid Esters of Oxysterols

Boyd and Mawer (114) reported the presence of material corresponding to fatty acid esters of 7α-OH-Chol in lipid extracts of human and rat serum. They also observed the same material in rat skin and liver. Hutton and Boyd (416) showed the formation of material with the chromatographic behavior of fatty acid esters of 7α-OH-Chol with a 100,000-g supernatant fraction of rat liver. Sakamoto (888) reported the enzymatic formation and hydrolysis of fatty acid esters (presumed to be esters of the 3β-hydroxyl group) of 7α-OH-Chol in subcellular fractions of rat liver. Gilbert et al. (336) observed the presence of diesters of 26-OH-Chol, mostly as oleate and palmitate esters, in atheroma of human aorta. Smith et al. (993) reported similar findings.

Brown et al. (138) reported the esterification of 25-OH-Chol upon incubation of [14C]25-OH-Chol with human fibroblasts. Drevon et al. (261) also observed the esterification of [3H]25-OH-Chol with rat hepatocytes as indicated by TLC mobility. When the putative ester of the labeled 25-OH-Chol was eluted from the TLC plate and saponified, almost all of the recovered radioactivity had the TLC mobility of 25-OH-Chol. Relatively slight (~5–10%) formation of material with the TLC mobility of fatty acid esters of 25-OH-Chol was observed upon extended (50–60 h) incubations of L929 fibroblasts, Chinese hamster lung cells, and CHO cells with labeled 25-OH-Chol (110). Lichtenstein and Brecher (569) reported the incorporation of [26,27-3H]25-OH-Chol into material with the TLC mobility of 25-OH-Chol. Smith et al. (993) reported similar findings.

Szedlacsek et al. (1082) reported that a number of oxysterols serve as substrates for lecithin:cholesterol acyltransferase (LCAT). With the use of discoidal bilayer particles containing dioleoyl-sn-glycero-3-phosphorylcholine, apoA-I, and individual oxysterols, esterification of the following oxysterols was observed: (25R)-26-OH-Chol, 25-OH-Chol, 7α-OH-Chol, 7β-OH-Chol, 7-keto-Chol, 5α,6α-epoxy-Chol, 5β,6β-epoxy-Chol, and 5α,6β-diOH-Chol. 26-OH-Chol (but not 7α-OH-Chol, 7β-OH-Chol, 25-OH-Chol, or 5α,6β-diOH-Chol) gave not only monoesters at C-3 (as indicated by MS studies) but also 3β,26-diesters. It was suggested that the 3β-hydroxy ester was an intermediate in the formation of the diesters of 26-OH-Chol under the
conditions studied. Lin and Morel (571) reported that incubation of [26,27-3H]25-OH-Chol with human serum at 37°C led to the formation of esters of the 25-OH-Chol. No formation of diesters was observed. The formation of the esters of 25-OH-Chol was ascribed to LCAT present in serum on the basis of inhibition of ester formation by known inhibitors of this enzyme. Esterification of another oxysterol, 3β-hydroxy-5α-cholest-8(14)-en-15-one, upon incubation with FCS has also been observed (unpublished data). These esterifications of oxysterols by serum are of obvious potential importance in the design and evaluation of a number of tissue culture experiments in which oxysterols are studied in media containing serum. Avoidance of these LCAT-catalyzed reactions can be affected by heat treatment of the serum or the use of delipidized serum (thereby removing the acyl donor for the LCAT reaction).

Cheng et al. (194) reported the formation of esters of 25-OH-Chol (with the TLC mobility of the 3β-oleate ester of 25-OH-Chol) upon incubation of [26,27-3H]25-OH-Chol with insect SF9 cells transfected with human acyl-coenzyme A:cholesterol acyltransferase (ACAT) cDNA. They further reported that addition of Chol (26 μM) increased the formation of the esters of the 25-OH-Chol by about threefold. This interesting result was interpreted as indicating that Chol is an activator of ACAT, with the idea that if Chol only served as a substrate (and not an activator of the enzyme), a reduction of 25-OH-Chol ester formation would have been observed.

Lange et al. (543) reported very rapid formation of fatty acid esters of 25-OH-Chol upon incubation of [26,27-3H]25-OH-Chol with rat hepatoma cells. The esterification of the labeled 25-OH-Chol was much more rapid than that of labeled Chol. The formation of esters of 25-OH-Chol (and of Chol) by the hepatoma cells was inhibited by progesterone (10 μM), monesin (2 μM), imipramine (70 μM), chloroquine (70 μM), nigericin (3 μM), and lysophosphatidylcholine (30 μM). Little or no inhibition of the formation of esters of 25-OH-Chol (or of Chol) was observed in in vitro incubations with 800-g supernatant fractions of rat hepatoma cells in the presence of the following compounds: progesterone (6 μM), monesin (1 μM), nigericin (3 μM), chloroquine (80 μM), or lysophosphatidylcholine (15 μM). Sandoz-58035 (1.5 μg/ml), an inhibitor of ACAT, markedly inhibited the in vitro esterification of 25-OH-Chol (and Chol). Morel et al. (672) observed rapid uptake and esterification of [26,27-3H]25-OH-Chol upon its incubation with J774 macrophages. The extent of esterification of the 25-OH-Chol was reported to be ~25% at all time points studied. The ACAT inhibitor Sandoz-58035 had no effect on the cellular uptake of 25-OH-Chol but markedly inhibited its esterification. The esters of the 25-OH-Chol were studied only by TLC and presumably represented monoesters. Gelissen et al. (326) reported high levels of fatty acid esters of 7-keto-Chol in mouse macrophage cells after incubation of the cells with acetylated LDL enriched with 7-keto-Chol (with almost all of the 7-ketosterol present as the free sterol; only ~1.0% was present as fatty acid esters). In the macrophage cells incubated with the acetylated LDL containing the 7-keto-Chol, ~90% of the cellular 7-keto-Chol was esterified.

The results of preliminary studies indicate that rat jejunal microsomes catalyze the oleoyl-CoA dependent esterification of (25R)-26-OH-Chol to give not only monoesters but also diesters of the oxysterol (unpublished data).

VI. ACTIONS OF OXYSTEROLS

A. Effect of Oxyesterols on Sterol Synthesis in Cultured Cells

In 1973, Kandutsch and Chen (466) first reported that certain oxygenated derivatives of Chol inhibited sterol synthesis, as judged by the incorporation of labeled acetate into DPS in mouse L cells and in primary cultures of fetal mouse liver cells incubated in chemically defined media. Their initial study (466) concentrated on three C-7 oxygenated derivatives of Chol, i.e., 7-keto-Chol, 7β-OH-Chol, and 7α-OH-Chol. In 1974, Brown and Goldstein (141) reported a marked inhibition, by 7-keto-Chol, of the synthesis of DPS from labeled acetate, but not mevalonate, by normal human fibroblasts. Subsequent studies from the Kandutsch laboratory, at times involving collaborations with others, extended these observations to oxysterols with oxygen functions at C-5 (469), C-6 (168, 469), C-9 (925), C-11 (152, 928), C-14 (936), C-15 (168, 921, 923, 924, 926, 927, 932-935, 938), and C-32 (931, 933, 937). Oxysterols that showed little or no inhibitory action on sterol synthesis included cholest-5-ene-3β,26-OH-Chol (with almost all of the 7-ketosterol present as the free sterol; only ~1.0% was present as fatty acid esters). In the macrophage cells incubated with the acetylated LDL containing the 7-keto-Chol, ~90% of the cellular 7-keto-Chol was esterified.
2,25-epoxylanosterol (data on identity and purity were not provided) inhibited the incorporation of [3H]acetate into DPS in cultured rat hepatocytes incubated for 17 h in serum-free medium. The potency (IC_{50}) of the epoxy-sterol was rather low, i.e., ~40 nM (estimated from a published graph). Rennert et al. (844) reported that 26-OH-Chol (12.4 nM) caused a marked inhibition of the incorporation of [U-14C]acetate into material with the TLC mobility of Chol in human granulosa cells incubated in a serum-free medium. Javitt and Budai (445) reported that 26-OH-Chol (6 nM) decreased (~12%) Chol synthesis in Hep G2 cells incubated in serum-free medium for 96 h as measured by the incorporation of D_{2}O into Chol. In the same study, 7α-OH-Chol, at concentrations of 6 or 12 nM, had no effect on Chol synthesis under these conditions. In another study from the same laboratory using the same methodology (647), 26-OH-Chol, at 0.63 nM, had no effect on the incorporation of D_{2}O into Chol in Hep G2 cells, whereas it caused a substantial lowering of Chol synthesis in human fibroblasts. 26-OH-Chol and 25-OH-Chol were highly active in inhibiting the incorporation of [2-14C]acetate into "cellular sterols" (as measured by radio-TLC) in rat and human arterial myocytes (218). Mambetisava et al. (611) observed that 3β-hydroxy-5α-cholest-8(14)-en-15-one, at 0.25 nM, caused a 50% inhibition of Chol biosynthesis from [2-14C]acetate in rabbit hepatocytes (as measured by radio-TLC).

B. Effects of Oxysterols on HMG-CoA Reductase in Cultured Cells

In 1973, Kandutsch and Chen (466) reported that three 7-oxygenated derivatives of Chol, i.e., 7-keto-Chol, 7β-OH-Chol, and 7α-OH-Chol, lowered the levels of HMG-CoA reductase activity in mouse L cells and in primary cultures of fetal mouse liver cells. Since this initial discovery, a very large number of oxysterols have been studied with respect to their action on this key regulatory enzyme in these cells. The studies in the Bar Harbor laboratory are notable in that they involved experiments in which cells were incubated in chemically defined media in the absence of added serum or serum lipoproteins. The potencies of various oxysterols in lowering HMG-CoA reductase activity and in the inhibition of sterol synthesis were, in many cases, a little higher in the mouse L cells than in primary cultures of fetal liver cells. However, large differences in the two cell types were observed for a few oxysterols. A striking example was 5α-cholestane-3β,5-diol, which had IC_{50} values for lowering reductase activity of 0.8 nM in mouse L cells and >75 nM in primary cultures of fetal mouse liver cells (469). Another example was 22-keto-Chol, which had IC_{50} values for lowering reductase activity of 3.5 nM in mouse L cells and 62 nM in primary cultures of fetal mouse liver cells (467).

Similar findings were made in the cases of 5α-cholesterol-8(14)ene-3β,15β-diol (L cells, IC_{50} 2.5 μM; liver cells, IC_{50} 16.1 μM) (921), 3β-hydroxy-5α-cholest-8(14)-en-15-one (L cells, IC_{50} 0.3 μM; liver cells, IC_{50} 4.0 μM) (921), 5α-cholesterol-8(14)-ene-3β,7a,15α-triol (L cells, IC_{50} 1.9 μM; liver cells, IC_{50} 18.0 μM) (921), and 14α-ethyl-5α-cholest-7-ene-3β,15α-diol (L cells, IC_{50} 0.2 μM; liver cells, IC_{50} 2.3 μM) (934). In general, the potencies of the oxygenated sterols with respect to lowering of the levels of HMG-CoA reductase activity were very similar to those for the inhibition of sterol synthesis, as measured by the incorporation of labeled acetate into DPS by mouse L cells or primary cultures of fetal mouse liver cells (168, 187, 466, 467, 921, 924, 925, 929, 931, 932, 935, 937, 938). Similar findings were made with Chinese hamster lung (Dede) cells (168). However, in a few cases, the reported potency of an oxysterol in inhibiting sterol synthesis was higher than that for lowering of HMG-CoA reductase activity. For example, this was the case in L cells for the following sterols: 7α-OH-Chol (469), 5α-cholestone-3,6-dione (469), 5α,6β-diOH-Chol (469), 5α,6α-epoxy-Chol (469), two 22-oxygenated derivatives of Chol (467), and several 14α-alkyl-substituted 15-oxygenated sterols (933, 934, 938).

In 1974, Brown and Goldstein (141) reported that incubation of human fibroblasts (previously incubated for 24 h with lipoprotein-deficient media) with 25-OH-Chol or 7-keto-Chol for 24 h at ~12.5 μM resulted in essentially complete suppression of the levels of HMG-CoA reductase activity, whereas Chol (13 μM) caused a 69% suppression of reductase activity. At a shorter incubation time of 4 h, Chol (13 μM) had no effect on reductase activity, whereas complete suppression of reductase activity was observed with 7-keto-Chol. Brown et al. (138) found that incubation of 25-OH-Chol, 7-keto-Chol, and 6-ketocolesterol with human fibroblasts (previously incubated in lipoprotein-deficient media for 24 h) for 5 h lowered HMG-CoA reductase activity. 25-OH-Chol was more potent than the ketosteroids, with an IC_{50} value of ~0.6 versus ~1.3 μM for the ketosteroids. Interestingly, higher concentrations of the oxysterols were reported to result in a virtually complete suppression of reductase activity at ~4 μM. In contrast, Chol and β-sitosterol had no effect on reductase activity under the conditions studied (highest concentration of Chol tested was 6.5 μM). Goldstein et al. (344) observed that a synthetic analog of 7-keto-Chol (SC-31769; 20-oxa-7-ketocolesterol) showed moderate potency in lowering the levels of HMG-CoA reductase activity in human fibroblasts (~80% lowering at 2.5 μM). At 12.5 μM, both SC-31769 and 25-OH-Chol showed an essentially complete lowering of reductase activity.

A very large number of oxysterols have been studied with regard to their potency in lowering HMG-CoA reductase activity in a variety of mammalian cells. Table 4 presents IC_{50} values, reported by the Kandutsch labora-
and Larsson (33) suggested that 3β-hydroxy-5α-chol-8(14)-en-15-one “most likely had to be 27-hydroxylated before being active.” This suggestion ignores the fact that this sterol, which undergoes little or no metabolism in CHO-K1 cells other than formation of its fatty acid esters (748), is highly active in these cells in the lowering of HMG-CoA reductase activity (650, 748, 809, 978, 1072–

In a prior study (187), the 22R- and 22S-isomers of 22-OH-Chol were reported to have the same potency in lowering reductase activity (i.e., IC50 ~3.5 μM), and a sample of (24RS)-24-OH-Chol was reported to show an IC50 value of 0.30 μM.

Among the most potent oxysterols were those with oxygen functions at C-15 or in the alkyl side chain. The major oxysterols present in plasma from normal human subjects, i.e., 26-OH-Chol, (24S)-24-OH-Chol, and 7α-OH-Chol, showed significant but differing potency in lowering HMG-CoA reductase, with IC50 values in the L cells for the 25R- and 25S-isomers of 26-OH-Chol of 0.26 and 0.16 μM; 0.8 μM for (24S)-24-OH-Chol, and 2.5 μM for 7α-OH-Chol. It is also noteworthy that several of the major oxysterols reported to be present in oxidized LDL, i.e., 7-keto-Chol, 5α,6α-epoxy-Chol, and 5α,6β-diol-Chol, showed only moderate or low potency in the L cells (i.e., 1.7, 1.9, 2.5, >25, and 13 μM, respectively).

3β-Hydroxy-5α-chol-8(14)-en-15-one is one of the most potent oxysterol regulators of HMG-CoA reductase and sterol synthesis in cultured mammalian cells. This oxysterol has been shown to have very high potency in the lowering of HMG-CoA reductase activity and/or in the inhibition of the incorporation of labeled acetate into DPS in a variety of cell types including mouse L fibroblasts (1022, 1103), primary cultures of fetal mouse liver cells (1022), Chinese hamster lung (Dede) cells (1022), CHO-K1 cells (650, 748, 805, 809, 978, 1072–1074, 1076, 1197, 1201), rabbit hepatocytes (611), human fibroblasts (33, 34), human transformed fibroblasts (33), human hepatocarcinoma (Hep G2) cells (810), human breast carcinoma cells (33), and human colon carcinoma cells (33). The 15-ketosterol did not lower the level of HMG-CoA reductase activity in human malignant melanoma cells (33) at the single concentration tested (0.12 μM). As shown in Table 4, the 3-keto analog of the 15-ketosterol as well as its 9α-fluoro derivative (and its 3-keto analog) also showed very high potency in lowering HMG-CoA reductase activity in the L cells. It is interesting that even the 3-deoxy analog (i.e., lacking an oxygen function at C-3) retained notable potency in lowering reductase activity. Analog of the 15-ketosterol with hydroxyl groups at C-24 (1072), C-25 (1071, 1072), or C-26 (485, 916) showed high potency, equivalent to that of the parent 15-ketosterol in lowering HMG-CoA reductase activity in CHO-K1 cells. Axelson and Larsson (33) suggested that 3β-hydroxy-5α-chol-8(14)-en-15-one “most likely had to be 27-hydroxylated before being active.” This suggestion ignores the fact that this sterol, which undergoes little or no metabolism in CHO-K1 cells other than formation of its fatty acid esters (748), is highly active in these cells in the lowering of HMG-CoA reductase activity (650, 748, 809, 978, 1072–

### Table 4. Lowering of 3-hydroxy-3-methylglutaryl-CoA reductase activity by C27 oxygenated sterols in mouse L cells and Chinese hamster lung (Dede) cells

<table>
<thead>
<tr>
<th>Sterol</th>
<th>L cells IC50, μM</th>
<th>Dede cells IC50, μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>3β-Hydroxy-5α-chol-8(14)-en-15-one</td>
<td>0.10 (1103)</td>
<td>0.15 (165)</td>
</tr>
<tr>
<td>5α-Chol-8(14)-ene-3,15-dione</td>
<td>0.11 (1103)</td>
<td>-</td>
</tr>
<tr>
<td>(24S)-Chol-5-ene-3β,20-diol</td>
<td>0.16 (1103)</td>
<td>-</td>
</tr>
<tr>
<td>Chol-5-ene-3β,25-diol</td>
<td>0.17 (1103)</td>
<td>0.6 (165)</td>
</tr>
<tr>
<td>9α-Fluoro-5α-chol-8(14)-ene-3,15-dione</td>
<td>0.20 (932)</td>
<td>-</td>
</tr>
<tr>
<td>3β-Hydroxy-5α-chol-8(14)-ene-3,11-diene-15-one</td>
<td>0.20 (1101)</td>
<td>-</td>
</tr>
<tr>
<td>3β-Hydroxy-5α-chol-8(14)-ene-13-onine oxime</td>
<td>0.20 (129)</td>
<td>-</td>
</tr>
<tr>
<td>7,7'-Azocholan-3β,25-diol</td>
<td>0.24 (1101)</td>
<td>-</td>
</tr>
<tr>
<td>15β-Hydroxy-5α,14β-chol-7-ene-3-one</td>
<td>0.25 (935)</td>
<td>-</td>
</tr>
<tr>
<td>(24R)-Chol-5-ene-3β,20-diol</td>
<td>0.26 (1103)</td>
<td>-</td>
</tr>
<tr>
<td>(24S)-Chol-5-ene-3β,24-diol</td>
<td>0.30 (1103)</td>
<td>0.5 (165)</td>
</tr>
<tr>
<td>3β-Hydroxy-5α-chol-8(14)-ene-3α,15-diene-15-one</td>
<td>0.30 (924)</td>
<td>-</td>
</tr>
<tr>
<td>5a-Chol-8(14)-ene-3β,15α-diol</td>
<td>0.31 (936)</td>
<td>-</td>
</tr>
<tr>
<td>5α-Chol-8(14)-ene-3β,15α-diol</td>
<td>0.31 (924)</td>
<td>-</td>
</tr>
<tr>
<td>5α-Chol-8(14)-ene-3β,15β-diol</td>
<td>0.30 (932)</td>
<td>-</td>
</tr>
<tr>
<td>3βa,7β-Dihydroxy-5α-chol-8(14)-ene-15-one</td>
<td>0.40 (932, 1103)</td>
<td>-</td>
</tr>
<tr>
<td>3β,25-Dihydroxycholest-5-ene-7-one</td>
<td>0.41 (924, 1103)</td>
<td>-</td>
</tr>
<tr>
<td>5α-Chol-7-ene-3β,15α-diol</td>
<td>0.50 (924, 1103)</td>
<td>-</td>
</tr>
<tr>
<td>5α-Chol-8(14)-ene-3β,15α-diol</td>
<td>0.50 (924, 1103)</td>
<td>-</td>
</tr>
<tr>
<td>5α-Chol-8(14)-ene-3β,15β-diol</td>
<td>0.50 (924, 1103)</td>
<td>-</td>
</tr>
<tr>
<td>5α-Chol-8(14)-ene-3β,15β-diol</td>
<td>0.50 (924, 1103)</td>
<td>-</td>
</tr>
<tr>
<td>5α-Chol-8(14)-ene-3β,15β-diol</td>
<td>0.50 (924, 1103)</td>
<td>-</td>
</tr>
</tbody>
</table>

Reference numbers are given in parentheses. * No inhibition at highest concentration tested.
1074, 1076, 1197, 1201). Furthermore, synthetic 3β-
hydroxy-25,26,26,27,27,27-heptafluoro-5α-cholest-
8(14)-en-15-one, in which side chain metabolism to form
the 26-hydroxy derivative is blocked by the fluorine sub-
titution, shows high potency, equivalent to the parent
15-ketosterol, in lowering HMG-CoA reductase activity in
both CHO-K1 cells and Hep G2 cells (1076).

Erickson et al. (285) noted that 25-OH-Chol lowered
the levels of HMG-CoA reductase activity in rat hepatocytes incubated in lipid-deficient medium. At concentra-
tions of 25, 50, and 124 μM, the lowering of reductase activity was sustained through a 3-h period, whereas in
cells incubated with 12.4 μM 25-OH-Chol, a marked
decrease in reductase activity was observed at 1 h, which
3 h returned to control levels. Preincubation of the cells
with cycloheximide followed by the addition of 25-OH-
Chol (12.4 μM) resulted in a marked and sustained
(through 3 h) decrease in the levels of reductase activity.
Incubation of hepatocytes with 25-OH-Chol (12.4 μM) for
1 h or with the combination of 25-OH-Chol plus cyclohexi-
midine resulted in a significant shortening of the half-life
(t1/2) of reductase activity in microsomes (27 min with
25-OH-Chol and 21 min with the combination of the oxy-
sterol and cycloheximide) relative to preparations ob-
tained from cells treated with cycloheximide alone (42
min). The metabolism of the 25-OH-Chol by the rat hepa-
tocytes under the incubation conditions was not studied.
However, the authors noted that “the rapid recovery of
activity observed in the 25-hydroxycholesterol experi-
ments may be explained by rapid metabolism of the sterol
to inactive products, permitting synthesis of new reduct-
ase.” Cycloheximide in the presence of 25-OH-Chol may
prevent the recovery of reductase activity either by inhib-
it the synthesis of new reductase or by preventing the
conversion of 25-OH-Chol to inactive products.

Field et al. (300) reported that 25-OH-Chol (in a micelle
preparation containing monolein and taurocholate) low-
ered the levels of HMG-CoA reductase activity in Caco-2
cells at concentrations of 2.5 and 25 μM (but not at 0.25 μM).
Under similar incubation conditions, Chol (commercial; no
data on purity provided) was reported to lower HMG-CoA
reductase activity. However, lowering of reductase activity
by Chol was only observed at very high concentrations (75
and 150 μM, but not at 25 μM). Field et al. (300) also
reported that 25-OH-Chol (2.5 μM), but not Chol (150 μM),
lowered the mass of HMG-CoA reductase as measured by
immunoblotting techniques. The level of mRNA in the
Caco-2 cells for the reductase was lowered (~50%) by 25-
OH-Chol at a concentration of 25 μM (but not at 2.5 μM).
However, it should be noted that the lowering of reductase
activity by 25-OH-Chol at 25 μM was much greater than the
lowering of mRNA for the reductase and that, at 2.5 μM,
significant lowering of enzyme activity, but not mRNA, was
observed. 25-OH-Chol (2.5 μM) accelerated the degradation
of the enzyme (t1/2 = 1.4 h) relative to that observed (t1/2 =
4.6 h) with cells incubated with micelles not containing the
oxysterol. 25-OH-Chol (2.5 μM) also caused a marked de-
crease in the synthesis of HMG-CoA reductase (as deter-
mined by the incorporation of [14C]methionine into immu-
nonprecipitable reductase). Field et al. (300) found no
evidence for a change in the state of phosphorylation of
HMG-CoA reductase in Caco-2 cells to account for the low-
ering of reductase activity caused by 25-OH-Chol in these
cells.

Zhang et al. (1224) reported on the effects of incuba-
tion of 25-OH-Chol and two of its metabolites, cholest-5-
ene-3β,7α,25-triol and 7α,25-dihydroxycholest-4-en-3-one,
on the level of HMG-CoA reductase activity in human
diploid fibroblasts and in a virus-transformed fibroblast
cell line. A 24-h incubation of the cells with the oxyge-
nated sterols was used. The three sterols appeared to have
approximately the same potency in the human diploid
fibroblasts. However, in a virus-transformed fibroblast
cell line, little or no suppression of HMG-CoA reductase
activity was observed with the 3β,7α,25-trihydroxysterol
and the 7α,25-dihydroxy-Δ4-3-ketosterol at a concentra-
tion (0.25 μM) at which 25-OH-Chol lowered reductase
activity. Pregnenolone (5 μM), which by itself had no
effect on HMG-CoA reductase activity in human diploid
fibroblasts, was reported to abolish the lowering effect
of 25-OH-Chol (0.06 and 0.25 μM) on reductase activity.
In contrast, pregnenolone had little or no effect on the
lowering of reductase activity caused by the 3β,7α,25-tri-
hydroxysterol or the 7α,25-dihydroxy-Δ4-3-ketosterol.

Zhang et al. (1224) also studied the effects of incub-
ation of 26-OH-Chol and two of its metabolites, i.e.,
cholest-5-ene-3β,7α,26-triol and 7α,26-dihydroxycholest-
4-en-3-one, on the levels of HMG-CoA reductase activity
in human diploid fibroblasts and in a virus-transformed fi-
broblast cell line. Whereas the 26-OH-Chol and the 7α,26-
dihydroxy-Δ4-3-ketosterol showed approximately the
same potency in lowering reductase activity in the diploid
fibroblasts, the 3β,7α,26-trihydroxysterol showed consid-
ervably less activity. In the virus-transformed fibroblast
cell line, the three sterols showed approximately the same
potency in lowering reductase activity. Pregnenolone (5
μM) abolished the effect of 26-OH-Chol in lowering re-
ductase activity in the human diploid fibroblasts, but it
had no effect on the suppressive action of the 3β,7α,26-
triol and the 7α,26-dihydroxy-Δ4-3-ketosterol on reduct-
ase activity. Zhang et al. (1224) concluded that their
results indicated that 7α-hydroxylation was “not directly
involved, positively or negatively, in the action of 25-
or 26-hydroxycholesterol as suppressors of HMG-CoA
reductase activity.” The effects of pregnenolone in abolish-
ing the suppressive action of 25-OH-Chol (1224) and 26-
OH-Chol (1224) on HMG-CoA reductase activity in human
fibroblasts are similar to the previously reported (759)
effect of progesterone in abolishing the suppressive ac-
tion of (24S)-24,25-epoxy-Chol on HMG-CoA reductase
activity in cultured intestinal epithelial (IEC-6) cells. However, in the latter cells, progesterone had no effect on the lowering of HMG-CoA reductase activity by 25-OH-Chol. In the intestinal cells, progesterone had no effect on the cellular uptake of either (24S)-24,25-epoxy-Chol or 25-OH-Chol (759). Progesterone itself was reported to increase HMG-CoA reductase activity in the IEC-6 cells (759). Shortly thereafter, Martin et al. (620) reported that 26-OH-Chol inhibited the synthesis of Chol, as measured by incorporation of labeled hydrogen of D$_{2}$O, in both CHO cells and human Hep G2 cells. In contrast, cholest-5-ene-reductase activity was observed at 0.25 M oxysterol.

The concentrations of the oxysterols used in these studies (10 μM for CHO cells and 5 μM for Hep G2 cells) appear to be quite high for incubations of cells in medium containing delipidated FCS. It should be noted that the oxysterols were added to the culture medium as a solution in hydroxypropyl-β-cyclodextrin. No characterization of the 3β,7α,26-triol as to identity or purity was presented. Additionally, no information on the source or purity of the 26-OH-Chol was provided. The lack of effect of the 3β,7α,26-triol on Chol synthesis in CHO-K1 cells and Hep G2 cells (620) differs from the reported effects of the triol on HMG-CoA reductase activity in either human diploid fibroblasts or a transformed line of human fibroblasts (1224). In the latter cells, lowering of reductase activity was observed at 0.25 μM oxysterol. Lowering of HMG-CoA reductase activity in human fibroblasts by the added 3β,7α,26-triol (0.25 μM) was also reported by Axelson et al. (34) and Axelson and Larsson (33). In these studies, the potencies of the 3β,7α,26-triol and 26-OH-Chol were similar. Zhang et al. (1225) reported results indicating that 7α,25-dihydroxycholesterol-4-en-3-one lowered HMG-CoA reductase activity in human diploid fibroblasts but that it was considerably less potent than 25-OH-Chol and 7α,25-diOH-Chol in this respect. In another study from the same laboratory (1224), the potency of the same three oxysterols appeared to be roughly similar. Interestingly, pregnenolone (5 μM) or dehydroepiandrosterone (5 μM) blocked the lowering of HMG-CoA reductase activity caused by 25-OH-Chol (but not that caused by 7α,25-diOH-Chol or 7α,25-dihydroxycholesterol-4-en-3-one) (1224). The mechanism of action of the steroids was not established. When added alone, pregnenolone and dehydroepiandrosterone were reported to have no effect on HMG-CoA reductase under the conditions studied. Zhang et al. (1224) reported findings indicating that 26-OH-Chol and 7α,26-dihydroxycholesterol-4-en-3-one had similar potency in lowering HMG-CoA reductase activity in human diploid fibroblasts, whereas 7α,26-diOH-Chol showed a lower potency. As with 25-OH-Chol, pregnenolone (5 μM) or dehydroepiandrosterone (5 μM) blocked the lowering of HMG-CoA reductase activity (but not that caused by 7α,25-diOH-Chol or 7α,25-dihydroxycholesterol-4-en-3-one) (1224).

In consideration of the actions of several potent oxysterols with α,β-unsaturated ketones [i.e., 7α,26-dihydroxycholesterol-4-en-3-one, 7α,25-dihydroxycholesterol-4-en-3-one, 3β,26-dihydroxycholesterol-5-en-7-one, and 3β,26-dihydroxy-5α-cholest-8(14)-en-15-one] in the sterol nucleus, Axelson and Larsson (33) noted that reduction of the olefinic bond led to almost complete loss of their potency in lowering HMG-CoA reductase activity. This idea does not apply to the case of the Δ$^{8(14)}$-15-ketosterol, since one of its saturated analogs (3β-hydroxy-25,26,26,27,27,27-heptafauoro-5α-cholestan-15-one) has been shown to retain the full activity of the parent Δ$^{8(14)}$-15-ketosterol in lowering HMG-CoA reductase activity (1075). Axelson and Larsson (32) also reported that studies of the time course of HMG-CoA reductase lowering in normal human fibroblasts by FCS (interpreted as LDL) was inversely related to the time course of the increases in the levels of 26-OH-Chol in the medium that were induced by the incubation of the cells with FCS.

The results of a number of studies have indicated that 24,25-epoxy-Chol lowers the levels of HMG-CoA reductase activity in mouse L cells (897, 1029, 1102) and in Hep G2 cells (259). The 24S-isomer appeared to be more potent than the 24R-sterol in lowering reductase activity in mouse L cells (1102); however, the reported variation precludes a more definitive statement.

The potencies of a number of 14α-alkyl-, 14α-hydroxymethyl-, and 4,4-dimethyl-substituted oxysterols in lowering HMG-CoA reductase activity in the L cells have also been determined (Table 5).

14α-Alkyl substituted oxygenated sterols represent analogs of lanosterol (and other 14α-methyl sterol precursors of Chol). The first sterols of this type to be studied as potential inhibitors of sterol synthesis were a series of 14α-methyl-15-oxo-sterol (921). Included were 3β-hydroxy-14α-methyl-5α-cholest-7-en-15-one, 3β-methoxy-14α-methyl-5α-cholest-7-en-15-one, 14α-methyl-5α-cholest-7-ene-3β,15α-diol, 14α-methyl-5α-cholest-7-ene-3β,15β-diol, 3β-methoxy-14α-methyl-5α-cholest-7-en-15α-ol, and 3β-methoxy-14α-methyl-5α-cholest-7-en-15β-ol. With the exception of 3β-methoxy-14α-methyl-5α-cholest-7-en-15-one, all of these synthetic 14α-methyl-15-oxo-sterol were highly active in lowering HMG-CoA reductase activity and in the inhibition of sterol synthesis in mouse L cells. It is noteworthy that the 14α-methyl-Δ$^{7}$-15-ketosterol showed the same potency (IC$_{50}$ ~0.3 μM) in L cells in lowering reductase activity and in the inhibition of sterol synthesis (as measured by the incorporation of labeled acetate into DPS). A number of other 14α-alkyl-substituted 15-oxo-sterol were prepared by chemical synthesis (770, 923, 938), were also found to be potent inhibitors of sterol synthesis (923, 934, 938). Frye et al. (317) subsequently reported that 3β-hydroxy-4,14α-trimethyl-5α-cholest-7-en-15-one lowered
HMG-CoA reductase activity in CHO-K1 cells, with a potency (0.5 μM) similar to that (0.8 μM) reported for the same compound in mouse L cells (933). The Δ7-C30-15-ketosterol not only lowered HMG-CoA reductase activity but also inhibited P-450DM. The lowering of reductase activity in CHO cells did not appear to depend on the inhibition of P-450DM since the sterol also lowered HMG-CoA reductase activity in a P-450DM-deficient cell line (AR45). 3β-Hydroxy-4,4,14,20-trimethyl-5α-cholest-8-en-7-one lowered the levels of HMG-CoA reductase activity in CHO-K1 cells with a potency (IC50 0.9 μM) similar to that (IC50 0.5 μM) for the corresponding Δ7-15-ketosterol.

(24S)-24,25-Epoxysterol lowered the levels of HMG-CoA reductase in mouse L cells (1102), rat intestinal epithelial cells (361, 758), rat hepatocytes (757), and CHO-K1 cells (757). The 24S-isomer appeared to be more potent than the 24R-sterol in lowering reductase activity in mouse L cells (1102); however, the reported variation precludes a more definitive statement. Panini et al. (757) reported a striking difference in the potency of the compound in rat hepatocytes and CHO-K1 cells. Inspection of their results indicates IC50 values of ~25 μM in the hepatocytes and ~1 μM in the CHO-K1 cells. In the intestinal epithelial cells, the IC50 value was reported to be 0.24 μM by the same investigators (758). In the latter cells, lanost-8-en-3β,25-diol and its 3-keto derivative showed IC50 values of 0.1 and 0.3 μM, respectively. Panini et al. (757) reported that (24S)-24,25-epoxysterol, at a high concentration (100 μM), lowered the levels of HMG-CoA reductase activity in cultured rat hepatocytes but had no significant effect on the steady-state levels of the mRNA for the reductase. Under the same conditions, 25-OH-Chol (25 μM) caused a comparable lowering of HMG-CoA reductase activity but lowered the levels of reductase mRNA. Ketoconazole was used to block metabolism of the (24S)-24,25-epoxysterol. Ketoconazole had no effect on the lowering of HMG-CoA reductase activity induced by the (24S)-24,25-epoxysterol in either rat hepatocytes or CHO-K1 cells, findings suggesting that the suppressive action of the epoxysterol on reductase activity did not require conversion to an active metabolite. The (24S)-24,25-epoxysterol was also reported to accelerate the degradation of HMG-CoA reductase in both rat hepatocytes and CHO-K1 cells.

14α-Hydroxymethyl sterols are generally considered to be obligatory intermediates in the enzymatic removal of the 14α-methyl group (carbon atom 32) of sterol precursors of Chol. The first demonstration that 14α-hydroxymethyl sterols are potent inhibitors of sterol synthesis was presented in 1976 (937). 14α-Hydroxymethyl-5α-cholest-7-en-3β-ol, shown in independent experiments to be convertible to Chol upon incubation with subcellular fractions of rat liver homogenate preparations (777, 1123), and 14α-hydroxymethyl-5α-cholest-6-en-3β-ol were shown to inhibit the synthesis of DPS and to lower the levels of HMG-CoA reductase activity in cultured mammalian cells (mouse L cells and primary cultures of fetal mouse liver cells). The IC50 values for these two measures of sterol synthesis were approximately the same. This

<table>
<thead>
<tr>
<th>Sterol</th>
<th>IC50 μM</th>
<th>Reference Nos.</th>
</tr>
</thead>
<tbody>
<tr>
<td>4,4-Dimethyl-5α-cholest-7-en-3β,15α-diol</td>
<td>0.1</td>
<td>933</td>
</tr>
<tr>
<td>3β-Acetoxy-14α-ethyl-5α-cholest-7-en-15α-ol</td>
<td>0.1</td>
<td>934</td>
</tr>
<tr>
<td>4,4-Dimethyl-5α-cholest-8(14)-en-15-one</td>
<td>0.2</td>
<td>933</td>
</tr>
<tr>
<td>15α-Hydroxy-4,4-dimethyl-14α-ethyl-cholest-7-en-3-one</td>
<td>0.2</td>
<td>933</td>
</tr>
<tr>
<td>14α-Ethyl-5α-cholest-7-en-3β,15α-diol</td>
<td>0.2</td>
<td>934</td>
</tr>
<tr>
<td>4,4-Dimethyl-14α-ethyl-5α-cholest-7-en-3β,15β-diol</td>
<td>0.2</td>
<td>933, 1103</td>
</tr>
<tr>
<td>4,4-Dimethyl-5α-cholest-7-en-3β,15α-diol</td>
<td>0.3</td>
<td>921, 934</td>
</tr>
<tr>
<td>3β-Hydroxy-14α-ethyl-5α-cholest-7-en-15-one</td>
<td>0.3</td>
<td>921, 934</td>
</tr>
<tr>
<td>4,4,14α-Tri methyl-5α-cholest-7-en-3β,15α-diol</td>
<td>0.3</td>
<td>933</td>
</tr>
<tr>
<td>3β-Hydroxy-4,4-dimethyl-14α-ethyl-5α-cholest-7-en-15-one</td>
<td>0.4</td>
<td>933</td>
</tr>
<tr>
<td>4,4-Dimethyl-14α-ethyl-5α-cholest-7-en-3β,15β-diol</td>
<td>0.4</td>
<td>933</td>
</tr>
<tr>
<td>14α-Hydroxy methyl-5α-cholest-7-en-3β,15α-diol</td>
<td>0.4</td>
<td>931</td>
</tr>
<tr>
<td>14α-Hydroxy methyl-5α-cholest-6-en-3β,15α-diol</td>
<td>0.5</td>
<td>931</td>
</tr>
<tr>
<td>4,4,14α-Tri methyl-5α-cholest-6-en-3β,15α-diol</td>
<td>0.5</td>
<td>933</td>
</tr>
<tr>
<td>14α-Hydroxy methyl-5α-cholest-7-en-3β,15β-diol</td>
<td>0.5</td>
<td>933</td>
</tr>
<tr>
<td>3β-Hydroxy-14α-ethyl-5α-cholest-7-en-15-one</td>
<td>0.6</td>
<td>934</td>
</tr>
<tr>
<td>4,4-Dimethyl-5α-cholest-5-en-3β,15α-diol</td>
<td>0.7</td>
<td>933</td>
</tr>
<tr>
<td>(24S)-24,25-Epoxysterol-4,4,14α-trimethyl-5α-cholest-8-en-3β-ol</td>
<td>0.7</td>
<td>1102</td>
</tr>
<tr>
<td>3β-Hydroxy-4,4,14α-trimethyl-5α-cholest-7-en-15-one</td>
<td>0.8</td>
<td>933</td>
</tr>
<tr>
<td>4,4,14α-Tri methyl-5α-cholest-7-en-3β,15α-diol</td>
<td>1.0</td>
<td>933, 1103</td>
</tr>
<tr>
<td>14α-Hydroxy methyl-5α-cholest-6-en-3β,15α-diol</td>
<td>1.0</td>
<td>931</td>
</tr>
<tr>
<td>4,4,14α-Tri methyl-5α-cholestane-3β,9β-diol</td>
<td>1.0</td>
<td>925</td>
</tr>
<tr>
<td>14α-Methyl-5α-cholest-7-en-3β,15β-diol</td>
<td>1.2</td>
<td>921, 934</td>
</tr>
<tr>
<td>4,4,14α-Tri methyl-5α-cholest-8-en-3β,32-di ol</td>
<td>1.3</td>
<td>933</td>
</tr>
<tr>
<td>4,4-Dimethylcholesterol-5-en-3β,7α-diol</td>
<td>1.5</td>
<td>764, 1103</td>
</tr>
<tr>
<td>3β-Hydroxy-4,4,14α-trimethyl-5α-cholest-5-en-7-one</td>
<td>1.5</td>
<td>764, 1103</td>
</tr>
<tr>
<td>(24R)-24,25-Epoxysterol-4,4,14α-trimethyl-5α-cholest-8-en-3β-ol</td>
<td>1.6</td>
<td>1102</td>
</tr>
<tr>
<td>4,4-Dimethyl-5α-cholest-5-en-3β,7α-diol</td>
<td>1.7</td>
<td>764, 1103</td>
</tr>
<tr>
<td>3β-Hydroxy-14α-ethyl-5α-cholest-7-en-15-one</td>
<td>1.9</td>
<td>934</td>
</tr>
<tr>
<td>14α-Hydroxymethyl-5α-cholest-7-en-3-one</td>
<td>2.0</td>
<td>931</td>
</tr>
<tr>
<td>14α-Methyl-5α-cholestane-3β,7α,15β-triol</td>
<td>2.0</td>
<td>931</td>
</tr>
<tr>
<td>9α-Hydroxy-4,4,14α-trimethyl-5α-cholestan-3-one</td>
<td>3.0</td>
<td>925</td>
</tr>
<tr>
<td>3β-Methoxy-14α-methyl-5α-cholest-7-en-15α-ol</td>
<td>3.0</td>
<td>921</td>
</tr>
<tr>
<td>14α-Hydroxy methyl-5α-cholest-7-en-3β,15α-diol</td>
<td>3.3</td>
<td>931, 1103</td>
</tr>
<tr>
<td>3β-Acetoxy-4,4-dimethyl-14α-ethyl-5α-cholest-7-en-15β-diol</td>
<td>3.5</td>
<td>933</td>
</tr>
<tr>
<td>14α-Ethyl-5α-cholest-7-en-3β,15β-diol</td>
<td>3.5</td>
<td>934</td>
</tr>
<tr>
<td>3β-Methoxy-14α-methyl-5α-cholest-7-en-15β-ol</td>
<td>3.7</td>
<td>921</td>
</tr>
<tr>
<td>14α-Butyl-5α-cholest-7-en-3β,15α-diol</td>
<td>3.6</td>
<td>934</td>
</tr>
<tr>
<td>14α-Propyl-5α-cholest-7-en-3β,15α-diol</td>
<td>3.6</td>
<td>934</td>
</tr>
<tr>
<td>14α-Hydroxymethyl-5α-cholest-8-en-3β-ol</td>
<td>0.8</td>
<td>931, 1103</td>
</tr>
<tr>
<td>3β-Hydroxy-14α-propyl-5α-cholest-7-en-15-one</td>
<td>8.1</td>
<td>934</td>
</tr>
<tr>
<td>14α-Propyl-5α-cholest-7-en-3β,15β-diol</td>
<td>8.1</td>
<td>934</td>
</tr>
<tr>
<td>14α-Butyl-5α-cholest-7-en-3β,15β-diol</td>
<td>&gt;10</td>
<td>934</td>
</tr>
<tr>
<td>3β-Hydroxy-14α-butyl-5α-cholest-7-en-15-one</td>
<td>&gt;10</td>
<td>934</td>
</tr>
<tr>
<td>14α-Ethyl-5α-cholest-7-en-15-one-3β,32-diol</td>
<td>&gt;10</td>
<td>934</td>
</tr>
<tr>
<td>3β-Hexadecanoyloxy-14α-ethyl-5α-cholest-7-en-5α-ol</td>
<td>&gt;10</td>
<td>934</td>
</tr>
</tbody>
</table>
study represented the first indication that the normal regulation of sterol synthesis in animal cells involved not only oxygenated metabolites of Chol but also oxygenated sterol precursors of Chol. These findings were subsequently also made with 14α-hydroxymethyl-5α-cholest-8-en-3β-ol and with the 3-keto analogs of the Δ7- and Δ8-14α-hydroxymethyl sterols (931). The same findings were also made with 14α-hydroxymethyl-5α-cholest-7-en-3β,15α-diol (and its Δ6-isomer) and the corresponding 3-keto analogs of these sterols (931). The concentrations of the sterols required to give 50% suppression of the levels of HMG-CoA reductase were, in general, similar to those required to cause a comparable inhibition of the synthesis of DPS from labeled acetate. Gibbons et al. (334) reported that lanost-8-ene-3β,32-diol and lanost-7-ene-3β,32-diol caused roughly comparable lowering of HMG-CoA reductase activity and inhibition of the synthesis of DPS in both L cells and in fetal mouse liver cells. Essentially identical results were observed in mouse liver cells in a subsequent study (933) in which the lanost-6-ene-3β,32-diol was shown to be more potent than either the corresponding Δ5- or Δ7-compounds. Gibbons et al. (334) also reported that the next putative intermediate in the removal of C-32, i.e., the corresponding aldehyde, showed similar actions on HMG-CoA reductase and on sterol synthesis. Gibbons et al. (334) studied the Δ5- and Δ7-14α-hydroxymethyl derivatives of 24,25-dihydroxysterols and the 32-aldehyde derivatives of the Δ8- and Δ7-lanosterols in Chinese hamster lung cells and in a mutant of those cells which was resistant to the effects of 25-OH-Chol on HMG-CoA reductase. Whereas the four sterols showed high potency in lowering reductase activity in the wild-type lung cells, no effect of the four C-32 oxygenated sterols on reductase activity was observed in the mutant. In the same series of experiments, lanost-8-ene-3β,32-diol also had little or no effect on the synthesis of DPS from labeled acetate in the mutant. In contrast, lanost-7-ene-3β,32-diol and both the Δ8- and Δ7-32-aldehydes showed significant inhibition (albeit somewhat less than that observed in the wild-type cells) of the synthesis of DPS in the mutant. The inhibition of the synthesis of DPS by the three latter sterols in the presence of no demonstrable effect on reductase activity was ascribed to an inhibition, by the oxyesters, of the metabolism of C30 plus C29 sterols (which precipitate poorly with digitonin). Leonard et al. (555) reported that 3β-hydroxylanost-8-en-32-al lowered the level of HMG-CoA reductase, and their results suggested that this oxyester lowers HMG-CoA reductase by suppressing HMG-CoA reductase translation and by increasing enzyme degradation. No effect of the sterol on steady-state levels of mRNA for the reductase was observed at a concentration (5 μM) at which lowering of HMG-CoA reductase activity, protein, and synthesis of the enzyme was observed along with an accelerated degradation of the enzyme. Comparable findings were made in a CHO mutant (189) that is deficient in the removal of the 14α-methyl group of lanosterol.

Cheng et al. (195) reported that penasterol, a 14α-carboxylic acid analog of lanosterol that was isolated from an Okinawan sponge, had in vitro antitumor activity (IC50 8 μM) against mouse LI210 leukemia cells. The effects of the compound on HMG-CoA reductase activity or on 14α-demethylase activity were not reported. Mayer et al. (627) reported that 3β-hydroxylanosta-8,15-diene-32-oic acid, a potent inhibitor of lanosterol 14α-demethylase, is highly active in lowering HMG-CoA reductase activity in Hep G2 cells in medium containing delipidated FCS. Reductase levels were reported to be reduced to 30–60% of control levels by 2–5 nM of the steroidal acid.

An oxime of 3β-hydroxy-5α-cholest-8(14)-en-15-one was found to be highly active in lowering the levels of HMG-CoA reductase activity in cultured mouse L cells (929). The synthetic oxime appeared to be a single compound. However, it was not established as to whether it was the Z- or E-isomer. The potency of the oxime in lowering reductase activity was essentially the same as that for inhibition of sterol synthesis as measured by the incorporation of labeled acetate into DPS. Administration of the oxime (0.15% in diet; 3.61 μmol/g diet) to male Sprague-Dawley rats had little or no effect on food consumption, changes in body weight, or serum Chol levels. Whether or not these findings were the result of a simple lack of absorption of the oxime was not established. Subsequently, Frye et al. (317) reported the syntheses of oximes and substituted oximes of 15-keto-, 7-keto-, and 32-aldehyde derivatives of 24,25-dihydroxysterols (or its Δ7-isomer). Although each of the oximes and oxime ethers appeared to be one compound, it was not established whether these were the E- or Z-isomers. The oxime of 3β-hydroxy-4,4,14α-methyl-5α-cholest-7-en-15-one not only lowered the level of HMG-CoA reductase activity in CHO-K1 cells (IC50 ~0.6 μM) but also inhibited lanosterol 14α-methyl demethylase (P450DM) activity with rat liver microsomal preparations (IC50 3.0 μM). Moreover, the oxime was reported to inhibit the incorporation of labeled acetate into C27 sterols in CHO cells, which was associated with an accumulation of radioactive C30 sterols (IC50 2.0 μM) as measured by radio-TLC. The oxime also lowered the levels of HMG-CoA reductase activity in a P450DM-deficient cell line (AR45), which indicated that the effects of the compound on HMG-CoA reductase were not dependent on the effects of the oxime on P450DM. In general, the decreases in HMG-CoA reductase activity in both wild-type CHO cells and AR45 cells paralleled demonstrated decreases in the levels of reductase protein as measured by immunoblot analysis. It is noteworthy that only the oxime showed high inhibitory potency; the oxime methyl ether and the oxime benzyl ether showed little or no activity on HMG-CoA reductase or P450DM activity. The potency of the oxime of 3β-hydroxy-4,4,14α-tri-
methyl-5α-cholest-7-en-15-one in lowering HMG-CoA reductase activity was essentially the same as that of the parent 15-ketosterol, a finding similar to that observed previously for 3β-hydroxy-5α-cholest-8(14)-en-15-one and its oxime (921, 929). In contrast, the oxime of 3β-hydroxy-4,4,14α-trimethyl-5α-cholest-8-en-7-one was considerably less potent in lowering HMG-CoA reductase activity in CHO cells than was the parent 7-ketosterol. The oxime of 4,4,14α-trimethyl-5α-cholest-7-en-32-αl showed high potency in lowering reductase activity in CHO cells and was 10 times more potent than the corresponding methoxime analog.

In consideration of the actions of the oximes of the various oxygenated sterols, recently reported enzymatic reactions of oximes should be noted. Several nonsteroidal oximes have been shown to be oxidized to the corresponding nitro compounds upon incubation with rat liver microsomes (219, 248). In addition, De Master et al. (248) reported the catalysis by rat liver microsomes of the dehydration of n-butylaldoxime to give the corresponding nitrile and nitrobutane. These reactions required NADPH and were reported to be catalyzed by cytochrome P-450. Boucher et al. (109) observed that the Z-isomers (but not the E-isomers) of benzaldoxime and 4-(hexyloxy)benzaldoxime were dehydrated to the corresponding nitriles in the presence of rat liver microsomes and NADPH or dithionite. Alkylaldoximes also were dehydrated to nitriles under the same conditions and did so more rapidly than aryldaldoximes. Ketoxyximes were not investigated in a similar manner, but it was reported that they did not give a Soret peak at 442 nm with microsomes as was observed in the cases of aldoximes. Jousserandot et al. (460) reported that several ketoxyximes (and aldoximes) were metabolized in a cytochrome P-450-dependent reaction to nitrile oxides plus the corresponding carbonyl product. If the generality of this metabolism extends to the oximes of the 15-ketosterols noted above, relatively rapid metabolism of the sterol oximes could yield the corresponding parent 15-ketosterols (which could be responsible for the observed biological effects).

As noted above, the addition of a wide variety of oxysterols to a variety of cells in culture has been associated with a lowering of HMG-CoA reductase activity. Very recently, Sudjana-Sugiaman et al. (1062) reported increased levels of HMG-CoA reductase activity (+58%, relative to control cells incubated in DMEM with 10% FCS and with a level of 54 ± 37 pmol·min⁻¹·mg protein⁻¹) in COS cells transfected with the cDNA for cyp7a. The cells showed the presence of cyp7a activity and demonstrable but low levels (11–67 ng/mg cell protein) of 7α-OH-Chol. The levels of free Chol and of LDL receptor activity were reported to be unaffected by the transfection. The same workers (1061) also observed that intraperitoneal administration of phenobarbital sodium (100 mg/kg body wt⁻¹·day⁻¹ at 9 A.M.) resulted in variable acute (48 h) effects on the levels of hepatic cyp7a activity and HMG-CoA reductase activity in different strains of rats. In three of nine strains examined, the levels for 7α-hydroxylase and HMG-CoA reductase were increased at 48 h. In one strain (Wistar F rats) in which this was observed, phenobarbital sodium caused a decrease in the level of HMG-CoA reductase in kidney. The increases in liver HMG-CoA reductase activity (and of cyp7a) were accompanied by changes in the level of mRNA.

It has been reported that oxysterols lower the levels of HMG-CoA reductase activity, at least in part, by a nuclear-dependent process, leading to accelerated degradation of HMG-CoA reductase. This process appears to be dependent on the synthesis of a macromolecule(s) that has not been identified. Very recently, Correll et al. (217) reported the identification of trans,trans-farnesol as the nonsterol product of mevalonate that is required for the accelerated degradation of HMG-CoA reductase. Specifically, trans,trans-farnesol, at a concentration of 50 μM (the only level tested), stimulated the degradation of HMG-CoA reductase in Met-18b-2 cells that were permeabilized by digitonin. Farnesyl pyrophosphate (FPP) (50 μM) also showed comparable action. However, the effect of FPP was blocked by the addition of NaF (a known inhibitor of phosphatases). NaF had no effect on the action of the trans,trans-farnesol. Nerolidol, cis,cis-farnesol, and geraniol (all at 50 μM) had little or no effect on the degradation of HMG-CoA reductase. Bradfute and Simoni (118) reported very closely related findings. Farnesyl acetate (38 μM) and the ethyl ether of farnesol (40 μM) inhibited the synthesis of HMG-CoA reductase and accelerated the degradation of HMG-CoA reductase in CHO-K1 cells. No effects on the levels of mRNA for HMG-CoA reductase were observed, leading to the suggestion that the decreased rate of synthesis of the reductase caused by the two farnesol derivatives resulted from changes in the translation of the mRNA for the reductase. In this study, farnesol itself was not tested.

Whether or not oxysterols affect the levels of farnesol in cells is not known. Indications of the substantial formation of farnesol or related isoprenoid alcohols have been noted previously. In an early study (915), the unusual finding was made that trans,trans-farnesol was the major labeled product in the NSL after incubation of an ascites tumor derived from a benzpyrene-induced sarcoma with [14C]mevalonate. The labeled farnesol was characterized by its lack of precipitability with digitonin and by its chromatographic mobility on a silicic acid column and on radio-GC. Bradfute et al. (117) isolated a CHO mutant that was deficient in squalene synthase. After incubation of the cells with [3H]acetate in lipid-poor serum for 24 h, most of the 3H in the NSL was found in farnesol, most of which was found in the culture medium. The labeled farnesol was characterized by the chromatographic behavior of the free alcohol, its catalytically re-
duced product, and its acetate derivative on reverse-phase HPLC and of the free alcohol on normal-phase HPLC. Farnesol, isolated from 20 plates under the same conditions, was also characterized by 1H-NMR and MS. It is noteworthy that the squalene synthase-deficient mutant was initially selected for resistance to the mevalonate-induced acceleration of the degradation of HMG-CoA reductase. In view of the findings of Correll et al. (217), increased levels of farnesol could stimulate increased degradation of HMG-CoA reductase. In this regard, it is important to note that the mutant cells showed findings compatible with increased degradation of HMG-CoA reductase when incubated with a mixture of 25-OH-Chol (6.2 μM) and Chol (65 μM) but little or no change when incubated with mevalonate (20 μM), whereas wild-type cells showed a very substantial decrease in HMG-CoA reductase. If increased levels of farnesol are responsible for the stimulation of the degradation of HMG-CoA reductase and if farnesol is involved in the accelerated degradation of the reductase induced by 25-OH-Chol, the levels of farnesol in the mutant treated with 25-OH-Chol could be anticipated to be quite high. Meigs et al. (637) presented additional results supporting the possible importance of farnesol in the acceleration of the degradation of HMG-CoA reductase. They found that farnesol, at concentrations between 20 and 50 μM, resulted in acceleration of the degradation of the reductase in CHO cells. At 30 μM farnesol, a reduction of the half-life of the reductase from 9.8 to 5.2 h was observed. Treatment of either CHO cells or squalene synthase-deficient cells with a high concentration of mevalonate (20 μM), known to cause an acceleration of the degradation of HMG-CoA reductase, resulted in increases in the intracellular levels of farnesol. Furthermore, CHO cells treated with a high concentration of compactin (50 μM) for 3 h showed a decrease in the intracellular level of farnesol. The authors noted that studies of the effects of farnesol are complicated by the fact that treatment of permeabilized (but not intact) cells with farnesol results in a substantial portion of HMG-CoA reductase protein becoming detergent insoluble, causing a decrease in immunoprecipitable reductase and an apparent increase in its degradation. These findings have implications with regard to the conclusions of Correll et al. (217) made in studies with permeabilized cells. Meigs and Simoni (638) reported the results of additional experimentation indicating that farnesol accelerates the degradation of HMG-CoA reductase. A nonhydrolyzable analog of FPP (SQ-32709) was shown to inhibit FPP activity in vitro with a lysate of CHO cells. After addition of SQ-32709 to transiently permeabilized cells, the inhibitor was shown to inhibit the mevalonate-dependent degradation of HMG-CoA reductase induced by 25-OH-Chol (2.5 μM). The levels of farnesol were not determined.

It should also be noted that incubation of Hep G2 cells with 25-OH-Chol (2.5 μM) has been reported (865) to result in a decreased synthesis of prenyltransferase and decreased levels of mRNA for the prenyltransferase. Because this enzyme catalyzes the formation of FPP, the precursor of farnesol, 25-OH-Chol could, by lowering of prenyltransferase, reduce the levels of farnesol. In the various studies cited above on the effects of farnesol on the degradation of HMG-CoA reductase, the metabolism of farnesol was not investigated. Known metabolites of farnesol, i.e., farnesol (206), farnesoic acid (206), and the various dicarboxylic products of farnesol metabolism reported by Gonzalez-Pacanowska et al. (346) and Bostedor et al. (106), might well be studied as to their effects on the degradation of the enzyme. The effect of oxysterols in accelerating the degradation of HMG-CoA reductase could conceivably involve Ca2+ metabolism. Several oxysterols have been reported to increase Ca2+ uptake in at least some mammalian cells (102, 103, 1228, 1229). Roitelman et al. (855) have reported that Ca2+ appears to be important in the acceleration of the degradation of HMG-CoA reductase caused by mevalonate. Information on the effect of farnesol on intracellular Ca2+ metabolism is obviously of interest. Very recently, Roullet et al. (866) reported that farnesol, but not geraniol or geranylgeraniol, induced a dose-dependent inhibitory effect on L-type Ca2+ currents in vascular smooth muscle cells. However, farnesol alone, at 25 μM, did not affect the basal level of free intracellular Ca2+ in these cells.

A recent study (480) with rats questioned the involvement of farnesol as a mediator in the degradation of HMG-CoA reductase. Administration of zaraogoric acid A (an inhibitor of squalene synthase) to rats resulted in an increase of the level of farnesol in liver (from ~0.1 μg to ~1 μg/g) and a marked reduction in the half-life of hepatic HMG-CoA reductase, findings consistent with the idea that increases in the levels of farnesol are involved in the degradation of the reductase. However, administration of mevalonolactone increased the levels of farnesol in liver but had no effect on the half-life of the reductase. Moreover, intragastric administration of farnesol (500 mg/kg body wt in corn oil) was reported to cause a very substantial increase in the level of farnesol in liver, but to have no effect on the half-life of the reductase. Mevinolin treatment (0.04% in diet for 2 days) and Chol feeding (2% in diet for 2 days) had no effect on the levels of farnesol in liver. Farnesol administration (as above) was reported to markedly increase the levels of farnesol in liver (from ~0.1 to >100 μg/g). Administration of mevalonolactone (1 mg/g body wt in corn oil) also increased the level of farnesol in liver, but the extent of the increase (from ~0.1 to ~0.7 μg/g) was not as high. In a subsequent study from the same laboratory, Lopez et al. (587) reported that whereas administration of farnesol (500 mg/kg) by intubation (presumably intragastric in corn oil as in Ref. 480) to male Sprague-Dawley rats on a chow diet had no effect on the half-life of hepatic microsomal HMG-CoA reduc-
tase (as determined by immunoassay), administration of farnesol to rats fed a chow diet containing mevinolin (0.04% in diet) was reported to cause a decrease in the half-life of HMG-CoA reductase protein and enzyme activity. The farnesol used in these studies was from a commercial source; the purity of this material was not reported.

Jingami et al. (453) presented evidence indicating that the acceleration of the degradation of HMG-CoA reductase by the mixture of Chol (26 μM) and 25-OH-Chol (2.5 μM) involved the transmembrane domains 4 and 5 of the reductase, since a mutant lacking both of these domains did not show accelerated degradation of the enzyme when incubated with the sterol mixture. They also observed that a mutant lacking the N-linked carbohydrate substitution did show the acceleration of the degradation of the enzyme upon incubation with the mixture of Chol and 25-OH-Chol and concluded that a glycosylated reductase is not essential for its accelerated degradation of the reductase induced by the sterol mixture. Chun and Simoni (208) attempted to determine the regions of HMG-CoA reductase that are involved in the regulation of the degradation of HMG-CoA reductase by 25-OH-Chol (and by LDL and mevalonate). Studies were made in CHO deletion mutants of the membrane and linker domains of Syrian hamster HMG-CoA reductase. Also studied were mutants in which the transmembrane regions were replaced with the first transmembrane span of bacteriorhodopsin. Replacement of the transmembrane span 4 of the reductase had no effect on degradation of the enzyme. However, replacement of spans 5 or 6 resulted in a protein possessing a normal rate of degradation but which was not accelerated by 10 μM 25-OH-Chol (or 20 μM mevalonate or LDL). Replacement of span 7 shortened the half-life of the reductase, and the degradation of this mutant protein was accelerated by 20 μM mevalonate but not by 10 μM 25-OH-Chol. Replacement of span 8 prolonged the degradation of this protein and mevalonate accelerated the degradation of the mutant enzyme, whereas 25-OH-Chol had no effect. Skalnik et al. (990) reported findings indicating that the membrane domain of HMG-CoA reductase is sufficient for both localization of the enzyme to the endoplasmic reticulum and its sterol-regulated degradation. Kumagai et al. (522) presented results indicating the importance of the second membrane-spanning domain in the regulated degradation of hamster HMG-CoA reductase. The degradation of HMG-CoA reductase does not appear to involve the ubiquitin system, and the regulated degradation of HMG-CoA reductase did not appear to involve the synthesis or activation of a protease in response to sterols but may involve an increased susceptibility of the reductase to proteases present in the endoplasmic reticulum (630).

Mitchell and Kochevar (656) reported that brefeldin A had no effect on the decrease in HMG-CoA reductase activity of UT-1 cells (a compactin-resistant mutant) induced by the mixture of Chol (31 μM) and 25-OH-Chol (0.5 μM). Their results indicated that brefeldin A had no effect on the dispersal and degradation of the crystalloid endoplasmic reticulum that is induced by the mixture of Chol and 25-OH-Chol. Ridgway and Lagace (852) found that brefeldin A (1 μg/ml) caused a dose-dependent inhibition of the suppression of the levels of mRNA for HMG-CoA reductase caused by 25-OH-Chol (6.2 μM) in CHO-K1 cells. The brefeldin A had this effect only when used at a concentration that caused disruption of the Golgi apparatus. Additional studies were carried out with marsupial kidney (PtK1) cells that were known to be resistant to the effects of brefeldin A in disrupting the Golgi apparatus. These cells displayed lowering of HMG-CoA reductase mRNA when incubated with 25-OH-Chol (6.2 μM). Brefeldin A did not appear to affect lowering of HMG-CoA reductase mRNA caused by 25-OH-Chol (as seen in the CHO-K1 cells). Monesin, an ionophore known to affect the Golgi apparatus, was also shown to antagonize the effects of 25-OH-Chol in lowering HMG-CoA reductase mRNA in CHO-K1 cells. The actions of brefeldin A and monesin were shown to be not due to stabilization of mRNA. The combined findings provided additional evidence for the importance of the Golgi apparatus in the regulation of HMG-CoA reductase caused by 25-OH-Chol.

C. Effects of Direct Addition of Oxysterols to Microsomes and Cell-Free Preparations

Brown and Goldstein (141) reported that direct addition of 7-keto-Chol (at concentrations up to 19 μM, the highest level tested) in ethanol, a cell-free extract of human fibroblasts, had no effect on the levels of HMG-CoA reductase activity. Erickson et al. (284) observed that direct addition of 7-keto-Chol (from 1 μM to 1 mM final concentration) in ethanol to microsomes (or to a 10,000-g supernatant fraction from which the microsomes were subsequently isolated) had no effect on the levels of microsomal HMG-CoA reductase activity. Corsini et al. (218) reported that 26-OH-Chol and 25-OH-Chol had no effect on HMG-CoA reductase activity when added to cell-free extracts of rat arterial smooth muscle cells. Panini et al. (758) observed that (24S)-24,25-epoxylanosterol, lanost-8-ene-3β,25-diol, and 25-hydroxylanost-8-en-3-one had no effect on HMG-CoA reductase activity when added directly to cell-free extracts of intestinal epithelial (IEC-6) cells, whereas each showed significant potency in lowering reductase activity when incubated with the cells. Direct addition of 3β-hydroxy-5α-cholest-8(14)-en-15-one (100 μM) to rat liver microsomes under a variety of conditions had no effect on the levels of HMG-CoA reductase activity (649). In contrast, the 15-ketosterol is highly active, at submicromolar concentrations, in the inhibition
of sterol synthesis and/or in the lowering of HMG-CoA reductase activity in a number of cell types (33, 34, 168, 611, 650, 748, 809, 810, 921, 978, 1022, 1072–1074, 1076, 1103, 1197, 1201). Furthermore, direct addition of the 15-ketosterol (100 μM) to the 10,000-g supernatant fraction of rat liver homogenates had no effect on the levels of cytosolic acetoacetyl-CoA thiolase or HMG-CoA synthase activity (649). Direct addition of 14α-ethyl-5α-cholest-7-ene-3β,15α-diol (100 μM), another potent oxysterol inhibitor of sterol synthesis, to rat liver microsomes or to a partially purified preparation of HMG-CoA reductase had no effect on the level of reductase activity (837).

Solaja et al. (1013) reported that synthetic lanosta-7,9(11)-dien-3α,15α,32-diol, 3β-hydroxylanosta-7,9(11)-dien-32-al, and 3β,32-dihydroxylanost-6-en-11-one inhibited HMG-CoA reductase activity of rat liver microsomes at the single high concentration (100 μM) studied. The Δ7,9(11),32-aldehyde was also reported to cause in vitro inhibition of sterol Δ14-reductase and Δ8-sterol isomerase activities at 200 μM. The IC50 value for inhibition of the former enzyme was reported as 86 μM. The Δ8-11-ketosterol was reported to inhibit sterol Δ8 isomerase activity at 200 μM.

D. Effects of Oxysterols on Enzymes Involved in Cholesterol Biosynthesis Other Than HMG-CoA Reductase

Certain oxygenated sterols have been shown to affect the levels of activity of enzymes other than HMG-CoA reductase. 25-OH-Chol has been shown to lower the levels of activity of cytosolic acetoacetyl-CoA thiolase, HMG-CoA synthase, and mevalonic kinase in CHO-K1 cells (180). Similarly, 3β-hydroxy-5α-cholest-8(14)-en-15-one was demonstrated to lower the levels of activity of not only HMG-CoA reductase but also of cytosolic acetoacetyl-CoA thiolase and of HMG-CoA synthase in CHO-K1 cells (650, 748). Taylor (1098) also reported that 25-OH-Chol and the 15-ketosterol were highly active in lowering HMG-CoA synthase activity in mouse L cells. (25R)-26-OH-Chol, 20α-OH-Chol, and (24S)-24-OH-Chol were also very potent in lowering HMG-CoA synthase activity (1098). Chol was reported to have no effect on this enzyme activity. For 10 oxysterols studied, a very high correlation was reported between the potencies of the oxysterols in decreasing HMG-CoA synthase activity and those for increasing the degradation of HMG-CoA reductase (1098). Correlations were also observed between the effects on HMG-CoA synthase activity in CHO-K1 cells and those on HMG-CoA reductase activity in L cells and between the effects on HMG-CoA synthase activity in CHO-K1 cells and the affinity of the sterols to the oxysterol binding protein in L cells (1098).

Sheares et al. (962) reported that 25-OH-Chol (5 μM) caused a 50% lowering of the levels of mRNA for FPP synthetase mRNA in Hep G2 cells in a medium containing 10% delipidated serum. Rosser et al. (865) also found that 25-OH-Chol (2.5 μM) lowered the levels of mRNA for FPP synthetase in Hep G2 cells and inhibited the synthesis of the enzyme in these cells. Faust et al. (292) reported that the addition of the combination of 25-OH-Chol (1.5 μM) and Chol (31 μM) reduced the levels of squalene synthetase activity (measured by the incorporation of labeled FPP into squalene) in fibroblasts from both normal (−90%) and homozygous FH (−86%) subjects. The experiments were carried out in cells incubated in medium containing lipoprotein-deficient serum for 24 h, followed by incubation for 48 h in the presence of the added sterols. It should be noted that, whereas both HMG-CoA reductase activity and squalene synthetase activity increased in normal fibroblasts upon incubation with medium containing lipoprotein-deficient serum, the time courses for the two activities were different. HMG-CoA reductase activity reached maximum values at −20–30 h; squalene synthetase reached a maximum at 48 h. Thus the effects of the sterols could result from the combination of blocking of the further rise in squalene synthetase activity plus a suppression of the elevated activity of the enzyme system. Nakamura et al. (691) reported that 25-OH-Chol blocked the increase in mRNA for squalene epoxidase that is induced by incubation of cells with medium containing lipoprotein-deficient serum. The IC50 for 25-OH-Chol in HeLa cells was −0.025 μM. In contrast, added commercial Chol showed an IC50 of −26 μM.

Certain oxygenated sterols have been shown not only to lower the levels of HMG-CoA reductase activity but also to inhibit the metabolism of lanosterol and 24,25-dihydrolanosterol. 14α-Ethyl-5α-cholest-7-ene-3β,15α-diol, a synthetic 15-oxygenated sterol (770, 923), is particularly notable in this regard. A second site of action, distal to mevalonate formation, was suggested by the presence of a significant discrepancy between the potency of the 14α-ethyl-Δ7-3β,15α-diol in the inhibition of sterol synthesis in cultured cells as measured by the incorporation of [1,14C]acetate into DPS and its potency in lowering the levels of HMG-CoA reductase activity in the same cells. With most of the oxygenated sterols studied, there was a very close correspondence between the two assays. However, the 14α-ethyl-Δ7-3β,15α-diol caused a 50% inhibition of sterol synthesis in mouse L cells and in primary cultures of fetal mouse liver cells at 0.05 and 0.06 μM, respectively (923, 934), whereas this 15-oxygenated sterol caused a 50% lowering of HMG-CoA reductase activity in the same two cell types, at 0.2 and 2.3 μM, respectively (934). The 14α-ethyl-Δ7-3β,15α-diol, upon incubation with the 10,000-g supernatant fraction of rat liver homogenate preparations, at a concentration of 1.0 μM, caused a 50% inhibition of the synthesis of DPS from labeled acetate and a 50% inhibition of the synthesis of DPS from labeled...
mevalonate (837). Purified Chol and 25-OH-Chol, at the same concentration (1.0 μM), had no effect on the synthesis of DPS from labeled acetate under the same conditions. Preincubation of the 14α-ethyl-Δ7-3β,15α-diol (100 μM) with rat liver microsomes had no effect on the level of HMG-CoA reductase activity or on the activity of purified HMG-CoA reductase (837).

Sonoda and Sato (1019) reported on the effects of a number of side chain-oxygenated derivatives of lanosterol and 24,25-dihydrolanosterol on the conversion of [24-3H]lanosterol to Chol by the 10,000-g supernatant fraction of rat liver homogenate preparations. Under the conditions studied, a single, relatively high (40 μM) concentration of the various oxygenated steroids, the C30 steroids oxygenated at C-24, C-25, and C-26, and the R- and S-isomers of 24,25-epoxylanosterol showed notable inhibitory action, i.e., 43–75% inhibition. Morisaki et al. (679) studied the effects of a number of 15-oxygenated sterols on the conversion of [24,25-3H]24,25-dihydrolanosterol to Chol by the 10,000-g supernatant fraction of rat liver homogenates (as judged by radio-TLC). A single high (40 μM) concentration of the various oxysterols was employed. Based on the amounts of 3H associated with dihydrolanosterol and Chol on TLC, the following percentage inhibition values were reported: 5α-cholest-8(14)-ene-3β,15β-diol, 5; 3β-hydroxy-5α-cholest-8(14)-ene-15-one, 42; 3β-hydroxy-14α-methyl-5α-cholest-7-en-15-one, 48; lanost-7-ene-3β,15β-diol, 51; 14α-ethyl-5α-cholest-7-ene-3β,15β-diol, 68; and 3β-hydroxy-14α-ethyl-5α-cholest-7-en-15-one, 72. Oxyesterols which, at 40 μM, showed very high inhibition were as follows: 3β-hydroxylanost-7-en-15-one, 87; lanost-7-ene-3β,15α-diol, 93; and 14α-methyl-5α-cholest-7-ene-3β,15α-diol, 96.

Sonoda et al. (1020) investigated the effects of a number of 14α-hydroxyxysterols on the conversion of labeled 24,25-dihydrolanosterol to Chol by the 10,000-g supernatant fraction of rat liver homogenates (as judged by radio-TLC). Reported values for the percent inhibition caused by 5 μM concentrations of the various sterols were as follows: lanost-8-ene-3β,32-diol, 42; lanost-7-ene-3β,32-diol, 39; lanost-6-ene-3β,32-diol, 35; lanostane-3β,32-diol, 4; 3β-hydroxylanost-8-en-32-al, 38; and 3β-hydroxylanost-7-en-32-al, 35.

Aoyama et al. (17) reported on the effects of 3β-hydroxy-4,4,14α-trimethyl-5α-cholest-8-en-7-one, 4,4,14α-trimethyl-5α-cholest-8-ene-3β,7β-diol, and 3β-hydroxy-4,4,14α-trimethyl-5α-cholest-8-en-11-one on lanosterol 14α-demethylase activity, using a reconstituted yeast system of cytochrome P-45014DM and NADPH-cytochrome P-450 reductase. The 14α-demethylase activity was monitored by the oxidation of NADPH. The 7-ketosterol inhibited the conversion of 24,25-dihydrolanosterol to the corresponding Δ8,14-sterol by the reconstituted system with an IC50 value of 0.2–0.3 μM. The 7α-hydroxysterol was less potent (IC50 2–3 μM), and the 11-ketosterol showed no inhibitory action under the conditions studied. The 7-ketosterol was found to be a competitive inhibitor of the 14α-demethylation of 24,25-dihydrolanosterol or lanosterol. Whereas the 7-ketosterol alone supported some NADPH oxidation (~20% of the rate observed with 24,25-dihydrolanosterol), no metabolism of the 7-ketosterol was detected. In contrast, the 7α-hydroxysterol was a less effective inhibitor of the demethylation of the 24,25-dihydrolanosterol than the 7-ketosterol, but it supported the oxidation of NADPH as effectively as 24,25-dihydrolanosterol. Under these conditions, the 7α-hydroxysterol was reported to be metabolized to 4,4-dimethyl-5α-cholesta-8,14-diene-3β,7α-diol, which was characterized (only) by GC-MS of its TMS derivative. Spectral studies of the P-450 system after incubation with the 7-ketosterol under aerobic conditions indicated the accumulation of an oxyferro intermediate, and it was concluded that the 7-ketosterol inhibited the reduction of the oxyferro intermediate of cytochrome P-45014DM. Sonoda et al. (1020) also reported that the 7-ketosterol (5 μM) inhibited (~85%) the conversion of labeled lanosterol to Chol in 10,000-g supernatant fractions of rat liver homogenate as judged by radio-TLC. 3β-Hydroxy-4,4,14α-trimethyl-5α-cholesta-5,8,11-trien-7-one showed comparable inhibitory action.

Ganoderic acid B (Fig. 8A) or its methyl ester, at a concentration of 40 μM, had no effect on the conversion of 24,25-dihydrolanosterol to Chol by the 10,000-g supernatant fraction of a rat liver homogenate (504).

However, a 27-nor-analog of ganoderic acid B (Fig. 8B), lacking the carboxylic acid function, showed significant inhibition of the conversion of 24,25-dihydrolanosterol to Chol at concentrations of 40 and 20 μM. The
degree of inhibition appeared to be slightly less than that of 7-keto-24,25-dihydrolanosterol (504).

Certain C_{27} oxygenated sterols have been reported to inhibit the demethylation of lanosterol or 24,25-dihydrolanosterol. Ortiz de Montellano et al. (734) reported that incubation of HTC cells (derived from Morris hepatoma 7288c) with a quite high concentration (30 \mu M) of 7\beta-OH-Chol, 7\alpha-OH-Chol, or (22R)-22-hydroxylesmossterol resulted in a moderate accumulation of labeled material (derived from [3H]mevalonic acid) with the TLC mobility of lanosterol.

### E. Oxysterol Binding Protein

In 1977, Kandutsch et al. (471) reported the presence of a protein species, subsequently designated as oxysterol binding protein (OBP), in the cytosol of mouse L cells that showed high binding affinity for 25-OH-Chol. Subsequent studies (473, 474, 1098, 1103) demonstrated that this protein showed high-affinity binding for a large number of oxysterols. Determination of the apparent binding affinities for a large number of oxygenated sterols revealed, in general, a quite close correlation between the in vitro binding affinity of an individual oxysterol with its potency in lowering HMG-CoA reductase activity in cultured mouse L cells (473, 474, 1098, 1103). Exceptions were noted in the cases of several oxygenated sterols with 3-keto functions which, despite having relatively high potency in lowering HMG-CoA reductase activity, showed no detectable binding to the OBP. These discrepancies were explained by the report of the metabolism of the 3-ketosteroids to the corresponding 3\beta-hydroxysterols (as judged by HPLC analysis) that had both high binding affinity and high potency in lowering reductase activity. Other discrepancies between the in vitro binding results and the potency in lowering reductase activity were noted in the cases of some (but not all) 4,4-dimethyl-substituted oxygenated sterols. Taylor et al. (1109) observed that, whereas the 24S- and 24R-isomers of 24,25-epoxy-Chol showed relatively high in vitro binding affinity to the OBP of L cells (comparable to their potencies in lowering HMG-CoA reductase activity in mouse L cells), neither the 24S- or 24R-isomers of 24,25-epoxylanosterol showed binding activity in the in vitro assay (despite their capacity to lower HMG-CoA reductase activity in the cells). OBP has been purified to apparent homogeneity from the cytosol of mouse L cells (1104), mouse liver (1104), and hamster liver (244), and the cDNA for the rabbit protein has been cloned and sequenced (243). mRNA for the OBP was detected in rabbit liver and brain (243). The cDNA for the human OBP has been cloned (560), and the chromosomal localization has been made for the mouse and human (560).

Whereas the idea that the liganded OBP might represent a repressor of transcription of the gene for HMG-CoA reductase appeared attractive, it has apparently been impossible to demonstrate appropriate specific binding of the liganded protein to DNA (851, 1104) or to show the presence of the liganded protein in the nucleus (851, 1104). Ridgway and co-workers (851, 853) reported that the liganded OBP is found concentrated in the Golgi membranes, and it was suggested that the protein might be involved in the “transport, metabolism, or regulatory actions of oxysterols.” In the course of the development of synthetic compounds that decrease the expression of the LDL receptor caused by 25-OH-Chol (113, 573), a recent study (113) has indicated the presence of a protein in the cytosol of hamster liver with high affinity for 25-OH-Chol, other than the OBP.

Lagace et al. (531) observed that overexpression of rabbit OBP in Chinese hamster ovary (CHO-K1) cells had notable effects on Chol ester formation. In comparison with mock-transfected cells, cells overexpressing the OBP showed a decrease in the formation of Chol esters (as assayed by the incorporation of [3H]oleate into Chol oleate) and decreased levels of ACAT activity in membranes and of ACAT mRNA. In three cell lines overexpressing the OBP, increases in mRNA for the LDL receptor, HMG-CoA synthase, and HMG-CoA reductase were observed. An increase in the incorporation of [14C]acetate into Chol (as judged by TLC) in the cells overexpressing the OBP was also observed. Thus overexpression of the OBP in CHO-K1 cells showed altered Chol metabolism, with decreased synthesis of Chol esters and increased synthesis of Chol. The transfected cells showed similar responses to 25-OH-Chol as controls with regard to its effects in increasing Chol ester formation, in increasing ACAT activity in cellular membrane preparations, and in decreasing the levels of mRNA for HMG-CoA synthase and HMG-CoA reductase. Ridgway et al. (850) recently reported that the OBP of CHO cells can be phosphorylated at multiple serine residues. The OBP was separated into two forms by SDS-PAGE, one hyperphosphorylated (101 kDa) and the other hypophosphorylated (97 kDa). 25-OH-Chol did not affect the phosphorylation status of the OBP or the in vitro binding of labeled 25-OH-Chol.

Brefeldin A, a fungal metabolite known to affect transport through the Golgi apparatus, inhibited phosphorylation of the OBP and also affected the dephosphorylation of the protein.

Bakos et al. (46) reported the presence of cytosolic OBP with high affinity for 25-OH-Chol in three clones derived from a human acute lymphoblastic leukemia cell line when they were grown in serum-free medium. For all three clones, the half-maximal concentration of 25-OH-Chol for lysis of the lymphocytes (20–40 nM) was correlated with the affinity of 25-OH-Chol for the OBP [dissociation constant (K_{D}) = 31 nM]. Ayala-Torres et al. (38) studied the binding characteristics of the OBP in a CEM...
C7 lymphoid cell line (the CEM line was originally established from a patient with acute lymphoblastic leukemia) and in a mutant cell line (M10) resistant to the effects of 25-OH-Chol on cell growth. The binding characteristics of 25-OH-Chol to the OBP in the two cell lines were essentially the same. The levels of mRNA for the OBP in the two cell lines were not affected by 25-OH-Chol (0.3 \( \mu \)M) after incubation for 1, 7, or 24 h. Whereas no differences in mRNA for OBP were observed in the two cell lines, the M10 cells grown in the presence of 25-OH-Chol (0.3 \( \mu \)M) showed decreased levels of mRNA (−50%) for cellular nucleic acid binding protein (CNBP) at 24 h (but not at 1 or 7 h). No comparable changes were seen in the parent CEM C7 cells. The levels of mRNA for CNBP were lowered in the M10 cells at 24 h at concentrations of 25-OH-Chol from 0.03 to 1.0 \( \mu \)M.

**F. Sterol Regulatory Element Binding Proteins**

Sterol regulatory element binding proteins, designated as SREBP-1a, SREBP-1c, and SREBP-2, are transcription factors that are synthesized as membrane-bound precursors and then undergo a specific, two-step cleavage (266, 408, 409, 714, 885, 904, 1183) before entering the nucleus, where they bind to sterol regulatory elements (SRE) in the promoter regions of the genes that encode the LDL receptor (891, 1051, 1180, 1215) and a number of important enzymes involved in sterol biosynthesis. These include HMG-CoA synthase (1183), HMG-CoA reductase (1149), FPP synthase (287, 431), squalene synthase (355, 356), lanosterol synthase (746), and lanosterol 14α-de-methylase (746). The SREBP also bind to the promoter regions of genes for other species involved in lipid metabolism. These include acetyl-CoA carboxylase (558), fatty acid synthase (58, 488, 604), glycerol-3-phosphate acyltransferase (288), stearoyl-CoA desaturase (487, 774, 1084), lepin (488), and adipocyte differentiation (488, 1120). An important review of the SREBP and their roles in the regulation of Chol metabolism were presented by Brown and Goldstein (143) in 1997.

The first step in the proteolytic cleavage of SREBP, to give their mature form which can then be translocated into the nucleus, occurs at a specific site (714) in the luminal loop. This cleavage requires the formation of a complex between the SREBP and another membrane protein, the SREBP cleavage activating protein (SCAP). Sterols (25-OH-Chol plus Chol) have been reported to suppress this first cleavage of SREBP, and this important regulatory event is considered to occur because the sterols abolish the ability of the SREBP-SCAP complex to activate the specific protease involved. SCAP is considered to be a sterol-sensitive species (143, 714) and has a membrane-spanning domain similar to the proposed sterol-sensitive domain in HMG-CoA reductase (714). Sterols are considered to decrease transcription of the genes involved in sterol synthesis, fatty acid synthesis, and the uptake of LDL by blocking the first proteolytic cleavage of SREBP (143) because of the loss of the ability of the SREBP-SCAP complex to activate the first proteolytic enzyme (408, 885, 886). A second proteolytic cleavage of the membrane-bound SREBP has been shown to be required for release from the endoplasmic reticulum (779). The initial proteolytic cleavage of SREBP-2 of CHO cells has been shown to be inhibited by treatment of the cells with sphingomyelinase (904), indicating that changes in the intracellular levels not only of sterols but also of sphingolipids can be involved in the regulation of SREBP processing.

Very recently, Thewke et al. (1112) reported that C18 fatty acids enhance the effect of sterols (25-OH-Chol plus Chol) in lowering the nuclear levels of mature SREBP-1 and SREBP-2 in CHO-K1 cells. The fatty acids studied were oleate (10 \( \mu \)M), stearate (10 \( \mu \)M), and the combination of oleate (10 \( \mu \)M) and stearate (10 \( \mu \)M). The fatty acids alone (i.e., without added sterols) were reported to have no effect on the nuclear levels of mature SREBP-1 or SREBP-2. Oleate (50 \( \mu \)M) potentiated the inhibition by 25-OH-Chol (2.5 \( \mu \)M) of the incorporation of \(^{3}H\)acetate into NSL, but oleate alone had little effect. Oleate (50 \( \mu \)M) suppressed the incorporation of \(^{3}H\)acetate into fatty acids, and 25-OH-Chol (2.5 \( \mu \)M) potentiated this effect. The addition of 25-OH-Chol (2.5 \( \mu \)M) alone (no added oleate) had a suppressive effect on the incorporation of the labeled acetate into fatty acid, and this inhibitory effect was much less than that caused by the addition of oleate or the combination of oleate and 25-OH-Chol. It is important to note that, as stressed by the authors, the effects of C18 fatty acid addition were dependent on the use of lipid-deficient culture medium. Under these conditions, C18 fatty acids appeared to potentiate the cytotoxicity of 25-OH-Chol with the CHO-K1 cells. It is interesting to note that in studies with the same cell line but with another oxysterol [3β-hydroxy-5α-cholest-8(14)-en-15-one] and with media containing FCS, oleate addition did not enhance the inhibitory effect of the oxysterol on cell growth (807). In fact, oleate (82 \( \mu \)M) reduced the inhibition of cell growth caused by the 15-ketosterol (a change from IC\(_{50}\) of 13 to 25 \( \mu \)M). A synthetic ACAT inhibitor abolished the effect of oleate and reduced the concentration of the 15-ketosterol to inhibit growth by 50% to 5 \( \mu \)M. The combined findings indicated that the exogenous oleate, the acyl-CoA form of which is a favored substrate for ACAT, provides additional fatty acid substrate for esterification of the 15-ketosterol, thereby reducing the levels of free 15-ketosterol in the cells and increasing the capacity of the cells to grow in otherwise inhibitory concentrations of this sterol.

Very recently, Worgall et al. (1205) reported that polyunsaturated fatty acids and/or oleate (but not satu-
rated fatty acids) reduced the transcription of SRE-dependent genes in CHO, Hep G2, and CV-1 cells. Oleate (150 μM) reduced the level of mRNA for HMG-CoA synthase in CV-1 cells to approximately the same level as that caused by the combination of Chol (26 μM) and 25-OH-Chol (2.5 μM). Oleate also reduced the level of SREBP-2. Polyunsaturated fatty acids were more potent than oleate in reducing SRE expression in CV-1 cells. The effect of the unsaturated fatty acids was considered to be “independent of but additive to exogenous sterols.” However, the possibility that the unsaturated fatty acid addition led to the formation of oxysterols by autoxidation of Chol during the 24-h incubations was not excluded.

Swinnen et al. (1081) reported that a synthetic androgen stimulates the expression of SREBP-1 and SREBP-2, increases the levels of the mature form of SREBP in the nucleus of the prostatic adenocarcinoma cell line LNCaP, and increases the levels of mRNA for HMG-CoA synthase, HMG-CoA reductase, and FPP synthase as well as those for acetyl-CoA carboxylase and fatty acid synthase. No effects of the androgen were observed in the androgen receptor-negative prostate cancer cell lines PC-3 and DU-145. Swinnen et al. (1080) also presented evidence for a SRE sequence in the promoter for the diazepam-binding inhibitor/acyl-CoA-binding protein in LNCaP prostate cancer cells and that this sequence can bind SREBP.

The genes for SREBP-1 and SREBP-2 have been cloned and sequenced (407, 655). Although mutations in SREBP have not been described in humans, mutations in SREBP-2 or in SCAP have been associated with resistance to the effects of 25-OH-Chol in hamster cell lines (408, 715). A Drosophila homolog (HLH106) of mammalian SREBP-1a has been identified (863, 1110). When expressed in mammalian cells, HLH106 was proteolytically processed in a SCAP-stimulated manner (863). Because de novo sterol synthesis is commonly considered to be absent in insects, the role of the HLH106 may involve regulation of fatty acid metabolism and perhaps the synthesis of isoprenoids including juvenile hormones. It will be of interest to study the effects of ecdysone (an oxygenated sterol) on the processing and actions of HLH106.

Shimomura et al. (973) showed that Chol feeding in hamsters resulted in decreases in the nuclear levels of mature SREBP-1c and SREBP-2 in liver. Administration of Chol (0.1–1%) reduced the level of nuclear SREBP-1c without affecting the levels of its precursor in membranes or the level of its mRNA. The authors proposed that Chol feeding affected SREBP-1c in liver by a reduction in the proteolytic processing of its precursor form. Dietary administration of Chol markedly lowered the levels of nuclear SREBP-2. At high levels of Chol supplementation, mRNA levels for SREBP-2 were also decreased. The authors proposed that Chol feeding affected SREBP-2 not only by decreasing the proteolytic processing of its precursor but also by decreasing its synthesis. In further studies from the same laboratory with mice, Horton et al. (402) demonstrated that fasting (24 h) resulted in profound decreases in the levels of nuclear SREBP-1 and SREBP-2 in liver. Fasting also resulted in decreases in liver mRNA levels for SREBP-1 and SREBP-2 as well as those for LDL receptor, HMG-CoA synthase, squaene synthase, acetyl-CoA carboxylase, fatty acid synthase, and stearoyl-CoA desaturase. All of the effects of fasting were reversed by refeeding.

Shimano et al. (972) studied mice with a disruption in the gene that codes for SREBP-1a and SREBP-1c. A substantial fraction (50–85%) of the homozygous gene-disrupted mice died in utero. Survivors showed increased levels of SREBP-2 in liver and increased hepatic sterol biosynthesis as indicated by the incorporation of 3H into DPS after the intraperitoneal administration of 3H-labeled water. The mice showed increased hepatic levels of mRNA for enzymes involved in sterol biosynthesis (HMG-CoA synthase, HMG-CoA reductase, FPP synthase, and squaene synthase) but not those for enzymes involved in fatty acid synthesis.

Very interesting results have been obtained from studies of transgenic mice expressing truncated versions of either SREBP-1a (970), SREBP-1c (971), or SREBP-2 (403). The livers of SREBP-2 transgenic mice were of normal weight but contained elevated levels of Chol (predominantly due to an increase in Chol esters) and triglycerides. The livers of SREBP-1a transgenic mice were considerably enlarged and contained markedly elevated levels of total Chol and triglycerides. The livers of SREBP-2 transgenic mice contained elevated levels of total Chol (which were not increased to the extent seen in the SREBP-1a animals). Only modest elevations of triglycerides were observed in the livers of the SREBP-1c and SREBP-2 transgenic mice. mRNA levels in liver for enzymes involved in sterol synthesis (HMG-CoA synthase, HMG-CoA reductase, and squaene synthase) were increased in the SREBP-1a and SREBP-2 transgenic mice (more so for the SREBP-2 animals). mRNA levels for the LDL receptor were also elevated in SREBP-1a and SREBP-2 mice. The levels of mRNA for enzymes involved in fatty acid synthesis were elevated in the SREBP-1a and SREBP-2 transgenic mice, with higher relative values for the SREBP-1a mice. At 1 h after the intraperitoneal injection of 3H-labeled water, the livers of the SREBP-1a and SREBP-2 transgenic mice showed increased incorporation of 3H into DPS (more marked in the case of the SREBP-2 mice) and increased incorporation of 3H into fatty acids in the SREBP-1c, SREBP-1a, and SREBP-2 mice (with a very large increase in the SREBP-1a mice). Interestingly, the mRNA in epididymal fat pads for enzymes involved in sterol biosynthesis and for the LDL receptor were elevated in SREBP-2 transgenic mice without significant changes in the levels of mRNA for enzymes involved in fatty acid synthesis (acetyl-CoA car-
boxylase and fatty acid synthase) and for stearoyl-CoA desaturase.

Parraga et al. (774) very recently reported the crystal structure of the DNA-binding domain of SREBP-1a bound to a portion of the SRE derived from the human LDL receptor gene promoter.

G. Oxysterols and Orphan Nuclear Receptors

In 1996, Janowski et al. (441) reported that certain oxysterols activate transcription via the nuclear receptor LXRα. (22R)-22-OH-Chol was reported to show the highest potency in activation of LXRα. The unnatural 22S-isomer, at the same concentration (10 μM), showed no activation. Other oxysterols that showed high activity were (20S)-20-OH-Chol, a 20,22-diOH-Chol (configuration at C-20 and C-22 not specified), a 24-OH-Chol (configuration at C-24 not specified), and 4β-OH-Chol. Oxysterols that activated LXRα but with lower potency than the aforementioned included 26-OH-Chol, 25-OH-Chol, and 7α-OH-Chol. In another series of experiments, the half-maximal effective concentrations (EC₅₀) for activation were reported as follows: (22R)-22-OH-Chol, 1.5 μM; (20S)-20-OH-Chol, 1.6 μM; 24-OH-Chol, 1.6 μM; 25-OH-Chol, 3.5 μM; and 7α-OH-Chol, 3.5 μM. Oxysterols that were reported to show little or no activation were a 7,25-di-OH-Chol (configuration at C-7 not specified) and 17α-OH-Chol. Chol, lanosterol, farnesol, and a number of C₂₁, C₁₉, and C₁₈ steroid hormones were reported to show no activity. It was stated that “the concentrations at which these sterols elicit an LXRα response are within their known physiological range.” In 1970, Dixon et al. (257) reported that (22R)-22-OH-Chol and (20α,22R)-20,22-diOH-Chol occur in bovine adrenal glands at levels of 7.33 and 5.14 μmol/kg, respectively. These values would not include fatty acid esters. No evidence for the occurrence of sulfate esters of the (22R)-22-OH-Chol or of the 20α,22R-diOH-Chol was obtained. In our own studies (521), the presence of (22R)-22-OH-Chol (free plus fatty acid esters) in plasma from normal human subjects was not observed (with a detection limit of ~0.003 μM). Using a scintillation proximity technique to measure ligand binding constants for LXRα and LXRβ, Janowski et al. (440) recently reported inhibition constant (Kᵢ), Kᵣ, and EC₅₀ values for a series of sterols oxidized in the side chain and/or in ring B. As expected, the Kᵢ and Kᵣ values (~0.1–2 μM) were lower than the EC₅₀ values (2–10 μM). The low Kᵢ and Kᵣ values give some plausibility to the statement that “oxysterols bind directly to LXR at concentrations that occur in vivo” and to “the hypothesis that naturally occurring oxysterols are physiological ligands for LXR.” High activation of LXRα and low Kᵢ values were observed for several oxysterols and C₂₄ steroids, including (24S)-24,25-epoxy-Chol, (24S)-5α,6α;24,25-diepoxychol, methyl 3β-hydroxychol-5-en-24-oate, and N,N-dimethyl-3β-hydroxychol-5-en-24-amide.

Lehman et al. (562) observed that certain oxysterols activated two nuclear orphan receptors, LXRα and LXRβ. With the sterols studied, the highest activation of LXRα was observed with (24S)-24-OH-Chol and 24-keto-Chol. Lower levels of activation were observed with (24S)-24,25-epoxy-Chol, (20S)-20-OH-Chol, (22R)-22-OH-Chol, and (24R)-24,25-epoxy-Chol. Each sterol was studied at a concentration of 10 μM. Little or no effect was observed with 7α, (22S)-22, (24R)-24, 25-, or 26-OH-Chol. Activation of LXRβ was highest with (24S)-24-OH-Chol, 24-keto-Chol, and (24S)-24,25-epoxy-Chol. Substantial activation was also reported for (24R)-24,25-epoxy-Chol, (20S)-20-OH-Chol, and (22R)-22-OH-Chol. Lower levels of activation were reported for (24R)-24-OH-Chol and 25-OH-Chol. Little or no activation of LXRβ was reported for 7α-OH-Chol, (22S)-22-OH-Chol, and 26-OH-Chol. Current evidence indicates that both LXRα and LXRβ are expressed in liver (552). LXRβ, also known as OR-1 (1105), has been reported to be expressed in fetal brain (463). Lehman et al. (562) also reported the identification of an LXR response element in the promoter region of the rat cyptα gene and suggested that LXR might play an important role in the regulation of Chol metabolism. Forman et al. (309) reported that the constitutive activity by LXRα is dependent on mevalonate biosynthesis. Competitive inhibitors of HMG-CoA reductase markedly reduced the constitutive activity of the heterodimer LXRα retinoid X receptor (RXR). Addition of mevalonate or certain oxysterols restored the transcriptional activity of LXRα. (20S)-20-OH-Chol and (22R)-22-OH-Chol showed high potency. In contrast, the unnatural (22S)-22-OH-Chol had no effect on LXRα. (20R,22R)-20,22-diOH-Chol also showed high potency, albeit not as high as that of (20S)-20-OH-Chol or (22R)-22-OH-Chol. The unnatural (20S,22S)-20,22-diOH-Chol also activated LXRα; however, its potency was considerably less than that of the (20R,22R)-isomer. Grøndahl et al. (351) reported activation of LXRα-dependent transcription by (22R)-22-OH-Chol, 25-OH-Chol, 26-OH-Chol, and “16-hydroxycholesterol.”

Peet et al. (788) reported very important studies on mice lacking LXRα. The LXRα knockout mice were reported to appear identical to wild-type littermates with regard to morphology, histology, and routine biochemical parameters when the animals were fed a standard chow diet (≥0.02% Chol). However, striking differences were observed when the mice were fed a diet enriched in Chol. On a diet containing 2% Chol, the null mice showed progressive increases in liver weight and hepatic Chol levels relative to the levels observed in wild-type mice. Furthermore, measurements made on pooled samples of plasma indicated increases in Chol, alanine transaminase, and aspartate transaminase in the LXRα−/− mice relative to wild-type mice. Upon Chol feeding (2%), LXRα−/− mice...
showed progressive accumulation of lipid deposits in liver and hepatocellular degeneration. Interestingly, Chol feeding was reported to have no effect on total body weight. When challenged with a Chol-containing diet, the LXRα−/− mice showed smaller increases in fecal bile acid excretion and hepatic cyp7a mRNA than the wild-type mice. The cyp7a promoter contains an LXR response element (552, 788). Peet et al. (788) proposed “that LXRα acts as a cholesterol sensor and upregulates expression of various components of cholesterol catabolism.” Chol feeding itself had no effect on the expression of LXRα or LXRβ in liver. Apart from differences related to Chol metabolism, the LXRα−/− mice differed from the wild-type mice in the expression of genes involved in fatty acid metabolism, with lower levels in the knockout mice for fatty acid synthase, stearoyl CoA desaturase, and SREBP-1.

Particularly intriguing was the observation by Janowski et al. (441) that 4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol activated LXRα with a potency comparable to those of 25-OH-Chol, 7α-OH-Chol, and 26-OH-Chol. The 4,4-dimethyl-∆8,14,24-sterol, isolated from human follicular fluid, was recently reported by Byskov et al. (150) to activate meiosis in mammalian oocytes. Other sterols reported (150) to activate meiosis were 4,4-dimethyl-5α-cholesta-8,14-dien-3β-ol and 4,4-dimethyl-5α-cholesta-8-en-3β-ol, and a sterol from bull testes with the suggested structure of 4,4-dimethyl-5α-cholesta-8,24-dien-3β-ol. Experimental variation (not defined) in the reported assay results appeared to be substantial. It should be noted that none of these sterols reported to activate meiosis contains an oxygen function other than that at C-3 and therefore would not be considered to be in the category of “oxysterols.” In considerations of this matter, it should be noted that the communication of Byskov et al. (150) indicates that the meiosis-activating sterols were not soluble in ethanol, a finding which would not be unanticipated for sterols of this type. Furthermore, before testing in the biological assay systems, the sterols were sonicated in medium, a procedure likely to result in autoxidation of the various unsaturated sterols. The activation of meiosis by the 4,4-dimethyl-∆8,14,24 sterol has now been demonstrated for the sterol prepared by chemical synthesis (351, 871). Activation was also demonstrated for the synthetic 4,4-dimethyl-∆8,24 sterol and 4,4-dimethyl-∆8(14),24 sterol (871). All of the synthetic sterols dissolved readily in ethanol, thus avoiding sonication before sample addition to the cultured oocytes (871). As noted above, Byskov et al. (150) reported the isolation of the 4,4-dimethyl-∆8,24 sterol from bull testes. This sterol and the 4,4-dimethyl-∆8,14,24 sterol (and possibly other related sterols) may play a role in the regulation of meiosis in male germ cells; however, definitive experimentation on this matter has not been published. Very recently, Strömstedt et al. (1055) found that, in the rat, lanosterol 14α-demethylase (cyp51) activity is higher in mature male germ cells than in those from prepubertal rats (in which postmeiotic germ cells are not present).

Another very recent communication by Yoshida et al. (1219) concerned the formation of two of the sterols reported to activate meiosis, i.e., 4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol and 4,4-dimethyl-5α-cholesta-8,24-dien-3β-ol. The formation of these sterols from lanosterol was studied in rat gonadal preparations. Unlabeled lanosterol was incubated with 10,000-γ supernatant fractions from rat ovary or testes in the presence of an NADPH-generating system and 1 mM KCN (to inhibit enzymatic removal of the 4-methyl functions). Identification of the sterols was based solely on capillary GC. Increases in the basal levels (before incubation) of peaks ascribed to the two sterols were taken as a measure of their formation from lanosterol. The sum of the amounts of formation of the two sterols was taken as a measure of cyp51 activity. Administration of pregnant mare’s serum as a source of gonadotrophin to immature rats was reported to cause an increase (+54%) in cyp51 activity in ovary, whereas little change was observed in liver. The mean values for four control and experimental rats were presented; however, no expression of the variation encountered was presented. The indication of the stimulation of cyp51 activity by gonadotrophin is particularly relevant to the possible physiological role of the proposed meiosis-activating sterols noted above. It should be noted that the highest levels of mRNA for cyp51 were observed in testis and ovary (1054).

Whereas 4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol was found to activate meiosis and to activate LXRα (351, 441), several oxysterols with high potency in the activation of LXRα have been found to have no effect on meiosis in mouse oocytes (351, 871). In one study, the oxysterols included (20R,22R)-20,22-diOH-Chol and (22R)-22-OH-Chol (871), both previously shown to have high potency in activating LXRα (309). In another study (351), (22R)-22-OH-Chol, 25-OH-Chol, 26-OH-Chol, and “16-hydroxysterol” were reported to activate LXRα but did not cause germinal vesicle breakdown in mouse oocytes.

Lala et al. (540) reported that certain oxygenated sterols, at a concentration of 10 μM, activate another orphan nuclear receptor, steroidalogenic factor 1 (SF-1), which has been reported to regulate multiple factors involved in steroid hormone production (490 and references cited therein, 1064, 1065). 25-OH-Chol, (25S)-26-OH-Chol, and (25R)-26-OH-Chol, in order of decreasing potency, were reported to activate transcriptional activity for SF-1. Other compounds reported to activate SF-1 transcriptional activity, but of lower potency, were 21-hydroxypregnenolone, pregnenolone, 7-keto-Chol, 7α-OH-Chol, 20,22-diOH-Chol (configurations at C-20 and C-22 not specified), and a compound designated as “6α-hydroxycholesterol.” Information on the source and purity
of the above compounds was not provided. EC50 values for 25-OH-Chol, (25S)-26-OH-Chol, and (25R)-26-OH-Chol were each reported at 5 μM, although a clear plateau in the activation was not evident in the experimental data from which these estimates of EC50 were made. Lala et al. (540) also observed that 25-OH-Chol (10 μM) weakly activated RXR and moderately activated LXR. (22R)-22-OH-Chol showed weak activation of SF-1 but strong activation of LXR, findings that differed from the strong activation of SF-1 and moderate activation of LXR by 25-OH-Chol. In contrast to the studies of Lala et al. (540), native promoters were used for experiments in CV-1 cells by Christenson et al. (204). Under these conditions, oxysterols produced much lower stimulation (~2-fold) of SF-1 transcriptional activity than observed by Lala et al. (540) (~10-fold). It is interesting to note that mutant mice, deficient in SF-1, have been reported by Sadovsky et al. (881) to die shortly after birth and to have no detectable adrenal glands and gonads.

H. Oxysterols as Regulators of Gene Transcription of Other Species

Rajavashisth et al. (832) cloned a gene for the cellular nucleic acid binding protein (CNBP), a protein which binds to single-stranded DNA containing SRE. In the Hep G2 cells, 25-OH-Chol (2.5 μM) was reported to cause a fourfold increase in CNBP binding activity (832). However, in CEM cell lines from human acute lymphoblastic leukemia cells, 25-OH-Chol (1 μM) was found to be a negative regulator of CNBP (38, 1113). At the present time, the function of the zinc finger protein CNBP is not known. Thompson and Ayala-Torres (1113) reported that 25-OH-Chol regulated the levels of gene transcription for c-myc in CEM cells. This protooncogene and its protein product c-Myc appear to be involved in regulation of the cell cycle and apoptosis (39, 1113). Recent work (39) indicated that treatment of CEM cells with 25-OH-Chol first results in repression of c-Myc, followed by a reduction in c-myc mRNA and the subsequent onset of cell death. Endo et al. (282) reported that certain oxygenated derivatives of Chol, designated as yakkasteroids, showed high binding affinity for a cytosolic-nuclear tumor promoter binding protein. 3β,5-Dihydroxy-5α-cholestan-6-one (designated as yakkasterone; Fig. 9) showed the highest binding affinity of a limited number of oxygenated sterols, all of which contained 6-keto or 6β-hydroxy functions.

The cytosolic-nuclear tumor promoter binding protein had previously been shown to bind 12-O-tetradecanoylphorbol 13-acetate (TPA) with very high affinity. Yakkasterone showed high activity in inhibiting the binding of [3H]TPA to the tumor promoter binding protein; however, it was inactive as an inhibitor of the binding of [3H]TPA to protein kinase C. The binding of other oxygenated sterols such as 7-oxygenated derivatives of Chol or of 20-, 22-, 24-, 25-, or 26-oxygenated derivatives of Chol was not reported.

I. Oxysterols and Antiestrogen Binding Activity

Certain oxygenated sterols have been reported to bind to intracellular antiestrogen-binding species, termed antiestrogen-binding site (AEBS). This high-affinity binding component(s) for synthetic antiestrogens such as tamoxifen, distinct from the estrogen receptor, was first demonstrated by Sutherland and co-workers (1067, 1068). Some of the studies of the distribution, properties, and possible functions of AEBS have been reviewed by Lazier and Bapat (548). With the exception of some oxygenated sterols (see below), high-affinity binding to AEBS appears to require an alkyl-substituted basic amino-terminal side chain (1191). Murphy et al. (687) reported that 7-keto-Chol and (to a lesser extent) cholest-4-en-3-one showed significant binding (albeit considerably less than that of tamoxifen and a number of other synthetic antiestrogens) to AEBS of chicken liver cytosol. Chol, 25-OH-Chol, 5α-cholestan-3-one, estrone, and hydrocortisone did not show significant binding. Hwang and Matin (419) extended these studies to the binding of a number of other sterols to AEBS of the 10,000-g supernatant fraction of rat liver. Sterols that showed significant binding were as follows (in order of decreasing binding affinity): 7-ketocholesterol, 7-ketocholestanoylacetate, 6-ketocholestanol, 6-ketocholestanoylacetate, 7-keto-Chol, 7-keto-Chol acetate, 5α,6β-diol-OH-Chol, 7α-OH-Chol, and cholest-4-en-3-one. Sterols showing no detectable binding under the conditions studied were Chol, cholest-5-en-3-one, 25-OH-Chol, 5α-cholestan, 5α-cholestan-3β-ol, 5α-cholestan-3α-ol, 5β-cholestan-3β-ol, 5α-cholestan-3-one, 5β-cholestan-3-one, 3β-chloro-5α-cholestan-6-one, 5α-cholestan-3β,6α-diol, and 5α-cholestan-3β,6β-diol. Androst-5-en-3β-ol, 3β-hydroxyandrost-5-ene-7,17-dione, and 3β-hydroxyandrost-5-ene-7,20-dione also showed no detectable binding. As noted above, the acetate derivatives of 7-ke-
J. Oxysterols and LDL Receptor

In 1975 Brown and Goldstein (142) first reported that 25-OH-Chol (1.5 μM) caused a suppression of the levels of \(^{125}\)I-LDL binding activity of human fibroblasts. No effect was observed at 4 h; however, thereafter LDL binding activity decreased. The suppression of LDL binding activity by 25-OH-Chol (1.5 μM) at the various time points (up to 48 h) was essentially the same as that caused by a mixture of 25-OH-Chol (1.5 μM) and Chol (31 μM). At these concentrations, the mixture of 25-OH-Chol and Chol suppressed binding and degradation of LDL by the fibroblasts. Basu et al. (52) reported that incubation of human fibroblasts with a mixture of 25-OH-Chol (1.5 μM) and Chol (36 μM) for 48 h caused a 90% decrease in high-affinity binding of \(^{125}\)I-LDL to isolated membranes obtained from the cells. Krieger et al. (514) observed that the mixture of 25-OH-Chol (2.5 μM) and Chol (32 μM) lowered the levels of degradation of \(^{125}\)I-LDL (−81%) by human fibroblasts. Goldstein et al. (341) reported that incubation of a mouse teratocarcinoma cell culture line with a mixture of 25-OH-Chol (2.5 μM) and Chol (31 μM) for 2 days in medium containing lipoprotein-deficient serum caused decreases in the levels of surface bound (−93%), internalized (−90%), and degraded (−94%) \(^{125}\)I-LDL. Schneider et al. (907) found that incubation of a mixture of 25-OH-Chol (1.5 μM) and Chol (27 μM) with human epithelioid carcinoma (A-431) cells for 24 h resulted in 77% decrease in the binding of \(^{125}\)I-LDL to the cells. Membranes prepared from the sterol-treated cells also showed decreased binding (−71%) to LDL (relative to membranes obtained from untreated cells). Russell et al. (877) reported first the cDNA cloning of the LDL receptor and that sterols reduced the levels of mRNA for the LDL receptor in cultured human epidermoid carcinoma A-431 cells.

Yamamoto et al. (1211) reported that incubation of SV40-transformed and normal human fibroblasts with a mixture of 25-OH-Chol (5 μM) and Chol (31 μM) for 24 h markedly lowered the levels of mRNA for the LDL receptor. Mehta et al. (636) reported that incubation of a mixture of 25-OH-Chol (2.5 μM) and Chol (26 μM) with *Xenopus* A6 kidney cells for 20 h reduced the level of mRNA for the LDL receptor. 25-OH-Chol (0.6 μM) has been reported to reduce the elevated levels of mRNA for...
the LDL receptor in CHO-K1 cells induced by transfer of the cells to medium containing delipidated serum (263). Ridgway (848) reported a concentration-dependent decrease in the level of mRNA for LDL receptor in CHO cells by 25-OH-Chol. The concentrations studied were from ~0.25 to 12.4 μM. The medium used was DMEM containing 5% “delipidated” FCS (prepared by ultracentrifugation). The maximal decrease (~60%) was observed at 1 μM and was not different at 2.5 and 5 μM. Dashti (239) observed that preincubation of Hep G2 cells with 25-OH-Chol (50 μM) in serum-free medium for 20 h caused modest decreases (20–25%) in LDL binding and LDL uptake. It was also reported (data not shown) that 25-OH-Chol inhibited LDL degradation (12–20%) and lowered the level of mRNA for LDL receptor (10%). Sorci-Thomas et al. (1021) reported that incubation of the mixture of 25-OH-Chol (10 μM) and Chol (130 μM) plus BSA (75 μM) with Hep G2 cells for 6 h in serum-free medium, followed by a 4-h incubation with 125I-labeled LDL, resulted in a significant lowering of the degradation of 125I-labeled LDL. LDL receptor activity (expressed as ng 125I-labeled LDL degraded/ng cell protein) was 167 ± 9 in the treated cells and 310 ± 12 for control cells. The combination of 25-OH-Chol (10 μM) and Chol (130 μM) was also reported to lower the level of mRNA for the LDL receptor in Hep G2 cells incubated in serum-free medium. After 6 h of incubation, the levels of mRNA for LDL receptor (in pg mRNA/μg RNA) were 2.6 ± 0.1 in treated cells and 7.1 ± 0.5 in the control cells. After 6 h (i.e., 12, 18, and 24 h), the levels of mRNA increased but did not return to initial or control levels. Cuthbert et al. (228) studied the effect of 25-OH-Chol (0.25 μM) on the levels of mRNA for the LDL receptor in freshly isolated human peripheral mononuclear cells. 25-OH-Chol (0.25 μM) lowered the levels of LDL receptor mRNA by 78 and 82% in cells incubated in vitro for 24 h in lipoprotein-deficient medium. This effect was not observed in cells incubated for <12 h. Ellsworth et al. (276) observed that 25-OH-Chol (25 μM), incubated with Hep G2 cells for 24 h in Eagle’s minimal essential media containing 10% FCS, caused a 40–50% reduction in the level of mRNA for the LDL receptor. In addition, 25-OH-Chol (25 μM for 24 h) reduced LDL receptor gene transcription by 40 ± 14% (SD). In separate experiments of shorter duration, 25-OH-Chol reduced mRNA levels by 15 and 50% at 3 and 6 h, respectively, whereas gene transcription was reduced by 45 and 63% at the same time points. In additional experiments, 25-OH-Chol (25 μM) had little effect on the turnover of mRNA for the LDL receptor (or the stability of the mRNA species). Tam et al. (1090) found that 25-OH-Chol (2.5 μM) lowered the levels of mRNA for the LDL receptor in Hep G2 cells, Hep 3B cells, and in normal human fibroblasts. The reduction in the mRNA levels was observed in cells incubated in media containing either lipoprotein-depleted serum or reconstituted “complete serum.” 25-OH-Chol (0.25 μM) markedly suppressed the levels of mRNA for the LDL receptor in peripheral blood mononuclear cells incubated in lipoprotein-deficient serum for 24 h (226). Stimulation of the cells with phytohemagglutinin did not affect the lowering of LDL receptor mRNA levels by 25-OH-Chol. Carlson and Kottke (158) reported that 25-OH-Chol decreased the number of LDL receptors in Hep G2 cells. Incubation of Hep G2 cells with 25-OH-Chol (5 μM) in a medium containing FCS (10%) was found to decrease 125I-LDL binding to the LDL receptor (total 125I-LDL associated with cells) by 38%. Under the same conditions, added Chol (26 μM) had no effect, as was also the case with cholic acid (24.5 μM) and several other bile acids. However, chenodeoxycholic acid (25.5 μM) caused a 49% increase under these conditions.

Stephan and Yuracek (1045) reported that 25-OH-Chol (30 μM) decreased LDL receptor activity in Hep G2 cells as determined by measurement of cell-associated LDL (using either 125I-LDL or LDL with a fluorescent label). 25-OH-Chol (30 μM) lowered cell-associated 125I-LDL (~55%), and mevinolin (1 μM) elevated cell-associated 125I-LDL (+26%). Cells were grown in DMEM with fetal bovine serum (8%) for 72 h. They were then changed to DMEM containing human serum albumin (0.5%) for 18 h in the presence or absence of 25-OH-Chol or mevinolin. Cell-associated LDL was measured after 2-h incubation at 37°C of the 125I-LDL.

Molowa and Cimis (663) reported on the effect of 25-OH-Chol on the levels of mRNA for the LDL receptor in Hep G2 cells. In one experiment, the rise in LDL receptor mRNA induced over 24 h by transfer of the cells to lipid-deficient media was reduced by 25-OH-Chol at concentrations of 1, 5, and 10 μM. At 10 μM, the levels of the mRNA species were only ~25% of control cells. However (and not commented on by the authors), 25-OH-Chol at lower concentrations resulted in a slightly higher level (~10–15%) of mRNA for the LDL receptor. In another experiment, the timing of the effect of 25-OH-Chol (1 μM) on the increase in LDL receptor mRNA induced by transfer of the cells to lipid-deficient medium was studied. The repression of the rise in the mRNA species was detected as early as 3 h and reached maximal levels (~50%) at 6 h (and did not change thereafter up to the last time point studied, i.e., 18 h).

Srivastava et al. (1032) showed that 25-OH-Chol reduced the levels of LDL receptor activity (cell-associated 125I-LDL) in a time-dependent and concentration-dependent (0.5–5 μM) manner in Hep G2 cells. 25-OH-Chol (6.2 μM) also reduced LDL protein, LDL receptor mRNA, and LDL transcription. Palmitic acid, but not oleic acid, also reduced LDL receptor activity (but without effects on LDL receptor protein, mRNA, or LDL receptor mRNA transcriptional activity). Palmitate enhanced the decreases in LDL receptor activity and LDL mRNA levels caused by 25-OH-Chol. The lowering of LDL receptor activity by
25-OH-Chol was ascribed to transcriptional control, whereas that caused by palmitate was ascribed to post-translational control (by an undetermined mechanism).

Metherall et al. (645) reported the isolation of two CHO mutants, resistant to the effects of 25-OH-Chol on cell growth, which showed little suppression (18 and 17%) of LDL receptor activity upon incubation of the cells with a mixture of 25-OH-Chol (1.5 μM) and Chol (26 μM) for 48 h. Control cells, from which the mutant cell lines were prepared, showed ~95% lowering of LDL-receptor activity under the same conditions. In separate experiments conducted under the same conditions, the mixture of sterols had no effect on the levels of mRNA species for the LDL receptor, HMG-CoA reductase, and HMG-CoA synthase. Under the same conditions, the levels of mRNA for the three proteins in the parent CHO cells were reduced 83, 63, and >90%, respectively.

The results of the above studies indicate significant effects of 25-OH-Chol on LDL metabolism, and these results have been commonly assumed to apply to other oxysterols. Pinkerton et al. (810) studied the effects of 25-OH-Chol on LDL metabolism, and these results have been commonly assumed to apply to other oxysterols. Pinkerton et al. (810) studied the effects of three oxysterols, (25R)-26-OH-Chol, 25-OH-Chol, and 3β-hydroxy-5α-cholest-8(14)-en-15-one, on LDL metabolism in Hep G2 cells. These oxysterols differed in their effects, as measured by the combination of cell-associated plus degraded 125I-LDL. The 26-hydroxycholesterol, at 0.1 to 75 μM, lowered LDL metabolism with an IC50 of ~0.1–2 μM. In contrast, the 15-ketosterol and the 25-OH-Chol, at 0.05 to 2.5 μM, caused an increase in LDL metabolism. At higher concentrations of these two oxysterols, decreases in LDL metabolism were observed. However, in the case of the 15-ketosterol, increasing the concentration above 40 μM resulted in increases in LDL metabolism, with an extraordinarily high value (9-fold increase) observed at 75 μM. The novel effects of the 15-ketosterol on LDL metabolism, with clear variations over different concentration ranges of the sterol, prompted studies of the effects of a recently synthesized (1076) analog of the 15-ketosterol, i.e., 3β-hydroxy-25,26,27,27,27-heptafluoro-5α-cholest-8(14)-en-15-one, in which its major side-chain metabolism is blocked by the fluorine substitution. The F7 analog of one of the compounds was reported to show no stimulation of the cellular uptake of 25-OH-Chol. Additional studies indicated that the new compounds increased the specific binding of labeled 25-OH-Chol but, in fact, resulted in an increase in the uptake of labeled 25-OH-Chol into the cells. The 3β-hydroxy epimer of one of the compounds was reported to show no stimulation of the cellular uptake of 25-OH-Chol. Additional studies indicated that the new compounds increased the specific binding of labeled 25-OH-Chol to cytosolic protein species of hamster liver. The evidence presented indicated that the protein species affected by the new compounds was distinct from the oxysterol binding protein.

26-OH-Chol (source and purity not presented), at 0.63 μM, has been reported to have no effect on the binding of 125I-LDL to either human fibroblasts or Hep G2 cells (647). The 26-OH-Chol, at the same concentration, decreased Chol synthesis in the human fibroblasts but not in the Hep G2 cells. Thus, for 26-OH-Chol, a concentration effective in the suppression of Chol biosynthesis in human fibroblasts, had no effect on LDL receptor binding in these cells. In another study (226), 26-OH-Chol was reported to lower mRNA levels for the LDL receptor in peripheral blood mononuclear cells incubated in lipoprotein-deficient serum. Although no data were presented, the 26-hydroxycholesterol was reported to be similar to 25-OH-Chol in...
this respect. Winegar et al. (1202) observed that 26-OH-Chol inhibited LDL uptake (as measured by cell-associated 125I-LDL) by Hep G2 cells with an IC50 value of 8 μM. In these experiments, the 26-OH-Chol was added through the use of β-cyclodextrin solubilized sterol. The conditions of the preparation of the β-cyclodextrin-sterol solution appeared to be critical, since the above results were obtained when cyclodextrin fully loaded with 26-OH-Chol (25 μM sterol solution) was used. When partially filled with 26-OH-Chol, i.e., 3 μM 26-OH-Chol stock solution, was used, little or no effect on LDL uptake was observed at 30 μM 26-OH-Chol in the medium. These studies did not include LDL degradation as part of their assay of LDL uptake. Their studies are further complicated by the fact that β-cyclodextrin itself had a stimulatory effect on “LDL uptake.” The oxysterol was incubated with the cells for 24 h, then fresh medium was added containing the 125I-LDL (but no oxysterol) for 3 h. (25R)-26-Aminocholesterol (0.16, 0.31, and 0.63 μM) caused significant decreases in 125I-LDL binding to human fibroblasts (647) and, at concentrations of 0.31 and 0.63 μM, decreased 125I-LDL binding to Hep G2 cells (647). At much higher concentrations (10 and 20 μM, but not 5 μM), (25R)-26-thiocholesterol was found to decrease 125I-LDL binding to human fibroblasts (647). In Hep G2 cells, the thiocholesterol had no effect on LDL binding at the same concentrations (647). In a subsequent study, Corsini et al. (218) observed different effects of 26-aminocholesterol on LDL metabolism in studies with human arterial myocytes. 26-Aminocholesterol, at concentrations of 1, 2, 3, and 5 μM, was reported to significantly increase 125I-LDL binding and 125I-LDL internalization but to have no significant effect on 125I-LDL degradation (except for a slight decrease at 5 μM). To the knowledge of this reviewer, the natural occurrence of the 26-amino and 26-thio derivatives of Chol has not been described. However, it should be noted that Ohmura et al. (726) recently isolated a 26-aminocholesterol derivative from the roots of Solanum abutiloides that the authors proposed to be an intermediate in the biogenesis of steroidal alkaloid in this plant.

Lorenzo et al. (589) studied the effect of 26-OH-Chol in blocking the rise of LDL receptor activity in human fibroblasts caused by incubation of the cells with lipoprotein-deficient serum. The 25R- and 25S-isomers of 26-OH-Chol, at a concentration of ~1 μM, caused a 50% inhibition of the increase in uptake and degradation of LDL induced by incubation of the cells in media containing 5% lipoprotein-deficient medium. Chol (recrystallized, purity not provided) at concentrations of 0.1, 2, and 6 μM had little effect (+10, +2, and −24%, respectively) on 125I-LDL degradation, whereas (25S)-26-OH-Chol, at the same concentrations, showed values which were +8.7, −62, and −96%, respectively. Bates et al. (53) reported that 22-OH-Chol, 22-hydroxy-25-fluorocholesterol, and 25-OH-Chol lowered LDL uptake in monkey arterial smooth muscle cells.

Panini et al. (757) reported that (24S)-24,25-epoxylanosterol, at a very high concentration (100 μM), had no effect on the levels of mRNA for the LDL receptor or for FPP synthetase in cultured rat hepatocytes incubated for 17 h in serum-free medium. Under the same conditions, 25-OH-Chol, at 25 μM, caused a 62 and 39% lowering of mRNA levels for LDL receptor and for FPP synthetase, respectively.

The possibility that metabolism of 25-OH-Chol is important in regulation of LDL receptor activity in liver was postulated by Dueland et al. (263). CHO-K1 cells were transfected with a plasmid encoding cyp7a. 25-OH-Chol (0.62 μM) caused substantial decreases in the elevated levels of mRNA for the LDL receptor at 24 h in both wild-type CHO-K1 cells and the cells expressing 7α-hydroxylase activity. Although the lowering of the mRNA for LDL receptor was sustained at 48 and 72 h in the wild-type cells, the lowered levels of LDL receptor mRNA by 25-OH-Chol were partially restored after 48 and 72 h of incubation. [26,27-3H]25-OH-Chol was incubated with the wild-type CHO-K1 cells and with cells expressing cyp7a activity for 24 h. On the basis of the recovery of a higher amount of the incubated [3H]25-OH-Chol at 24 h in the cells and medium in the case of the wild-type cells, it was postulated that the 25-hydroxysterol was metabolized to inactive species (with respect to effect on the LDL receptor), presumably 7α-hydroxylated species, in the cells containing the cyp7a activity. The chemical nature of the postulated metabolites was not determined. More recent studies by Toll et al. (1119) have demonstrated that whereas COS cells transfected with human liver cyp7a cDNA showed significant 7α-hydroxylation of Chol, they did not show hydroxylation of 25-OH-Chol. Furthermore, Toll et al. (1119) provided additional results indicating that the 7α-hydroxylation of 25-OH-Chol is catalyzed by an enzyme other than cyp7a.

It is commonly assumed that the levels of HMG-CoA synthase, HMG-CoA reductase, FPP synthase, and the LDL receptor are coordinately controlled by gene transcription. Cuthbert and Lipsky (227) studied the mRNA levels for HMG-CoA reductase, HMG-CoA synthase, and LDL receptor in resting and mitogen-stimulated (phytohemagglutinin) T lymphocytes. Mitogenic stimulation of T lymphocytes cultured in lipoprotein-depleted medium resulted in increased levels of mRNA for HMG-CoA synthase and for LDL receptor. However, no significant effect on the levels of mRNA for HMG-CoA reductase was observed under these conditions. In resting lymphocytes in the presence of LDL, the levels of mRNA for all three enzymes were reduced. In contrast, LDL addition to mitogen-stimulated lymphocytes blocked the increase in mRNA levels of HMG-CoA synthase and HMG-CoA reduc-
tase; however, expression of the LDL receptor gene was enhanced.

Rudling (874) reported that when mice were fed a diet containing high levels of Chol (0.6 or 1.7% by weight), cholic acid, and saturated fat, the levels of mRNA for HMG-CoA reductase, cyp7a, and the LDL receptor were lower than control chow-fed mice. It should be noted that in one experiment the mRNA for LDL receptor was not reduced by the high-fat diet. Feeding a different diet containing commercial Chol, but without added cholic acid or saturated fat, at levels of 0.4, 1.7, and 5% in diet with 10% corn oil in chow diet to mice for 10 days reduced the levels of mRNA for HMG-CoA reductase and LDL receptor relative to animals fed chow diet with 10% corn oil. The extent of the reduction in mRNA levels for HMG-CoA reductase upon Chol feeding was greater than that for LDL receptor. The levels of mRNA for cyp7a appeared to be relatively insensitive to Chol feeding. Significant elevation (+70%) was observed only at a level of 5% Chol in diet. Feeding of mevinolin (0.25% in diet) plus colestipol (2% in diet) for 10 days resulted in increases in mRNA levels for LDL receptor (+69%), cyp7a (+214%), and HMG-CoA reductase (+445%). Thus feeding of mevinolin plus colestipol increased the levels of mRNA for all three proteins; however, the magnitude of the increases for the three mRNA species varied markedly. Spady et al. (1025) reported that the rates of LDL uptake and Chol synthesis are independently regulated in liver in vivo in rats and hamsters. When Chol synthesis was either markedly decreased by Chol feeding (0.5% in diet) or markedly increased by cholesteryamine administration (2% in diet), little or no effect on LDL uptake by liver was observed in female rats or female hamsters. In male hamsters, Chol feeding reduced Chol synthesis but had little or no effect on LDL uptake by liver. However, in the male hamster, the markedly increased hepatic Chol synthesis induced by cholesteryamine (+17.1-fold) was associated with only a modestly increased receptor-dependent LDL uptake (2.6-fold) by liver. Grove et al. (352) found that oncostatin-M, a glycoprotein produced by a human macrophage cell line, caused a rapid increase in LDL receptor activity and LDL receptor protein in Hep G2 cells. Oncostatin-M (100 ng/ml) caused an increase in LDL receptor levels that reached maximal levels (~40%) at 8 h. At the same concentration, oncostatin-M had no effect on the incorporation of [14C]acetate into NSL from 2 to 8 h. At 20 h, a 55% lowering of acetate incorporation into NSL was observed. Thus, under these conditions, a coordination in the effect of oncostatin-M on “sterol biosynthesis” and LDL receptor activity was not observed.

A series of elegant studies from the laboratory of Brown and Goldstein and co-workers (127, 1180, 1183, 1215) resulted in the identification of SREBP-1 which, upon cleavage by a protease, generates a 68-kDa fragment that activates the transcription of the gene for the LDL receptor (as well as that for HMG-CoA synthase) by binding to the sterol responsive element-1 (SRE-1). The proteolytic cleavage of SREBP-1 was reduced by sterols [i.e., the combination of 25-OH-Chol (2.5 μM) and Chol (26 μM)] in HeLa cells (1183). 25-OH-Chol itself (0.25 μM) or 20α-OH-Chol (0.25 μM) was found to be highly active in lowering the levels of the mature form (proteolyzed) at SREBP-1 in nuclei, whereas 7-keto-Chol was less active. Chol itself had relatively low activity.

There is not complete agreement that oxysterols and Chol lower LDL receptor due mainly to inhibition of gene transcription. Winegar et al. (1202), on the basis of studies with Hep G2 cells, have suggested that “changes in the intracellular free cholesterol pool” are “most likely responsible for the regulation of SREBP-1-mediated transcription” and not formation of 26-OH-Chol (i.e., for the decreased LDL receptor activity induced by LDL or Chol). This conclusion was based largely on results with cyclosporin under certain experimental conditions. Very recently, Makar et al. (607) presented evidence indicating that, in the human leukemic T cell line Jurkat, the LDL promoter contains both SRE-dependent and SRE-independent response elements and that the SRE-dependent response element in these cells may not involve SREBP-1 or SREBP-2.

Takagi et al. (1088) reported on studies of gene transcription for the LDL receptor in JEG-3 choriocarcinoma cells transfected with the LDL receptor promoter chloramphenicol acetyltransferase. Serum, LDL, and serum plus 25-OH-Chol reduced receptor activity. Ketoconazole (50 μM) abolished the effects of serum and LDL but not that of serum plus 25-OH-Chol. The authors proposed that ketoconazole blocks the formation of an effector molecule from LDL Chol.

The effects of the combination of 25-OH-Chol and Chol on another lipoprotein receptor, i.e., the LDL receptor-related protein, have also been studied (510). The biosynthesis of this protein, a cell-surface receptor involved in the uptake of apoprotein E-rich lipoproteins such as β-VLDL, was unaffected by incubation of fibroblasts with the combination of 25-OH-Chol (5 μM) and Chol (26 μM). The levels of mRNA for the protein were also reported to be unchanged after incubation of the cells with the combination of 25-OH-Chol and Chol. These findings are in contrast to those made for the LDL receptor, for which inhibition of its synthesis and lowering of its mRNA were observed upon incubation with the sterols.

In 1984, Goldstein and Brown (342) proposed coordinate regulation of HMG-CoA reductase activity and LDL receptor activity not only in cultured cells but also in animals and reviewed evidence in support of this hypothesis. It is also commonly believed that all oxysterols that are active in the lowering of HMG-CoA reductase activity also lower LDL receptor activity and further that the
oxysterols act as suppressors of gene transcription not only for the reductase but also for the LDL receptor (844). Ridgway (848) reported that 25-OH-Chol (from ~0.25 to 12.4 μM) caused dose-dependent decreases in the levels of mRNA for LDL receptor, HMG-CoA reductase, and HMG-CoA synthase in CHO cells. Incubations were carried out in DMEM containing 5% delipidated FCS. The decreases in mRNA for HMG-CoA synthase (>90%) were greater than those for the LDL receptor (~60%) and HMG-CoA reductase (~65%).

That the coordination of levels of HMG-CoA reductase activity and the LDL receptor activity is not invariant is indicated by a number of studies. Panini et al. (760) reported that incubation of Hep G2 cells with 4,4,10-tetramethyl-trans-decal-3β-ol (TMD; 20 μg/ml), an inhibitor of the cyclization of 2,3-epoxysqualene, for 24 h in lipoprotein-deficient serum (after a preincubation of the cells for 24 h in the lipoprotein-deficient serum) had no significant effect on the levels of HMG-CoA reductase activity. However, incubation of the Hep G2 cells with the TMD at the same concentration under the same conditions resulted in increases in the levels of LDL binding, internalization, and degradation. Panini et al. (757) also reported that, whereas (24S)-24,25-epoxylanosterol (100 μM) lowered HMG-CoA reductase activity (~86%) in rat hepatocytes incubated in serum-free medium, it had no significant effect on the levels of mRNA for HMG-CoA reductase and the LDL receptor in rat hepatocytes incubated in serum-free media. In contrast, 25-OH-Chol (25 μM) lowered the levels of mRNA for both the reductase (~63%) and the LDL receptor (~62%). As noted previously, the effects of 25-OH-Chol and 3β-hydroxy-5α-cholest-8(14)-en-15-one on LDL metabolism in Hep G2 cells differed at different concentrations (810), with elevations at low concentrations (0.1–2.5 μM) and with decreases at intermediate concentrations. At higher concentrations, the 15-ketosterol (but not the 25-hydroxysterol) showed a marked increase in LDL metabolism (9-fold increase at 75 μM). In contrast, each of these oxysterols caused a concentration-dependent decrease in the levels of HMG-CoA reductase activity. Mayer et al. (627) reported that 3β-hydroxylanost-8,15-diene-32-carboxylic acid, a potent inhibitor of lanosterol 14α-demethylase, is highly active in lowering HMG-CoA reductase activity in Hep G2 cells in medium containing delipidated FCS but had no detectable effect on LDL receptor activity (in cells incubated in medium containing 1% human serum albumin) as measured by cell-associated 125I-LDL. Assays were made after an 18-h incubation with the steroidal acid and a subsequent 3-h incubation with 125I-LDL in the presence or absence of added unlabeled LDL (300 μg/ml). The LDL receptor-mediated association of the labeled LDL was defined as the difference in the cell association of the 125I-LDL in the presence or absence of the unlabeled LDL (300 μg/ml). These experiments involve blocking of the rise in LDL receptor induced by transfer of the cells to lipid-deficient media. Mayer et al. (627) also reported that, under presumably similar assay conditions, “other oxysterol compounds ... [for example 3β-hydroxycholest-8(14)-en-15-one] did cause a decrease in receptor activity.” No data on these experiments were presented.

Larsen et al. (545) reported that a seco-oxysterol analog (Fig. 11) which, at 10 μM, showed significant activity in the inhibition of HMG-CoA reductase transcription in transfected Hep G2 cells and which lowered the levels of HMG-CoA reductase mRNA and HMG-CoA reductase activity in Hep G2 cells, caused a stimulation (+476%) of LDL receptor binding activity in the same cells. Liao and Floren (568) reported that whereas tumor necrosis factor stimulated LDL receptor activity in Hep G2 cells, it had little or no effect on the incorporation of labeled acetate into Chol. Taniguchi et al. (1094) reported that progesterone (32 μM) caused a noncoordinate change in the levels of mRNA for the LDL receptor (“in-duced 3-fold”) and HMG-CoA reductase (“suppressed to 34% of control”) in Hep G2 cells grown in serum-free medium. In the same experiment, progesterone was reported to cause a 76% decrease in the level of mRNA for cyp7a. Taniguchi et al. (1094) also found that either chenodeoxycholic acid (100 μM) or deoxycholic acid (100 μM) caused markedly increased levels of mRNA for the LDL receptor (~7- and 4-fold, respectively) in Hep G2 cells at 8 h. In contrast, the two bile acids had no effect on the levels of mRNA for HMG-CoA reductase. Corsini et al. (218) reported on the effects of varying concentrations of 26-OH-Chol, 25-OH-Chol, and 26-aminocholesterol on LDL metabolism in human arterial myocytes. Whereas all three sterols caused a lowering of HMG-CoA reductase activity in both human and rat arterial myocytes, the three sterols gave different responses on LDL metabolism in the human cells. Both 26-OH-Chol and 25-OH-Chol caused significant lowering of the levels of internalized 125I-LDL at all concentrations studied (1, 3, and 7.5 μM), whereas 26-aminocholesterol caused significant increases at each of the concentrations studied (1, 2, 3, and 5 μM). Different results were reported for the effects of the three sterols on the binding of LDL to the cells. 26-Aminocholesterol increased binding at each concentration studied (1, 2, 3, and 5 μM). Neither 26-OH-Chol nor 25-OH-Chol caused a sig-

![FIG. 11. Structure of a seco-oxysterol analog that shows significant activity in inhibiting transcription of 3-hydroxy-3-methylglutaryl-CoA reductase.](Image 367x642 to 521x712)
significant lowering of LDL binding. 25-OH-Chol, at 1 and 7.5 μM, caused a significant increase in LDL binding; however, no significant effect was observed at 3 μM. In contrast, 26-OH-Chol had no effect on LDL binding at 1 and 3 μM but caused a significant increase at 7.5 μM. Different results were observed on LDL degradation. 26-Aminocholesterol had no significant effect at 1, 2, and 3 μM; however, a slight decrease was observed at 5 μM. 26-OH-Chol caused a slight decrease in the levels of degraded 125I-LDL at 1 μM, but had no effect at 3 and 7.5 μM. In contrast, 25-OH-Chol caused significant decreases in the levels of degraded 125I-LDL at each of the concentrations studied (1, 3, and 7.5 μM).

Sudjana-Suglam et al. (1062) reported no change in LDL receptor activity in COS cells transfected with the cDNA for cyp7a. The cells showed demonstrable levels of the enzyme and of 7α-OH-Chol. However, the levels of 7α-OH-Chol were low, i.e., 11–67 ng/mg cell protein. Horton et al. (401) reported a clear dissociation of the effects of Chol feeding on hepatic Chol synthesis and on hepatic receptor-dependent transport of LDL in the rat, i.e., dietary Chol had a profound suppressive effect on Chol synthesis, whereas it had no effect on receptor-dependent LDL transport by liver. Kushwaha et al. (526) reported studies in baboons indicating differences in the effects of feeding a high-Chol (1.7 mg/kcal), high-fat (40% of calories) diet. Whereas the levels of mRNA for HMG-CoA reductase in liver were lower on the high-Chol diet, reductase mRNA was essentially unchanged at 6 h and 5 days. HMG-CoA reductase mRNA was essentially unchanged at 6 h and 5 days (the only time points studied).

K. Oxysterols and Cholesterol Ester Formation and Hydrolysis

1. Microsomes or cell-free preparations

In 1978, Goldstein et al. (344) reported that addition of 25-OH-Chol (12.4 or 124 μM), added in acetone, had no effect on the incorporation of [14C]oleoyl CoA into Chol esters by a cell-free extract from human fibroblasts. In contrast, 7-keto-20-oxacholesterol, at concentrations of 12.5 and 125 μM, showed significant inhibition of Chol ester formation. Drevon et al. (261) reported that direct addition of 25-OH-Chol (2.5 and 25 μM) to microsomes isolated from rat hepatocytes caused a stimulation of ACAT activity (as determined by the incorporation of [14C]oleoyl CoA into Chol esters). Erickson et al. (286) observed that 25-OH-Chol (100 μM), added in ethanol, increased ACAT activity of rat liver microsomes (as determined by the incorporation of [14C]oleoyl into Chol esters). Lichtenstein and Brecher (569) reported that 25-OH-Chol (26 μM) stimulated the oleoyl CoA-dependent esterification of Chol by rat liver microsomes. Microsomes preincubated with 25-OH-Chol and then reisolated through a sucrose gradient ultracentrifugation showed retention of 25-OH-Chol and a concentration-dependent increase in Chol ester formation.

Field and Mathur (299) found that preincubation of rabbit intestinal microsomes with 25-OH-Chol (25 μM), added in ethanol, stimulated ACAT activity (as measured by the incorporation of [14C]oleoyl CoA into Chol esters). The stimulation was dependent on the concentration of 25-OH-Chol and was reported to occur within 5 min. 25-OH-Chol also stimulated the incorporation of added [14C]Chol into Chol esters. Enrichment of the Chol concentration in the intestinal microsomes by their incubation with Chol-containing liposomes resulted in an increase in ACAT activity; however, the ability of 25-OH-
Chol to stimulate ACAT activity decreased, and at a Chol concentration of 49 or 66 μg/mg protein, no significant stimulation of ACAT activity by 25-OH-Chol was observed. Liza et al. (584) reported that 25-OH-Chol (124 μM) stimulated ACAT activity (incorporation of [14C]oleoyl CoA into Chol esters) of rat liver microsomes. 2-Hydroxypropyl-β-cyclodextrin (6 mM) also stimulated ACAT activity, and the compound was suggested to act by making endogenous Chol in microsomes more accessible to the enzyme. 25-OH-Chol (124 μM) also stimulated ACAT activity assayed in the presence of 2-hydroxypropyl-β-cyclodextrin (6 mM) or Chol (250 μM) in 2-hydroxypropyl-β-cyclodextrin (6 mM). Bhuvaneswaran et al. (75) studied the effects of 25-OH-Chol on ACAT activity of rat liver microsomes. In accord with numerous previous reports, 25-OH-Chol stimulated the esterification of endogenous Chol. Half-maximal stimulation was observed at 17 μM. The results of detailed studies of the effects of the 25-OH-Chol on Chol transfer processes were interpreted as indicating that the effect of 25-OH-Chol in increasing the levels of ACAT activity in vitro were not related to an effect of the 25-OH-Chol to increase the availability of the Chol substrate to ACAT but rather to some other undetermined mechanism.

3β-Hydroxy-5α-cholest-8(14)-en-15-one has been shown to inhibit ACAT activity of rat hepatic and jejunal microsomes with IC50 values of 10 and 3 μM, respectively (648). The 15-ketosterol was shown to serve as an efficient substrate for ACAT of rat liver and jejunal microsomes (648) and to cause an inhibition of the oleoyl-CoA-dependent esterification of Chol. The high potency of the 15-ketosterol in inhibiting ACAT of rat jejunal microsomes in vitro has been confirmed in a number of studies (485, 916). One metabolite of the 15-ketosterol, i.e., (25R)-3β,26-dihydroxy-5α-cholest-8(14)-en-15-one (485, 916), also inhibited ACAT activity in rat jejunal microsomes; however, it was less potent than the parent 15-ketosterol (55% inhibition at 10 μM).

Liza et al. (584) reported that 25-OH-Chol (124 μM) had no significant effect on Chol ester hydrolyase activity in lysosomes, microsomes, or cytosol of rat liver. 3β-Hydroxy-5α-cholest-8(14)-15-one has been shown to be a weak inhibitor of pancreatic Chol ester hydrolyase with Chol oleate as substrate (1046).

2. Cultured cells

In 1975, Brown et al. (138) reported that certain oxysterols, i.e., 25-OH-Chol, 7-keto-Chol, and 6-ketocholesterol, stimulated the formation of labeled Chol esters from endogenous [14C]Chol in human fibroblasts incubated in lipoprotein-deficient medium containing sodium oleate (0.1 mM). 25-OH-Chol was more potent than the two ketosterols in this respect. Under the same conditions, added Chol or β-sitosterol had no effect. After incubation of normal human fibroblasts with 25-OH-Chol (12.4 μM) for 5 h, a subcellular particulate fraction isolated from the cells also showed markedly increased incorporation of label of [14C]oleoyl-CoA into material with the TLC behavior of Chol esters. Drevon et al. (261) also observed that 25-OH-Chol (25 μM) caused a stimulation of the incorporation of [3H]oleate into Chol ester by rat hepatocytes. However, this effect varied markedly with time of exposure of the cells to 25-OH-Chol. At 15 min, the stimulatory effect was maximal; it was much less at 4 h, and by 18 h, no stimulatory effect on Chol ester formation was observed. 7-Keto-Chol (25 μM) also stimulated the incorporation of labeled oleate into Chol esters. Chol (26 and 52 μM) had no stimulatory effect on the incorporation of [3H]oleate into Chol esters. Long-term (20-h) incubation of 25-OH-Chol (25 μM) with rat hepatocytes in the presence of added oleate (50 μM) had no effect on the levels of Chol esters in the cells. At earlier time points (2 and 6 h), an elevation of Chol esters (relative to control cells) was observed. Goldstein et al. (344) reported that three synthetic analogs of 7-keto-Chol with modifications in the side chain (Fig. 12) had different effects on cholesterol ester formation than did 7-keto-Chol.

SC-31769 inhibited the incorporation of [14C]oleoyl CoA into Chol esters (as judged by TLC) after incubation with a cell-free extract of human fibroblasts (344); SC-31769, at 12.5 and 125 μM, reduced the formation of Chol esters to ~35 and ~15% of the control value, respectively. 25-OH-Chol (12.4 and 124 μM) had no effect on Chol ester formation. In another experiment, the analogs inhibited Chol ester formation with cell-free extracts of human fibroblasts as follows: SC-31769 (2.5 μM), 75%; SC-31448 (2.3 μM), 74%; and SC-31082 (2.3 μM), 56%. SC-31769 (12.5 μM) inhibited the LDL-induced stimulation of the incorporation of [14C]oleate into Chol esters. SC-31769 (12.5 μM) caused a stimulation of Chol ester formation by 10.220.33.3 on August 12, 2017 http://physrev.physiology.org/ Downloaded from
tion. In incubations of the fibroblasts in the presence of added LDL, the three analogs of 7-keto-Chol (SC-31769, 25 μM; SC-31448, 23 μM; and SC-31082, 23 μM) markedly inhibited the formation of Chol esters from the \[1^{14}\]C]oleate, whereas 7-keto-Chol (25 μM) and 25-OH-Chol (25 μM) had little or no effect.

Bates et al. (53) reported that different oxysterols had different effects on the incorporation of labeled oleate into Chol esters in human fibroblasts and in monkey arterial smooth muscle cells incubated with media containing lipoprotein-deficient serum. 25-OH-Chol, at 12.4 and 25 μM, markedly stimulated Chol ester formation. No effect was observed at 2.5 μM. 7-Keto-Chol (at 2.5, 12.5, and 25 μM) stimulated Chol ester formation. However, 22-OH-Chol (at 2.5, 12.4, and 25 μM) and 22-hydroxy-25-fluorocolesterol (at 2.4, 12, and 24 μM) had no effect on Chol ester formation. In the smooth muscle cells, 25-OH-Chol (at 2.5, 12.4, and 25 μM) clearly stimulated Chol ester formation; however, 22-OH-Chol (at 2.5, 12.4, and 25 μM) and 22-hydroxy-25-fluorocolesterol (at 2.4, 12, and 24 μM) had no effect on Chol oleate formation. When the cells were incubated in media containing hyperlipidemic serum (1%), which induced a marked increase in the incorporation of \[1^{14}\]C]oleate into Chol esters, 22-hydroxy-25-fluorocolesterol (at 2.4, 12, and 24 μM) markedly suppressed the incorporation of labeled oleate into Chol esters in both human fibroblasts and monkey smooth muscle cells. Under these conditions (i.e., hyperlipidemic serum), 22-OH-Chol and 22-hydroxy-25-fluorocolesterol showed almost the same degree of inhibition of Chol ester formation from labeled oleate. Zhang et al. (1221) also reported that different oxysterols had different effects on Chol ester formation from \[1^{14}\]C]oleic acid in cultured mouse peritoneal macrophages. 7-Keto-Chol (12.5 μM) and 25-OH-Chol (12.5 μM) (which was considerably more potent) each caused a marked stimulation of Chol ester formation. “7-Hydroxycholesterol” (12.5 μM) and “5,6-epoxycholesterol” (12.5 μM) had a slight stimulatory effect, whereas (22R)-22-OH-Chol (12.5 μM) had no effect. Acetyl-LDL stimulated Chol ester formation in the peritoneal macrophages. At the same concentrations as above, 7-keto-Chol caused a further increase (as did, to a lesser extent, 5,6-epoxy-Chol). 25-OH-Chol had no effect on the Chol esterification induced by acetyl-LDL. However, (22R)-22-OH-Chol and “7-hydroxycholesterol” caused an inhibition.

Cao et al. (154) reported on the effects of incubation of several oxysterols on the incorporation of labeled oleate into Chol esters (as judged by radio-TLC) in a murine macrophage cell line (J774A.1). Incubations were carried out for 12 h in medium containing lipoprotein-deficient FCS plus added LDL. Under these conditions, 5β,6β-epoxy-Chol (0.8 μM) stimulated (+71%) Chol ester formation. Less active in this respect were the 5α,6α-epoxysterol (+39%), cholest-3,5-dien-7-one (+30%), and 25-OH-Chol (+17%). Chol (0.8 μM) had no effect, and 5α,6β-diOH-Chol had a slight inhibitory action (~14%). The authors also reported the effects of the same oxysterols on the cellular levels of free Chol, free desmosterol, and “steryl esters” (assumed to be a mixture of esters of Chol and desmosterol) after 24-h incubation of the cells with medium containing lipoprotein-deficient FCS plus LDL. The HPLC separation of desmosterol from Chol was incomplete. Moreover, the material believed to be desmosterol and Chol appeared to be incompletely separated from other ultraviolet-absorbing (210 nm) material such as to preclude an accurate determination of the levels of desmosterol and Chol. Under the conditions used, esters of Chol were eluted only shortly after free Chol. 5β,6β-Epoxy-Chol (0.8 μM) was reported to cause an increase in total sterols, an increase in free Chol, a marked decrease in free desmosterol, and a marked increase in steryl esters. In contrast, 25-OH-Chol (0.8 μM), 5α,6α-epoxy-Chol (0.8 μM), or 5α,6β-diOH-Chol (0.8 μM) had no effect on each of the above. Cholesta-3,5-dien-7-one (0.8 μM) increased “steryl esters” and decreased the level of free desmosterol.

Ridgway (848), as part of studies of other matters, reported a concentration-dependent stimulation, by 25-OH-Chol, of the formation of labeled Chol esters from \[1^{14}\]C]oleate in CHO cells. The studies were carried out in DMEM containing 5% “delipidated” FCS (prepared by ultracentrifugation). The amount of labeled Chol esters was maximal (over 3 times control) at 12.4 μM 25-OH-Chol. Ridgway and Lagrange (852) observed that 25-OH-Chol caused a marked stimulation of the incorporation of \[3^{14}\]H]oleate into Chol esters in CHO-K1 cells and in marsupial kidney PtK1 cells. Brefeldin A (1 μg/ml) caused a similar stimulation of Chol ester formation in CHO-K1 cells as 25-OH-Chol. Incubation of CHO-K1 cells with both 25-OH-Chol and brefeldin A resulted in a further increase in Chol esterification; however, effects of the two compounds on Chol ester formation were not 100% additive. Brefeldin A, at the concentration used, was shown to disrupt the Golgi apparatus (852). Thus an intact Golgi apparatus does not appear to be required for observation of the stimulation of Chol ester formation from \[3^{14}\]H]oleate in CHO-K1 cells.

Kusuhara et al. (528) reported that 25-OH-Chol stimulated Chol ester formation in Caco-2 cells as measured by the incorporation of \[1-1^{14}\]C]oleate into material with the TLC mobility of Chol esters. Significant stimulation by 25-OH-Chol was observed at concentrations of 0.1, 1, and 10 μM. Cycloheximide (10 μg/ml) and actinomycin D (5 μg/ml) had no effect on the stimulation of Chol ester formation caused by 25-OH-Chol, suggesting that this stimulation does not require protein synthesis. Staurosporine (1 or 10 nM), an inhibitor of protein kinases, caused a reduction in the stimulation of Chol ester formation induced by 25-OH-Chol. Staurosporine, at the
same concentrations, had no effect on the levels of Chol ester formation in the absence of added 25-OH-Chol. Incubation of the Caco-2 cells with 25-OH-Chol resulted in an increased level of ACAT activity in microsomes prepared from the cells. Dashti (239) reported that incubation of Hep G2 cells with 25-OH-Chol (50 μM), while having no effect on the levels of total Chol in cells or in the culture medium, had a marked effect on the state of esterification of Chol. The levels of Chol esters in the cells and medium increased 104 and 219%, respectively. The levels of free Chol in cells and medium decreased 43 and 50%, respectively. Progesterone (16 μM), an inhibitor of ACAT (286, 306, 344, 569, 652, 987), caused a partial reversal of the effects of 25-OH-Chol on the levels of free Chol in cells and on the levels of Chol esters in cells and in the medium. In contrast, purified Chol, even at very high levels (i.e., 259 μM) had little or no effect on the levels of Chol esters in cells or in medium or on the levels of free Chol in the medium. In contrast to 25-OH-Chol, purified Chol caused a substantial increase in the levels of free Chol in the cells.

Bhadra et al. (74) observed that incubation of the 5α,6α- and 5β,6β-epoxides of Chol (25 μM) with human fibroblasts for 24 h in the presence of [14C]oleate led to increased levels of incorporation of the [14C] into Chol esters as judged by TLC. No data were presented to show that “cholesterol esters” did not contain esters of the epoxysterols. Cholesta-3,5-dien-7-one (26.2 μM) was reported to cause a slight (+30%) stimulation of the incorporation of [14C]oleic acid into material with the TLC behavior of Chol esters. Mahfouz et al. (605) reported that 5α,6β-dioH-Chol (23.8 μM) increased the incorporation of [14C]oleic acid into Chol esters in porcine kidney cells after 6-h incubation with the labeled oleate. However, after incubation with [14C]oleate for 1 or 12 h, no significant effect of the 5α,6β-dioH-Chol was observed.

Matsuda et al. (623) studied the effects of the combination of 25-OH-Chol and Chol on ACAT in Hep G2 cells. The cloning of human ACAT cDNA (173) prompted investigations of possible transcriptional regulation of ACAT in Hep G2 cells by the sterol mixture. Incubation of the cells with the mixture of 25-OH-Chol (2.4 μM) and Chol (26 μM) in medium containing 3% lipoprotein-deficient newborn calf serum resulted in 1) elevation of the Chol ester levels in the cells, 2) increased levels of incorporation of labeled oleate into Chol oleate, and 3) increased levels of microsomal ACAT activity. In contrast, the sterol mixture had no effect on the levels of ACAT mRNA. Furthermore, the levels of ACAT activity in a reconstituted system were not affected by incubation of the cells with the sterol mixture, a finding suggesting that the sterol mixture did not affect the level of ACAT protein. In the same study, the mixture of 25-OH-Chol and Chol was reported to decrease binding and degradation of 125I-LDL and to decrease the levels of mRNA for the LDL receptor and HMG-CoA reductase.

Chang et al. (172) demonstrated that the stimulation of ACAT activity in human fibroblasts or Hep G2 cells by 25-OH-Chol (25 μM) or by LDL (160 μg/ml) was not accompanied by significant increases in ACAT protein as studied by immunoblot analysis. These results indicated that modulation of the levels of ACAT protein is probably not important in the oxysterol regulation of ACAT in these cells. Cheng et al. (194) studied the effects of oxysterols on Chol ester formation in insect Sf9 cells transfected with human ACAT cDNA. Uninfected Sf9 cells had no detectable “ACAT-like activity.” Addition of 25-OH-Chol to the transfected cells caused a very marked stimulation of the incorporation of [3H]oleate into Chol oleate. The concentration required to give half-maximal activation was ~2.5–5.0 μM. Other oxysterols tested (7-keto-Chol, 6-ketocholestanol, and 7α-OH-Chol) did not show this effect. 25-OH-Chol (2.5–25 μM) had no effect on the levels of ACAT protein of the cells. 25-OH-Chol increased ACAT activity when added to cell extracts of the transfected Sf9 cells. The maximal activation by 25-OH-Chol (3.5- to 7-fold) occurred very rapidly (within 10 min). The concentration required to give half-maximal activation was essentially the same (i.e., 2.5–5.0 μM) as observed with the intact cells. 7-Keto-Chol (250 μM) and 6-ketocholestanol (250 μM) also increased ACAT activity in vitro, but considerably less than the increases caused by 25-OH-Chol. 7α-OH-Chol (25 and 250 μM) was reported to cause very marked decreases in ACAT activity in vitro.

Green et al. (349), while screening a mouse macrophage cDNA for receptors for oxidized LDL in a Xenopus oocyte expression system, obtained a clone that encoded a protein that was identical (with the exception of one amino acid residue) in sequence to that reported for mouse ACAT by another laboratory (1142). Injection of the mouse ACAT mRNA into oocytes was reported to induce specific binding of oxidized LDL. The mechanism of this very interesting phenomenon is not clear. The authors noted that a direct interaction of oxidized LDL with ACAT would require a surface localization of ACAT on the eggs. In consideration of this matter, the results of Chang et al. (172) showing localization of ACAT by indirect immunofluorescent microscopy in the endoplasmic reticulum (and not plasma membrane) of human melanoma cells, human skin fibroblasts, and CHO cells appear to exclude this possibility unless the frog eggs are uniquely different in their intracellular localization of ACAT. It appears that overexpression of ACAT may indirectly cause an increase in oxidized LDL binding proteins on the Xenopus cell surface, a possibility also noted by Green et al. (349).
3. Whole animals

Needleman et al. (698) demonstrated that dietary administration of 3β-hydroxy-5α-cholest-8(14)-en-15-one (0.1% in a chow diet) to rats for 8 days resulted in a marked (77%) reduction in the levels of ACAT activity in jejunal microsomes. Under these conditions, no effect of 15-ketocholesterol on the level of microsomal Chol (a substrate in the assay of ACAT) was observed. The level of the free 15-ketocholesterol in the microsomes of the treated animals was quite low (0.43 μg/mg protein) relative to that of free Chol (74.1 μg/mg protein). Erickson et al. (284) studied the effect of short-term (18 or 66 h) dietary administration of 7-keto-Chol (0.05, 0.1, and 0.5% by weight in diet) to male Sprague-Dawley rats on the levels of Chol and Chol esters in microsomes. No effect on the levels of free Chol was observed. In contrast, increases in the levels of Chol esters in microsomes were observed in each case except in livers from animals receiving the 7-keto-Chol at a level of 0.05% in diet for 66 h. In these experiments, Chol and Chol esters were separated by TLC and subjected to colorimetric analysis with a ferric chloride color reagent (which was reported to give no color response with 7-keto-Chol). Erickson et al. (285) observed little or no effect of short-term (18 or 66 h) dietary administration of 25-OH-Chol (0.1% in chow diet) to male Sprague-Dawley rats on the levels of Chol or Chol esters in microsomes from liver.

A substantial body of evidence indicates that ACAT is very important in the intracellular metabolism of Chol. A very large number of inhibitors of ACAT have been studied with the goal of inhibition of the intestinal absorption of Chol and/or the inhibition of Chol ester formation in arterial cells. Several major advances in this field were made recently. The native enzyme has never been obtained in a pure form. In 1993, Chang et al. (173) cloned human ACAT, and Uelmen et al. (1142) subsequently isolated mouse ACAT cDNA from a liver cDNA library. The predicted mouse ACAT protein was 87% identical to that for the human enzyme. Administration of a high-Chol high-fat diet to C57BL/6J mice for 3 wk increased ACAT activity in liver microsomes (in 1 of 2 experiments) and increased hepatic ACAT mRNA levels (in both of 2 experiments) (1142). Interestingly, the high-Chol diet had no effect on ACAT mRNA in small intestine in one experiment and modestly decreased the level in another experiment. No effect of the high-Chol diet was observed on the levels of adrenal ACAT mRNA. The levels of ACAT mRNA in mouse aorta appeared to increase on Chol feeding; however, the differences were not statistically significant. Pape et al. (763) isolated a partial rabbit cDNA (ACATn) that showed >90% homology with the human ACAT nucleotide sequence. In agreement with the results of Uelmen et al. with mice (1142), administration of a high-Chol high-fat diet to rabbits resulted in a substantial increase (−2 times) in liver ACAT mRNA. Whereas feeding the high-Chol high-fat diet to rabbits had no significant effect on ACAT mRNA in small intestine and adrenal, an approximately twofold increase was observed in aorta (763). The levels of ACAT mRNA in different organs of C57BL/6J mice on a chow diet were very different (1142). Low levels were observed in liver, adipose tissue, brain, lung, jejunum, duodenum, muscle, kidneys, and heart. Higher levels were detected in thymus, adrenal gland, and testes. Ovary and especially preputial gland contained very high levels of ACAT mRNA. Pape et al. (763) reported that mRNA for ACAT was found in a number of organs of the rabbit. The relative abundances of rabbit ACAT mRNAac were adrenal > omental fat > small intestine > brain > pancreas, spleen > lung > heart > aorta > liver > kidney > skeletal muscle. Adrenal was reported to contain 20-, 30-, and 50-fold more ACAT mRNAac than small intestine, aorta, and liver, respectively. A number of studies have now indicated the existence of multiple forms of ACAT (167, 492, 539, 639, 640). A very major advance was recently described by Chang et al. (174), with the purification to homogeneity of recombinant human ACAT-1. The availability of the purified protein will permit detailed studies of the mechanism of action of this important regulatory enzyme.

L. Oxysterols and Dolichol Synthesis

Mills and Adamany (653) reported that 25-OH-Chol (25 μM) strongly inhibited the incorporation of labeled acetate not only into sterol but also into dolichol of dolicholpyrophosphoryl oligosaccharides by bovine aortic smooth muscle cells. This finding led to the suggestion that “HMG-CoA reductase may function as a rate-controlling enzyme in the biosynthesis of not only sterols but also the dolichols.” 25-OH-Chol (25 μM) had no effect on the incorporation of [5-3H]mevalonate into either sterol or dolichol. These studies involved incubations carried out in medium containing 10% FCS. Under these conditions, sterol synthesis and the levels of HMG-CoA reductase activity would be presumably very low. James and Kandutsch (434) also reported that 25-OH-Chol inhibited the incorporation of labeled acetate into DPS and into dolichol by mouse L cells. However, 25-OH-Chol was a more potent inhibitor of sterol synthesis. At low concentrations of 25-OH-Chol, i.e., 0.6 and 1.2 μM, sterol synthesis was inhibited by 66 and 87%, respectively. In contrast, at the same concentrations of 25-OH-Chol, little or no inhibition of dolichol synthesis (6 and 17%, respectively) was observed. Moreover, at a high concentration of 25-OH-Chol (~12.5 μM) sterol synthesis was inhibited by 99%, whereas dolichol synthesis was inhibited by ~68% (a value that was only slightly different from that observed at 2.4 μM). Under these conditions, the differences in the
inhibition of the incorporation acetate into DPS and into dolichol by 25-OH-Chol cannot simply be ascribed to suppression of HMG-CoA reductase. James and Kandutsch (434) proposed that 25-OH-Chol affects the concentration of a common intermediate, distal to mevalonic acid, which has different Michaelis values for reactions leading to dolichol and Chol. It should be noted that the extent of incorporation of labeled acetate into dolichols is usually only a small fraction of that into Chol (or more properly DPS), 0.03–0.11% in various cultured cell lines (434) and in mouse liver slices (435). However, more substantial incorporation into dolichols has been observed during mouse spermatogenesis (435, 818), in mouse preputial gland (817), at certain times in developing mouse brain (436), and in mouse spleen after induction of anemia by administration of phenylhydrazine (816). James and Kandutsch (437) reported that various treatments (Chol feeding, starvation, cholestyramine treatment) that modulate the incorporation of acetate into DPS also affected the conversion of acetate to dolichol by mouse liver slices. However, in each case, the magnitude of the modulation of dolichol synthesis appeared to be less than that for sterol synthesis. A limitation of this study was in the use of pooled samples of liver (from 4 mice) which precluded any statistical evaluation of these interesting findings.

M. Oxysterols and Prenylation of Proteins

Relatively little is known about the effects of oxysterols on the prenylation of proteins. Repko and Maltese (845) reported findings indicating that preincubation of murine erythroleukemia cells with 25-OH-Chol (25 μM) for 12 h did not appear to cause the accumulation of nonprenylated proteins. It should be noted that the cell incubations were carried out using media containing 10% FCS, a condition for which very low levels of HMG-CoA reductase activity could be anticipated. Leonard et al. (557) reported findings with CHO-K1 cells incubated in medium containing 5% delipidized FCS for 4 h in the presence or absence of 25-OH-Chol (1.24 μM). Under these conditions, no effect of the 25-OH-Chol on the incorporation of [35S]methionine into pro-p21ras (an unprenylated precursor form of mature p22ras) was observed. These results were in contrast to those obtained with a high concentration (24 μM) of mevinolin wherein an accumulation of the pro-p21ras was observed. At lower concentrations of mevinolin, at which HMG-CoA reductase activity was only partially inhibited, no accumulation of the pro-p21ras was reported. It was suggested that with partial inhibition of mevalonate formation, the endogenously synthesized FPP used for prenylation can be selectively used for prenylation rather than for sterol synthesis. Pomerantz et al. (813) found that incubation of arterial smooth muscle cells with 7-keto-Chol (25 μM) for 1 wk resulted in decreases in membrane heterotrimeric G protein expression as measured by immunoblot assay on membrane preparations. Under the conditions studied, the 7-ketosterol reduced Gαi levels to ~20% that of controls and the Gβ subunit to ~50% that of control cells. A direct assay of prenylation of the G proteins was not made.

N. Oxysterols and Cholesterol Efflux

Maor and Aviram (612) reported results suggesting an impairment (approximately ~40%) of the HDL₂-mediated efflux of Chol on incubation of oxidized LDL (containing 7-keto-Chol as the major oxysterol) with J774A.1 mouse macrophages. The results of studies with intact cells and lysosomal extracts indicated that incubations with the oxidized LDL led to an impairment (relative to that observed with LDL or acetylated LDL) in the degradation of its protein component but not of its sterol ester component. Kilsdonk et al. (484) reported that preincubation of [3H]Chol-loaded cells with 25-OH-Chol (12.4 μM) and ovalbumin caused an inhibition (~27%) of the efflux of [3H]Chol from L cells incubated in the presence of HDL₃. Other oxysterols tested showed lesser inhibition under similar conditions. These included 7-keto-Chol, 7α-OH-Chol, 7β-OH-Chol, and (22S)-22-OH-Chol. 5α,6α-Epoxy-Chol and 5α,6β-diOH-Chol showed no inhibitory action. Thus different oxysterols varied in their effects on Chol efflux from L cells under the conditions studied. It is noteworthy that the concentrations of the oxysterols used in this particular experiment were rather high (12–12.5 μM). However, in separate experiments, 25-OH-Chol was found to cause slight, but significant, inhibition of Chol efflux at 0.12 and 1.24 μM using two different assay methods. The inhibition of Chol efflux by 25-OH-Chol varied with respect to cell type. L cells appeared to be most sensitive in this regard. Lesser inhibition was observed in Fu5AH hepatoma cells (a rat hepatoma line) and J774 macrophages. At a concentration of 12.4 μM, 25-OH-Chol had no effect on Chol efflux from EA.Ly 926 cells (a human endothelial cell line). Direct addition of 25-OH-Chol (12.4 μM) to L cells in the presence of HDL₃ (i.e., no preincubation of the cells with the oxygenated sterol) caused a slight but significant inhibition of Chol efflux at 6 and 8 h (but none at 1, 2, 3, or 4 h). Direct addition of 25-OH-Chol (12.4 μM) to isolated plasma membrane vesicles of L cells had no effect on Chol efflux.

Gelissen et al. (326) observed that the efflux of Chol was impaired in mouse macrophage foam cells enriched with 7-keto-Chol (by incubation of the cells with acetylated LDL enriched with 7-ketocholesterol). Efflux of 7-keto-Chol itself was reported to be much less than that of Chol. Further studies (518) with mouse macrophage cells enriched with 7-keto-Chol [by incubation of the cells...
with oxidized LDL prepared by oxidation of LDL in the presence of cupric chloride (10 μM for 24 h at 37°C) indicated higher efflux of Chol than 7-keto-Chol when BSA or BSA plus apo A-I were used as acceptors. In contrast, incubation of the 7-keto-Chol-enriched cells with BSA and hydroxypropyl-β-cyclodextrin led to a markedly increased efflux of 7-keto-Chol relative to that of Chol.

Gesquière et al. (329) reported that HDL₃ preparations enriched with oxysterols reduced the increased efflux of Chol from mouse macrophage cells (P388D₁) induced by incubation of the cells with native HDL₃. Native HDL₃ was reported to contain 7-keto-Chol and 7β-OH-Chol at levels of 5 and 6 ng/mg HDL₃ protein, respectively, and nondetectable levels (<0.5 ng/mg HDL₃ protein) of 7α-OH-Chol and 25-OH-Chol. Enrichment of HDL₃ with a mixture of oxysterols was achieved by oxidation of HDL₃ in the presence of CuSO₄ (50–100 μM) for 16–24 h at 37°C. The oxidized HDL₃ was reported to show high mean levels of the following oxysterols (in ng/mg HDL₃ protein): 7-keto-Chol (2, 234), 7α-OH-Chol (257), 7β-OH-Chol (201), and 25-OH-Chol (49). Enrichment of HDL₃ with individual oxysterols (7-keto-Chol, 7β-OH-Chol, or 25-OH-Chol at levels of 1,170, 1,960, and 1,340 ng/mg HDL₃ protein, respectively, was reported to have been effected by incubation of the individual oxysterols with a solution of HDL₃ for 2 h at 37°C. Identification and quantitation of the oxysterols was based on capillary GC. Chol efflux from cells was significantly decreased by the oxidized HDL₃ or by HDL₃ enriched with the individual oxysterols. The magnitudes of the decreases were not high, i.e., 7.6% for the oxidized HDL₃ and 17, 16, and 14% for HDL₃ preparations enriched with 7-keto-Chol, 25-OH-Chol, and 7β-OH-Chol, respectively. In further studies, with comparable levels of the individual oxysterols in HDL₃, 7-keto-Chol, and 25-OH-Chol were found to be more potent than 7β-OH-Chol in reducing Chol efflux from the cells. Under the same conditions of study, the authors reported that no significant transfer of the oxysterols of HDL₃ to cells was observed. However, no experimental details on this matter were presented.

Caveolae appear to be involved in the efflux of free Chol from cells containing these cell surface invaginations (301). Oxysterols have been reported to regulate this caveolae-mediated efflux of Chol (301). Specifically, 5α,6α-epoxy-Chol has been reported to inhibit the efflux of free Chol from human fibroblasts. This effect has been ascribed to an inhibition of the transport of free Chol to caveolae (and not to a direct inhibition of the efflux of free Chol from the caveolae) (301). Other oxysterols studied, i.e., 5β,6β-epoxy-Chol, 7-keto-Chol, and 7α-OH-Chol, showed roughly comparable extents of inhibition of the transport of free Chol to the surface of the cells. The same oxysterols also were reported to show roughly comparable (~50%) reductions of caveolin mRNA levels, suggesting a direct involvement of caveolae and caveolin in the efflux of Chol from the fibroblasts. It is important to note that this relationship would not be involved in all cells, since a number of cell types appear to lack caveolae and caveolin. The levels of the oxysterols studied were very high, i.e., reported as 10% (wt/wt) relative to plasma free Chol. Hailstones et al. (371) reported that incubation of Madin-Darby canine kidney (MDCK) cells with 25-OH-Chol (1.2 μM) plus Chol (26 μM) for 3 days had no effect on the levels of caveolin protein. Under the same conditions, no effect on the level of caveolin mRNA was observed other than that resulting from a 24% reduction of cellular Chol levels. Studies with bacterially expressed caveolin (lacking bound Chol) showed significant binding of Chol (28 μg Chol/mg protein) (686). A very recent study (1114) indicated the occurrence of two types of arterial smooth muscle cells, a contractile type and a “synthetic” type, which differed in morphology and in the numbers of caveolae. The synthetic cells had few caveolae, and it was suggested that, whereas caveolin was demonstrable in Golgi-like structures, these cells had a low capacity to transport Chol to the plasma membrane via caveolin with the eventual result of Chol ester accumulation in lipid droplets and foam cell formation.

Individual oxysterols have also been reported to differ in their efflux from phospholipid monolayers (1111). Under the conditions studied, little transfer of Chol from the membranes was observed in the presence of HDL₃, HDL₂, LDL, VLDL or small unilamellar vesicles or large unilamellar vesicles. However, significant rates of transfer of different oxysterols were observed. The oxysterols (in order of increasing rates of transfer) were 7-keto-Chol, 7β-OH-Chol, 7α-OH-Chol, and 25-OH-Chol. This order was the same irrespective of the nature of the lipoprotein added or of the type of vesicle. With the different lipoproteins, the highest rates of transfer were observed with HDL₂. The rates of transfer of 25-OH-Chol from the monolayer in the presence of HDL₃ were particularly high with a t₁/₂ of ~7 min under the conditions studied. 7-Keto-Chol has been reported to show a much higher rate of desorption from monolayers to β-cyclodextrin than did Chol (728).

Morel et al. (672) reported that the rate of efflux of 25-OH-Chol from J774 macrophages was much greater than that of Chol. The efflux of 25-OH-Chol was increased by the presence of HDL and BSA. In contrast, the efflux of Chol from the macrophages was unaffected by BSA. Kritharides et al. (517) reported decreased release of 7-keto-Chol (relative to Chol) to medium containing apo A-I in mouse macrophage cells preincubated with oxidized LDL (24 h at 37°C with 10 μM cupric chloride). However, the methodology presented for the identification and measurement of the 7-keto-Chol (reverse-phase HPLC) was not convincing. Babiker et al. (40) observed that albumin increased the secretion of labeled 26-OH-
Chol and, more strikingly, 3β-hydroxycholest-5-en-26-oic acid from human pulmonary macrophages that were pre-labeled with [4-14C]Chol. In contrast, albumin had no stimulatory effect on the levels of labeled Chol recovered in the culture medium. Addition of LDL, HDL, or apoA-I increased the amounts of 26-oxygenated sterol products and labeled Chol that were recovered in the culture medium. 25-OH-Chol (12.4 μM) was reported to decrease the amount of 26-oxygenated sterol products recovered in culture medium of human lung alveolar macrophages. Lange et al. (544) also reported that 25-OH-Chol or 7-keto-Chol; 3β-hydroxycholest-5-en-3β-ol from prelabeled human erythrocytes to plasma culture medium of human lung alveolar macrophages. The amount of 26-oxygenated sterol products recovered in the culture medium. 25-OH-Chol (12.4 μM) plus Chol (129 μM) on the levels of mRNA for apoA-I, apoB, and apoE in Hep G2 cells incubated in serum-free medium. Carlson and Kottke (158) reported that incubation of Hep G2 cells (medium containing 10% FCS) with the 15-ketosterol for 42 h. The 15-ketosterol, at a concentration of 0.25 μM, also inhibited the secretion of labeled apoE (−51% and −43% in 2 experiments). It was reported that the 15-ketosterol did not decrease the synthesis and secretion of total protein. In hepatocytes isolated from hypercholesterolemic rabbits, the 15-ketosterol, at a concentration of 0.25 μM, had no effect on the secretion of labeled apoB and apoE. The 15-ketosterol was also reported to inhibit the secretion of newly synthesized apoB by human Hep G2 cells (55% inhibition at 0.25 μM).

P. Oxysterols and Fatty Acid Synthesis

The studies of Kandutsch and co-workers (169, 186, 466, 921, 929, 934, 935, 937, 938) and others (758) have indicated that, for quite a large number of oxysterols, little or no effect on the incorporation of labeled acetate into fatty acids was observed at concentrations at which the oxysterols caused a major depression of the incorporation of acetate into DPS. Very recently, the results of two studies (478, 588) indicated that, under certain conditions, the synthesis of fatty acids may be coordinated with that of Chol. Lopez et al. (588) reported that Hep G2 cells incubated in media containing lipoprotein-deficient serum (10%) and mevinolin (1 μM) have high levels of mRNA for acetyl coenzyme A carboxylase, a key regulatory enzyme in fatty acid synthesis, and that these high levels were markedly lowered after incubation of the cells under the same conditions in the presence of a mixture of Chol (26 μM) and 25-OH-Chol (2.5 μM) for 24 h. The authors proposed that SREBP-1 is involved not only in the transcriptional regulation of Chol metabolism but also that of fatty acid synthesis. Kawabe et al. (478) reported an interaction between regulation of sterol metabolism and that of fatty acid synthase (FAS), an enzyme catalyzing the formation of palmitic acid from acetyl-CoA and malonyl-CoA in the presence of NADPH. These workers observed a 1.6-fold increase in FAS mRNA in Hep G2 cells incubated in the presence of lipoprotein-deficient serum (10%) and compactin (1 μM). Incubation of the cells with 25-OH-Chol (5 μM) in the presence of FCS (10%) lowered the levels of LDL receptor mRNA at 6 and 8 h but had no effect on the levels of FAS mRNA at the same time points. Incubation of the cells with TAN1607A (an inhibitor of squalene synthase), at concentrations of 1, 3, 10, and 30 μM, showed increases in the levels of mRNA species for HMG-CoA reductase, squalene synthase, and the LDL receptor. TAN1607A also increased the levels of FAS mRNA, but only at the higher concentrations (10 and 30 μM). These combined results were interpreted as indicating that the transcriptional regulation of FAS is related to the regulation of Chol metabolism but that the former is
less sensitive to the regulatory factors than is the transcriptional regulation of Chol metabolism. Mahfouz et al. (606) reported that 5α,6β-dioH-Chol, at 24 μM (the single concentration studied), stimulated the incorporation of [3H]acetate into free fatty acids (+67%) and total fatty acids (+52%) in LLC-PK cells (porcine kidney cells). Under the same conditions, incorporation of [3H]acetate into material with the TLC behavior of free Chol was inhibited (−90%).

Q. Oxysterols and Triglyceride Metabolism

25-OH-Chol (25 μM) had no effect on the incorporation of [14C]oleic acid into triglycerides in rat hepatocytes under conditions with which significant stimulation of the incorporation of [3H]oleate into Chol ester was observed (261). 25-OH-Chol (2.5, 12.4, and 25 μM), 22-OH-Chol (2.5, 12.4, and 25 μM), and 22-hydroxy-25-fluorocholesterol (2.4, 12, and 24 μM) had no effect on the incorporation of [14C]oleate into triglycerides in monkey arterial smooth muscle cells (53). Ridgway (848), as part of studies of other matters, reported that 25-OH-Chol (from ~0.6 to 12.4 μM) had little or no effect on the incorporation of [1-14C]oleate into triglycerides in CHO cells. Ridgway (848) also reported data indicating that several oxy-sterols, i.e., 25-OH-Chol (6.2 μM), (22R)-22-OH-Chol (6.2 μM), 19-OH-Chol (6.2 μM), 7β-OH-Chol (6.2 μM), 7-keto-Chol (6.3 μM), and Chol (6.5 μM) had no effect on the incorporation of [1,14C]oleate into triglycerides in CHO-K1 cells. Mahfouz et al. (605) observed that 5α,6β-dioH-Chol (24 μM) increased the incorporation of [14C]oleate into triglycerides in porcine kidney cells. High concentrations of 5α,6α-epoxy-Chol (25 μM) and cholesta-3,5-dien-7-one (26 μM) had slight stimulatory effects (+18 and +7%, respectively) on the incorporation of [1-14C]oleic acid into triglycerides (as measured by TLC) in human fibroblasts (74). 5β,6β-Epoxo-Chol (25 μM) had no significant effect on the incorporation of labeled oleic acid into triglycerides. In contrast, 7-keto-Chol (at 2.5, 12.5, and 25 μM) had no effect on the incorporation of labeled acetate into cellular triglycerides in cultured hepatocytes (507). However, a decreased incorporation of the labeled acetate into triglycerides of VLDL in the medium was observed with 7-keto-Chol at 12.5 and 25 μM.

Seillan (945) studied the effects of 20-OH-Chol and (22R)-22-OH-Chol on the incorporation of [1-14C]acetate into various cellular lipids in bovine aortic media cells. Results were expressed as a percentage distribution in cell lipids. The expected dose-dependent lowering of acetate incorporation into Chol (as judged by TLC) was observed. The decreased incorporation into Chol by (22R)-22-OH-Chol was −11, −84, −85, and −85% at 1, 5, 10, and 15 μM, respectively. The decreased incorporation into Chol by 20-OH-Chol was −53, −89, −89, and −90% at 1, 5, 10, and 15 μM, respectively. The 22-hydroxy and 20-hydroxy sterols were reported to have significant but opposite effects on the incorporation of [1-14C]acetate into triglycerides (and into phospholipids) (as analyzed by TLC). (22R)-22-OH-Chol increased the incorporation of labeled acetate into triglycerides while 20-OH-Chol was reported to cause a decrease. The significant effects of the 22-hydroxysterol were +14, +41, and +175%, at 5, 10, and 15 μM, respectively. The significant effects of the 20-hydroxysterol were −19, −34, −47, and −57% at 1, 5, 10, and 15 μM, respectively. The changes in incorporation of acetate into triglycerides were accompanied by opposite effects on the incorporation of acetate into phospholipids. Seillan (945) also reported that 20-OH-Chol and 5α,6β-dioH-Chol decreased the incorporation of [1-14C]arachidonate into triglycerides (and increased the incorporation of arachidonate into phospholipids) in bovine aortic media cells. It was also reported that the same effects observed with arachidonate incorporation were also observed with oleate incorporation when (22R)-22-OH-Chol and 20-OH-Chol were studied. 5α,6β-dioH-Chol (24 μM; the single concentration studied) had no significant effect on the incorporation of [3H]acetate in material with the TLC behavior of triglycerides by LLC-PK cells (606). Under the same conditions, inhibition of the incorporation of the labeled acetate into material with the TLC behavior of Chol was observed, and stimulation of the incorporation of the [3H]acetate into free fatty acids, total fatty acids, and material with the TLC behavior of phospholipids and diglycerides was observed. The same authors (606) also reported that tril (23.8 μM) stimulated the cellular uptake of [U-14C]glycerol but had no significant effect on its incorporation into triglycerides. Carlson and Kottke (158) reported that preincubation of Hep G2 cells with 25-OH-Chol (5 μM) stimulated (+148%) the secretion of labeled triglycerides (derived from [3H]glycerol) and also stimulated (+244%) the secretion of apolipoproteins (158). Under the same conditions, mevinolin (1 μg/ml) depressed the secretion of labeled triglycerides (−34%) but had little (−15%) effect on the secretion of apolipoproteins (158).

R. Oxysterols and Sphingolipid Metabolism

Ridgway (848) reported that 25-OH-Chol (6.2 μM) increased the incorporation of [G-3H]serine into sphingo-myelin and glucosylceramide in CHO cells using DMEM with 5% “delipidated” FCS. In contrast, these increases were not observed with a number of other commercial oxysterols tested, i.e., (22R)-22-OH-Chol (6.2 μM), 20-OH-Chol (6.2 μM), 19-OH-Chol (6.2 μM), 7-keto-Chol (6.25 μM), 7β-OH-Chol (6.2 μM), or Chol (6.5 μM). The incorporation of [3H]serine into sphingomyelin appeared to be
inhibited by (22S)-22-OH-Chol (6.2 μM). Other oxysterols (or Chol) including 25-OH-Chol (6.2 μM) and (22S)-22-OH-Chol (6.2 μM) had no effect on the incorporation of [3H]serine into phosphatidylserine, phosphatidylethanolamine, or ceramides. 25-OH-Chol was also reported to increase the incorporation of [3H]palmitate into long-chain bases by a factor of 2 (similar to the increase observed on the incorporation of [3H]serine into sphingosine and sphinganine). 25-OH-Chol (6.2 μM) also stimulated the incorporation of [methyl-3H]choline into sphingomyelin but with no effect on the incorporation of the labeled choline in phosphatidylcholine. Under the conditions studied, 25-OH-Chol (6.2 μM) had no effect on the incorporation of [3H]serine into ceramides, phosphatidylserine, or phosphatidylethanolamine. Cells treated with 25-OH-Chol (6.2 μM) for 6 or 18 h showed no changes in the mass levels (nmol/mg cell protein) of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, or diglyceride. Sphingomyelin mass levels were increased (+52%) at 18 h but were unchanged at 6 h. 25-OH-Chol-treated cells showed lower levels of ceramide mass (pmol/nmol lipid phosphorus) than controls (ethanol alone) at 3 and 6 h but no differences at 1 and 18 h. Ridgway (848) also reported that cells treated with 25-OH-Chol displayed increased conversion of [3H]sphinganine-labeled ceramide into sphingomyelin and increased conversion of added [3H]sphinganine-labeled and [3H]sphingosine-labeled N-hexanoyl ceramide into sphingomyelin. 25-OH-Chol (6.2 μM) had no effect on the uptake of the [3-3H]sphinganine into the CHO cells. Incubation of the cells in the presence of 25-OH-Chol (6.2 μM) had no effect on the activities of serine palmitoyltransferase, sphingomyelin synthase, or sphinganine N-acetyltransferase when membrane preparations from the cells were assayed in vitro. In experiments carried out in media containing lipoprotein-deficient serum, addition of LDL, FCS, or Chol (26 μM) did not increase the levels of incorporation of [3H]serine into sphingomyelin, glucosylceramide, or ceramide. However, in these experiments, the addition of 25-OH-Chol (6.2 μM) stimulated the incorporation of [3H]serine into sphingomyelin (+51%) (relative to LDL alone) but had no effect on the incorporation into glucosylceramides or ceramides. Addition of the combination of LDL plus 25-OH-Chol (6.2 μM) stimulated the incorporation of [3H]serine into sphingomyelin (+15%) and into glucosylceramides (+98%) but had no effect on the incorporation into ceramides. Addition of the combination of LDL, Chol (26 μM), and 25-OH-Chol (6.2 μM) increased the incorporation of [3H]serine into sphingomyelin (+67%) but had no effect (relative to LDL + Chol) on the incorporation into glucosylceramides and ceramides. The addition of 25-OH-Chol (6.2 μM) plus FCS increased the incorporation of [3H]serine into sphingomyelin (+194%) and glucosylceramide (+77%) (relative to FCS alone) but had no effect on the incorporation into ceramides. It is also noteworthy that, under the conditions studied, 25-OH-Chol (6.2 μM), Chol (25.9 μM), LDL, or FCS each caused a very marked stimulation of ACAT activity (as assayed by incorporation of [1-14C]oleate into Chol esters). Recent experiments by the same group (532) involving overexpression of OBP in CHO cells indicated that OBP mediates the stimulation of sphingomyelin synthesis by 25-OH-Chol.

Ridgway (849) observed that two ceramides, N-acetyl-β-erythro-sphingosine and N-hexanoyl-β-erythro-sphingosine (from 2 to 10 μM) inhibited Chol esterification in CHO cells. This effect of the ceramides was also observed with cells incubated in the presence of either 25-OH-Chol (6.2 μM) or LDL. The C2-ceramide (10 μM) also markedly lowered ACAT activity in membranes from the CHO cells that were incubated either in the absence or presence (6.2 μM) of 25-OH-Chol. Long-chain ceramides (either the N-hexadecanoyl ceramide or that prepared from bovine sphingomyelin) added in ethanol at 10 μM were inactive on ACAT activity with membranes from the CHO cells. In recent experiments extending this work, Ridgway et al. (853) linked sphingomyelin hydrolysis to dephosphorylation of OBP and indirectly to enhanced Chol esterification and to OBP translocation to the Golgi apparatus.

Gupta and Rudney (362) reported that treatment of several cell types [rat intestinal epithelial cells (IEC-6), human skin fibroblasts, and human hepatoma (Hep G2) cells] with sphingomyelinase (commercial) caused a lowering of HMG-CoA reductase activity. This effect was suggested to be due to increased levels of oxysterols. Ketoconazole, a known inhibitor of the cytochrome P-450 and of the formation of certain oxysterols, was found to prevent the lowering in HMG-CoA reductase activity caused by treatment of the cells with sphingomyelinase. Treatment of the rat intestinal cells with sphingomyelinase was reported to be associated with increased incorporation of [3H]acetate into polar sterols (as judged by TLC and reverse-phase HPLC). Ketoconazole (30 μM) caused a decrease in the incorporation of [3H]acetate by the intestinal cells into polar sterols with or without subsequent sphingomyelinase treatment. This interesting idea should be studied further, since identification of the polar sterols was not made (or demonstration that the labeled materials were, in fact, sterols). The products of the sphingomyelinase-catalyzed hydrolysis of sphingomyelin, i.e., phosphocholine and ceramide, had no effect on the levels of HMG-CoA reductase activity in the IEC-6 cells. Sphingosine at 5 and 10 μM was reported to increase the levels of reductase activity in the cells. However, the results of a subsequent study (808), showed that β-erythro-sphingosine, but not β-threo-sphingosine, caused a reduction in the levels of HMG-CoA reductase activity in CHO-K1 cells. β-Erythro-sphingosine (IC50, 10 μM) suppressed the rise in reductase activity induced by
transfer of the cells to lipid-deficient media and also lowered the elevated levels of reductase activity induced by transfer of the cells to lipid-deficient media. Direct addition of \( \beta \)-erythro-sphingosine to rat liver microsomes (from 1 to 100 \( \mu \)M) had no effect on the levels of HMG-CoA reductase activity.

Chatterjee (182) observed that treatment of human fibroblasts with neutral sphingomyelinase (which catalyzes the conversion of sphingomyelin to phosphocholine and ceramide) resulted in a stimulation of Chol ester synthesis (as measured by the incorporation of labeled oleic acid into Chol esters). Subsequently, Schek et al. (904) reported that treatment of CHO cells with sphingomyelinase inhibits the proteloiytic cleavage of SREBP-2 (thereby preventing the nuclear entry of SREBP-2 and the subsequent transcription of genes induced in sterol metabolism). Sphingomyelinase-treated cells showed increased levels of ceramide and decreased levels of the mature (proteolyzed) form of SREBP-2. Addition of \( \beta \)-acetyl-sphingosine (up to 100 \( \mu \)M) was reported to have no effect on the level of mature SREBP-2. No effect of sphingomyelinase treatment was observed with 25-RA cells (a line of CHO cells with a mutation in the SREBP gene).

Maor et al. (613) reported that 7-keto-Chol (25 \( \mu \)M) caused an inhibition of lysosomal sphingomyelinase activity in a cell-free system [an extract of a lysosomal-rich fraction of a mouse macrophage-like cell line (J774A.1)]. Under the same conditions, the 7-steroid had no effect on the hydrolysis of phosphatidylcholine. Mahfouz et al. (606) reported that 5\( \alpha \),6\( \beta \)-diOH-Chol (24 \( \mu \)M) increased the incorporation of \( ^{32} \)P-phosphate into sphingomyelins (+43% at 6 h and +37% at 12 h), as well as a number of other phospholipid classes, in cultured porcine kidney cells.

S. Effects of Oxysterols on Membranes

In 1991, Luu and Moog (598) presented a short review of oxysterols and their possible actions in modifying membrane properties of cells. Oxysterols can affect membrane fluidity (598, 847, 858, 1111) and the permeability of membranes for cations (395), albumin (104), and glucose (1111), as well as affecting membrane-bound protein kinase C activity (669). Holmes and Yoss (395) reported that 25-OH-Chol increased the permeability of liposomes to Ca\(^{2+}\), with a maximum effect at \( \sim 5 \) mol\%. At a level of 10 mol\%, 25-OH-Chol stimulated Ca\(^{2+}\) uptake, whereas Chol had no effect. 25-OH-Chol (10 mol\%) also stimulated the uptake of Na\(^+\) by liposomes (\( \sim 20 \) times that of Chol-containing liposomes).

Richert et al. (847) reported that 25-OH-Chol and 7\( \beta \)-OH-Chol decreased membrane fluidity in HTC hepatoma cells as measured by changes in fluorescence polarization using 1,6-diphenyl-1,3,5-hexatriene. 25-OH-Chol was more potent than 7\( \beta \)-OH-Chol in this regard. The changes in fluorescence polarization induced by the oxysterols were correlated with the effects of the two sterols in lowering HMG-CoA reductase activity in the cells. Significant increases in fluorescence polarization were observed with 25-OH-Chol at concentrations of 40, 80, 160, and 320 \( \mu \)M, whereas 7\( \beta \)-OH-Chol had no effect at 40 \( \mu \)M but increased fluorescence polarization at levels of 80, 160, and 320 \( \mu \)M. The increases caused by the 7\( \beta \)-OH-Chol were less than those caused by 25-OH-Chol. From the graphical data presented, the \( IC_{50} \) values for lowering HMG-CoA reductase activity were \( \sim 30 \) and 100 \( \mu \)M for 25-OH-Chol and 7\( \beta \)-OH-Chol, respectively. Hagihara et al. (370) studied the effects of Chol analogs with hydroxy functions at C-22 or C-23 on the interaction with multilamellar liposomes composed of dipalmitoylphosphatidylcholine as studied by differential scanning calorimetry and permeability of a fluorescent dye (carboxyfluorescein). (23\( S \))-25-OH-Chol, (23\( R \))-23-OH-Chol, and (22\( R \))-22-OH-Chol showed behavior similar to that of Chol. However, (22\( R \))-22-OH-Chol appeared to show little or no interaction with the phospholipid membrane preparation. Varying the length of the alkyl side chain resulted in no significant difference from Chol on the interaction of the sterol with the phospholipid membrane preparation.

Theunissen et al. (1111) studied the membrane properties of four oxysterols, i.e., 7-keto-Chol, 7\( \alpha \)-OH-Chol, 7\( \beta \)-OH-Chol, and 25-OH-Chol. The results of studies with mixed monolayers with dioleoylphosphatidylcholine indicated that 7-keto-Chol, 7\( \alpha \)-OH-Chol, and 7\( \beta \)-OH-Chol showed a condensing effect which, however, was less than that of Chol. 25-OH-Chol was reported to show no condensing effect. The oxysterols also differed with regard to their effects on glucose permeability, which was studied by following the release of glucose from egg phosphatidylcholine liposomes containing 4 mol\% phosphatidic acid and varying levels of oxysterol (up to 50 mol\%). Whereas Chol (and to a lesser extent 7-keto-Chol, 7\( \beta \)-OH-Chol, and 7\( \alpha \)-OH-Chol) reduced permeability of glucose, 25-OH-Chol caused a marked increase in permeability to glucose. This effect was observed at even relatively low concentrations of 25-OH-Chol (i.e., 2.5 and 5 mol\%). Rooney et al. (858) reported that 20\( \alpha \)-OH-Chol (\( \sim 10 \)% of membrane sterol) resulted in immobilization of lipid acyl chains of erythrocyte membranes as studied by Fourier transform IR. Pannecoucke et al. (761) reported \( ^{2} \)H-NMR studies of the effects of certain oxysterols (15 mol\%) in artificial membrane preparations containing 1-myristoyl-2\([^{3} \)H\(_{2}\)]myristoyl-sn-glycero-3-phosphocholine and Chol (15 mol\%). 25-OH-Chol, (22\( S \))-22-OH-Chol, 7\( \beta \)-OH-Chol, and 7\( \alpha \)-OH-Chol were reported to have ordering effects on the acyl chains of the phospholipid in
the membrane preparations. These results are similar to those reported by Theunissen et al. (1111) with monolayers with the exception that 25-OH-Chol had no condensing effect in the latter studies. Panneconucque et al. (761) also reported results that were interpreted as suggesting that, with 25-OH-Chol, at 15 or 30 mol%, two regions were formed in the membrane preparation, one rich in 25-OH-Chol and one that was 25-OH-Chol poor.

Boissonneault et al. (104) studied the effects of several oxysterols on albumin transfer in endothelial cells obtained from pulmonary arteries of pigs. The cells were incubated with oxysterols (or mevinolin) for 24 h in media containing 5% FCS. The cells were then washed and incubated for 1 h in serum-free medium containing BSA. The medium was then analyzed for albumin levels. A number of oxysterols were shown to enhance "transmonolayer movement of bovine serum albumin." These included (in order of decreasing potency) 5α,6β-diol-Chol, 7β-OH-Chol, 25-OH-Chol, 7-keto-Chol, and 5α,6α-epoxy-Chol. The most potent, the triol, was active at micromolar concentrations and showed maximal effectiveness at 25 μM. Pure Chol was reported to have no effect at concentrations as high as 130 μM. The various oxysterols were also tested with regard to effects on the incorporation of [14C]acetate into DPS. All of the oxysterols reduced sterol synthesis. Recrystallized Chol (130 μM) had no effect on sterol synthesis. There was not a high correlation between effects on albumin transfer and inhibition of sterol synthesis. In a separate experiment, the triol (25 μM) increased albumin transfer markedly (+660%), and sterol synthesis was depressed (11% of control). Mevinolin (12 μM) did not increase permeability to albumin, but it suppressed sterol synthesis to 0.9% that of the control cells. Moog et al. (669) reported the effects of 7β,25-diOH-Chol on protein kinase C activity of spleen cells. The dihydroxysterol (6 and 1.5 μM) lowered the levels of protein kinase C activity in a particulate fraction (membranes) of cells. In studies with peritoneal macrophages, the dihydroxysterol was reported to inhibit the phosphorylation of two endogenous substrates of protein kinase C. Direct addition of the sterol to a purified preparation of protein kinase C from lymphocytes was reported to have no effect on enzyme activity.

T. Effects of Oxysterols on Platelets

Several groups have reported that oxygenated derivatives of Chol have effects on platelet aggregation and related processes. The variability of observed effects may be related to differences in methodologies and/or species studied.

Shimada et al. (969) studied the effects of a number of oxygenated derivatives of Chol on the aggregation of bovine platelets induced by thrombin or ADP. Ethanol solutions of the oxysterols were added to Tris-acid citrate dextrose (ACD) (pH 7.35), and the resulting mixture was added to platelet-rich plasma (final concentration of ethanol of 1%). After incubation of the platelets with the oxysterol for 30–60 min, thrombin or ADP was added to induce aggregation. All sterols were added at a concentration of 25 μM. Thrombin-induced aggregation was inhibited by 5α,6β-diol-Chol and 3β,5-dihydroxy-5α-cholestan-6-one. A stimulation was observed with sterols having hydroxyl functions in the side chain, specifically (20R)-20-OH-Chol, (20S)-20-OH-Chol, (22R)-22-OH-Chol, (22S)-22-OH-Chol, (24R)-24-OH-Chol, (24S)-24-OH-Chol, and 25-OH-Chol. Little or no effect on thrombin-induced platelet aggregation was observed with Chol, 7-keto-Chol, 5α-cholestan-3β,6β-diol, 6-ketocholestanol, and 5α,6α-epoxy-Chol. Markedly different effects of the oxysterols were observed on platelet aggregation induced by ADP. At the same concentration (25 μM), none of the sterols noted above stimulated platelet aggregation except for (22S)-22-OH-Chol. Moreover, two sterols, i.e., (24S)-24-OH-Chol and 25-OH-Chol, which stimulated thrombin-induced platelet aggregation, were reported to inhibit ADP-induced aggregation of platelets. More detailed studies with the 22R- and 22S-isomers of 22-OH-Chol indicated that both isomers caused a concentration-dependent stimulation of platelet aggregation induced by thrombin. The 22R-isomer appeared to be slightly more active than the 22S-isomer. Under the same conditions, added Chol (up to and including 100 μM) had no effect. In striking contrast, the 22R-isomer had little effect on platelet aggregation induced by ADP (with possible slight inhibition at the higher concentrations studied, i.e., 75 and 100 μM). The 22S-isomer of 22-OH-Chol showed a clear concentration-dependent stimulation of platelet aggregation induced by ADP. Added Chol (up to and including 100 μM) had no effect on platelet aggregation under the same conditions. The stimulation of the thrombin-induced platelet aggregation by (24S)-24-OH-Chol (50 μM) required only a short period of preincubation with the oxysterol (with maximum stimulation after only ~10 min). Similar results were observed with the stimulation and inhibition of ADP-induced platelet aggregation by (22S)-22-OH-Chol (50 μM) and (24S)-24-OH-Chol (50 μM), respectively. Under the conditions studied, added Chol (50 μM) or ethanol (1%) had no effect on platelet aggregation, induced by either thrombin or ADP, over the preincubation periods examined (up to 120 min). Saito et al. (884) reported that (22R)-22-OH-Chol caused a lysis of bovine platelets, as measured by the release of lactate dehydrogenase. Little or no lysis was observed at 25 μM (or lower concentrations) after incubation of the oxysterol (added in ethanol to Tris-ACD buffer) with platelets for 20 min at 37°C. At concentrations of 50, 75, and 100 μM, the extent of lysis of the platelets was ~10, 18, and ~35%, respectively. Very interestingly, (22S)-22-OH-Chol had little or no effect at various concentrations up to and including 100 μM.
The oxysterols studied were 26-OH-Chol, 7-Keto-Chol (at 10 \( \mu M \)) and 5,6,6\( \beta \)-diOH-Chol (at 20 \( \mu M \)). 25-OH-Chol (at 20 \( \mu M \), but not at 5 \( \mu M \)) showed inhibition of thrombin-induced platelet aggregation, whereas no effect was observed with 7\( \beta \)-OH-Chol (5, 10, and 20 \( \mu M \)) and 7-keto-Chol (5 and 10 \( \mu M \)). In the case of ADP-induced platelet aggregation, (22S)-22-OH-Chol (at 2.5, 5, and 10 \( \mu M \)) and 5,6\( \beta \)-diOH-Chol (at 20 \( \mu M \) but not at 5 and 10 \( \mu M \)) were reported to be stimulatory. 7-Keto-Chol (at 10 \( \mu M \) but not at 5 \( \mu M \)) and 25-OH-Chol (at 5, 10, and 20 \( \mu M \)) were inhibitory. 7\( \beta \)-OH-Chol (at 5, 10, and 20 \( \mu M \)) had no effect. Purified Chol was stated to have no effect on platelet aggregation. Experiments involving preincubation of the oxysterols complexed with lipid-free BSA for 30 min at 22\( ^\circ \)C were reported to show stimulation of thrombin-induced platelet aggregation and serotonin release by (22S)-22-OH-Chol and 5,6\( \beta \)-diOH-Chol, inhibition of aggregation and serotonin release by 25-OH-Chol, and no effect on aggregation or serotonin release with 7-keto-Chol and 7\( \beta \)-OH-Chol. Significant stimulation of ADP-induced aggregation was reported for (22S)-22-OH-Chol but not 5,6\( \beta \)-diOH-Chol, 7\( \beta \)-OH-Chol, and 7-keto-Chol. 25-OH-Chol was reported to be inhibitory.

Selley et al. (948) reported that certain oxysterols potentiated the aggregation of human platelets induced by ADP, thrombin, or collagen. No assessment of the purity of the oxysterols was presented. The oxysterols were added to platelet-rich plasma in ethanol. It was stated that the concentrations of ethanol used “did not adversely influence agonist-induced activation responses.” The oxysterols studied were 26-OH-Chol, 7\( \alpha \)-OH-Chol, 5\( \alpha \),6\( \beta \)-diOH-Chol, 7-keto-Chol, cholesta-3,5-dien-7-one, 5\( \alpha \),6\( \alpha \)-epoxy-Chol, and 5\( \beta \),6\( \beta \)-epoxy-Chol. Under the conditions studied, i.e., preincubation of the platelet-rich plasma with the oxygenated sterol for 60 min before the addition of agonists, all oxysterols were reported to be active in augmenting platelet aggregation induced by the agonist. The oxysterol concentrations employed were rather high, i.e., 5–100 \( \mu M \). The potencies of the various oxysterols on platelet aggregation induced by the different agonists appeared to vary. For example, 26-OH-Chol, at 10 \( \mu M \), was reported to cause a substantial increase in platelet aggregation induced by thrombin, whereas the same oxysterol at 25 \( \mu M \) resulted in only a slight increase in platelet aggregation induced by ADP. Selley et al. (948) observed that either 7-keto-Chol or 5\( \beta \),6\( \beta \)-epoxy-Chol, at concentrations of 100 \( \mu M \), caused increases in the release of arachidonate from platelet membranes (in the presence of collagen). The observed effects with the 7-keto-Chol and the 5\( \beta \),6\( \beta \)-epoxide (+14 and +15%, respectively) appeared to be slight. All of the oxysterols studied were reported to potentiate the production of thromboxane A2 (assayed as thromboxane B2) induced by ADP, thrombin, and collagen. However, the results presented indicated that, with ADP as agonist, thromboxane B2 levels were lower with 26-OH-Chol (25 \( \mu M \)) than in controls. With regard to potentiation by the oxysterols of platelet aggregation induced with the agonists, 26-OH-Chol was stated to be the most active “while 7\( \alpha \)-hydroxycholesterol and cholestanetriol were more active than 7-ketoocholesterol, cholesta-3,5-diene-7-one, cholesterol 5\( \alpha \)-epoxide, and cholesterol 5\( \beta \)-epoxide.”

The results of Selley et al. (948) with human platelets differ from those of Blache and Bontoux (98) who reported that 7-keto-Chol and 25-OH-Chol had an inhibitory effect on the aggregation of rat platelets induced by ADP and thrombin. Both studies indicated a potentiation by 5\( \alpha \),6\( \beta \)-diOH-Chol. The results of Selley et al. (948) with human platelets also differ from those of Shimada et al. (969) with bovine platelets, in which it was reported that 5\( \alpha \),6\( \alpha \)-epoxy-Chol and 7-keto-Chol had no effect on platelet aggregation induced by thrombin, and 5\( \alpha \),6\( \beta \)-diOH-Chol inhibited this process. It is very important to note that the in vitro effects of the various oxygenated sterols on platelet aggregation reported by Selley et al. (948) were observed at quite high concentrations, i.e., from 10 to 100 \( \mu M \). The authors noted that these concentrations of oxysterols were less than reported plasma levels of certain oxysterols in normal human subjects and cited the results of Breuer and Björkhem (122). From these results, Selley et al. (948) calculated the millimolar concentrations of 7\( \alpha \)-OH-Chol, 7-keto-Chol, 5\( \beta \),6\( \beta \)-epoxy-Chol, 5\( \alpha \),6\( \alpha \)-epoxy-Chol, and 5\( \beta \),6\( \beta \)-epoxy-Chol in plasma to be 286, 175, 189, 60, and 48 \( \mu M \), respectively. These values are incorrect as they should be 0.286, 0.175, 0.189, 0.060, and 0.048 \( \mu M \), respectively. This situation results from an obvious typographical error in Table 3 of the paper by Breuer and Björkhem (122) as can be noted from the discussion of their results. Also cited by Selley et al. (948) for the physiological and medical relevance of their results on the effects of oxysterols on platelet aggregation was the extraordinarily high value reported by Gray et al. (348) of 11,300 \( \mu M \) for 5\( \alpha \),6\( \alpha \)-epoxy-Chol in a sample of pooled serum from patients with hypercholesterolemia. In the 1996 paper of Selley et al. (948) they stated that “no reliable data on the concentration of oxysterols in serum from patients with atherosclerosis have been reported.” It should be noted that Björkhem et al. (86) in 1988 reported no elevation of unesterified 5\( \alpha \),6\( \alpha \)-epoxy-Chol in patients with atherosclerosis or in FH or familial combined hyperlipidemia. Moreover, the levels of the free epoxide in plasma reported by Björkhem et al. (86) for normal human subjects appear to be clearly erroneously high in
view of the very low levels of the 5α,6α-epoxide (free plus esterified) shown to be present in normal human plasma (41, 273, 521).

U. Oxysterols and Signal Transduction

It has been proposed that oxygenated sterols might be involved in signal transduction. Lahoua et al. (534) observed that several oxysterols potentiated the effect of FCS in the stimulation of arachidonic acid release in NRK 49F cells. 7α-OH-Chol, 7β-OH-Chol, and 25-OH-Chol were active in this respect, whereas 7-keto-Chol and Chol had no effect at various concentrations including the highest levels tested (25 μM for 7-keto-Chol and 26 μM for Chol). 7α-OH-Chol and 7β-OH-Chol showed activity at the lowest concentration studied (1.5 μM). 7α-OH-Chol appeared to be more potent than its 7β-hydroxy isomer at all concentrations studied (from 1.5 to 25 μM). At concentrations of 6.2, 12.4, and 26 μM, 7β-OH-Chol and 25-OH-Chol had essentially the same effect. The stimulation of arachidonate release by the oxysterols was only observed in the presence of FCS. In the presence of FCS, 25-OH-Chol not only stimulated its release but also the formation of the prostaglandins PGE₂ and PGF₂α (as measured by radioimmunoassay). The effects of 25-OH-Chol on prostaglandin formation did not appear to be due to an effect on cyclooxygenase activity. Most of the effect of 25-OH-Chol on arachidonate release appeared to be due to an effect on arachidonate release from phosphatidylinositol.

The stimulating effect of oxysterols on arachidonate release and on prostaglandin formation occurred in the absence of Ca²⁺ in the external medium or when cellular Ca²⁺ stores were depleted (533). The oxysterols (25-OH-Chol, 7α-OH-Chol, and 7β-OH-Chol, at a concentration of 12.4 μM) showed no Ca²⁺ ionophoretic properties by themselves. However, the oxysterols potentiated the arachidonate release induced by ionomycin (a Ca²⁺ ionophore) (533). It was concluded that the oxysterol effects on arachidonate release and on prostaglandin biosynthesis are probably mediated by a mechanism other than one involving a direct effect on Ca²⁺ metabolism (533). The results of this study and a subsequent investigation (535) suggested that the effects of oxysterols on arachidonate release are synergistic, but not fully dependent on direct effects of the oxysterols on protein kinase C or on Ca²⁺ metabolism. Moog et al. (669) reported that 7β,25-diOH-Chol caused a decrease in the protein kinase C activity of mouse spleen lymphocytes, which were stimulated by concanavalin A. No effect of the oxysterol was observed in a cell-free system. The 7β,25-dihydroxysterol was also reported to decrease the phosphorylation of a substrate of protein kinase C in phorbol myristate acetate-stimulated mouse peritoneal macrophages. However, in these studies, no expression of variation or statistical treatment of data was presented.

Lahoua et al. (535) observed that 7α-OH-Chol (12.4 μM) and 25-OH-Chol (12.4 μM) potentiated the increase in arachidonate release and PGE₂ formation induced by the phorbol ester TPA (an activator of protein kinase C) in NRK 49F (normal rat kidney, fibroblastic clone 49F) cells. 7-Keto-Chol (12.5 μM) had no effect on arachidonate release either in the presence or absence of the phorbol ester. On the basis of further studies with 25-OH-Chol, the authors suggested that the stimulation by the oxysterol of arachidonate release and PGE₂ biosynthesis involved activation of phospholipase A₂, which is mediated by protein kinase C. The authors further suggested that the action of 25-OH-Chol in these processes might involve effects on binding of growth factors to their receptors that then affected protein kinase C and Ca²⁺ flux. Kawamura and Kummerow (479) noted that 25-OH-Chol stimulated prostacyclin formation by human umbilical arterial endothelial cells as measured by radioimmunoassay of the levels of 6-keto-PGF₁α, an hydrolysis product of PGI₂. This stimulation was observed at concentrations of 25-OH-Chol of 2, 10, and 20 μM. In contrast, Peng et al. (791) claimed that certain oxysterols caused an inhibition of prostacyclin (PGI₂) formation in human endothelial cells in culture. Incubation of 25-OH-Chol (25 μM), 7-keto-Chol (25 μM), 5α,6α-epoxy-Chol (25 μM), or 5α,6β-diOH-Chol (24 μM) for 12 or 24 h resulted in an inhibition of PGI₂ formation (as judged by immunooassay of 6-keto-PGF₁α, with a commercial assay kit). No inhibition was observed with the same oxysterols at 50% of the concentrations noted above. No effect of Chol addition (13 or 26 μM) was observed. Peng et al. (791) also reported that 25-OH-Chol (25 μM), 7-keto-Chol (25 μM), and 5α,6β-diOH-Chol (24 μM) increased the adhesion of platelets to endothelial cell monolayers. Chol (26 μM) was reported to have no effect.

Astruc and Lahoua (26) reported that certain oxysterols potentiated the epidermal growth factor-induced release of arachidonic acid and prostaglandin (PGE₂) synthesis in NRK cells (fibroblast clone 49F). The concentration of the sterols evaluated (5 μg/ml) corresponded to 12.4 μM for dihydroxysterols, 12.5 μM for 7-keto-Chol, and 12.0 μM for 7-keto-25-hydroxycholesterol. Three of the oxysterols evaluated were inactive [7-keto-Chol, 20α-OH-Chol, (22S)-22-OH-Chol]. Compounds that were active under the conditions studied were the following: 7β,25-diOH-Chol, 7-keto-25-hydroxycholesterol, (22R)-22-OH-Chol, 7α-OH-Chol, 7β-OH-Chol, and 25-OH-Chol. The oxysterols had little or no effect on the binding of epidermal growth factor to the NRK cells. Small but statistically significant increases in the epidermal growth factor high-affinity binding site number was found for cells incubated with 25-OH-Chol, 7β,25-diOH-Chol, and 7-keto-25-hydroxycholesterol. No effect was observed for the following: 7-keto-Chol, 7α-OH-Chol, 7β-OH-Chol, 20α-OH-Chol, (22S)-22-OH-Chol, (22R)-22-OH-Chol, and Chol. The combined results were interpreted as indicating that “the po-
tentating effect of oxysterols on arachidonic acid release seems to be exerted downstream to the growth factor receptor (as demonstrated here with epidermal growth factor) and probably at the PKC (protein kinase C) level, but not exclusively.

Wohlfeil and Campbell (1204) observed that incubation of bovine coronary artery endothelial cells with 25-OH-Chol (25 μM) for 48 h resulted in a marked stimulation of the incorporation of [14C]arachidonic acid into prostaglandins. The major labeled product corresponded chromatographically with 6-keto-PGF 1α. The 25-hydroxysterol was also reported to increase the incorporation of the labeled arachidonate into 15- and 11-hydroxyeicosatetraenoic acids and into 12-hydroxyheptadecatrienoic acid. The stimulation of the metabolism of arachidonic acid into the indicated products was blocked by incubation with indomethacin (10 μM). Similar stimulation of the formation of these metabolites of arachidonic acid was not observed with samples of 20-OH-Chol (25 μM) or (22S)-22-OH-Chol (25 μM). The stimulation, by 25-OH-Chol, of the conversion of arachidonic acid into prostaglandins (as judged by TLC) was observed at 7.4 and 25 μM but not at 2.5 μM. The authors reported that “concentrations exceeding 10.0 μg/ml” (25 μM) “resulted in cell death within 12 h of treatment”. Using rabbit polyclonal antibodies prepared against a peptide fragment corresponding to the carboxy-terminal end of prostaglandin G/H synthase-2, 25-OH-Chol induced an increase in the level of the enzyme in the endothelial cells as measured by immunoblot analysis (1204). The concentration of the 25-OH-Chol was not specified but probably corresponded to 25 μM used in other experiments. 25-OH-Chol was reported to have no effect on the levels of prostaglandin G/H synthase-1. The authors claimed that “this is the first time that an oxysterol has been shown to directly modulate endothelial eicosanoid production by enzyme induction.”

Deckert et al. (247) recently reported that 7-keto-Chol and 7β-OH-Chol caused an inhibition of the acetylcholine-induced, endothelium-dependent relaxation of rabbit aortic segments. The concentrations of the two oxysterols, added in ethanol with fatty acid-deficient BSA, were very high, i.e., 150 μM for the 7-ketosterol and 149 μM for the 7β-hydroxyxysterol. Under the same conditions, 19-OH-Chol (149 μM), 5α,6α-epoxy-Chol (149 μM), and Chol (155 μM) were reported to have no effect. 7-Keto-Chol (150 μM) had no effect on the relaxation of the arterial segments induced by sodium nitroprusside.

Martinez-Sales et al. (621) reported that a 24-h feeding of a laboratory chow diet supplemented with 2% “cholesterol oxidation products” to rats resulted in a slight increase (+34%; P = 0.05) in prostacyclin formation by aortic tissue preparations. No determination of the chemical composition of the oxidized Chol preparation was presented. Prostacyclin formation was assayed by the conversion of labeled arachidonic acid to material with the TLC behavior of 6-keto-PGF 1α.

Endo et al. (282) studied the possible role of certain oxysterols in signal transduction processes involved in tumor promotion by TPA. These workers reported that 3β,5α-dihydroxy-5α-cholestan-6-one showed no detectable binding to protein kinase C in an in vitro competition binding assay with labeled TPA. However, this sterol and its 1α-hydroxy analog strongly inhibited the binding of TPA to a cytosolic tumor promoter binding protein that binds strongly to TPA. The association constant of the 3β,5α-dihydroxy-6-ketosterol to the binding protein was reported to be 5 × 10^8 M⁻¹. Modification of the side chain of the 3β,5α-dihydroxy-6-ketosterol decreased the inhibitory effect on the binding of labeled TPA to the binding protein.

Very considerably more information is available on the effects of an oxygenated derivative of vitamin D, i.e., 1α,25-dihydroxyvitamin D₃ on signal transduction processes (995, 1165 and references cited therein). Slater et al. (995) reported that 1α,25-dihydroxyvitamin D₃ causes a direct activation of protein kinase C, with half-maximal activation at 16 ± 1 nM. Vitamin D₃ itself had no significant effect on the enzyme activity. The activating effect of 1α,25-dihydroxyvitamin D₃ was observed with purified protein kinase C (with various isoforms of the enzyme) in the presence of substrate, cofactors, and lipid vesicles. Synergistic effects were observed with either diacylglycerol or 4β-TPA. Slater et al. (995) concluded that “protein kinase C acts as a membrane-bound receptor for 1,25-D₃ and as such may be responsible for many of the non-genomic cellular responses to the hormone.” Vazquez and de Boland (1165) reported that 1α,25-dihydroxyvitamin D₂ was highly active in rapidly stimulating Ca²⁺ uptake by myoblast cells in culture and have indicated the involvement of protein kinase C in this stimulation through experiments with inhibitors of the enzyme.

The formation of oxysterols may also involve signal transduction. As noted previously, Stravitz and co-workers (1049, 1050) have reported findings indicating that protein kinase C appears to be involved in the lowering of transcription of the cyp7a gene caused by hydrophobic bile acids in primary cultures of rat hepatocytes. Activation of protein kinase C with phorbol 12-myristate 13-acetate (0.1 μM) decreased mRNA for cyp7a (−71%) and transcriptional activity (−60%). Taurocholate, at a physiologically relevant concentration (25 μM), decreased mRNA for the 7α-hydroxylase, an effect which could be blocked by preincubation of the cells with inhibitors of protein kinase C. Hydrophobic bile acids were also reported to cause an increase in the amount of protein kinase C that was associated with membranes. The repression of cyp7a mRNA levels in rat hepatocytes by taurocholate was recently suggested to involve calcium-independent isoforms of protein kinase C (1050).
V. Oxysterols and Gap Junctional Communication

Chol has been reported to increase gap junctional communication between hepatoma cells (610, 646) and between human smooth muscle cells (1231). Chang et al. (171) reported that 5α,6α-epoxy-Chol, 5β,6β-epoxy-Chol, and 5α,6β-diol-Chol inhibited gap-junctional intercellular communication in Chinese hamster V79 cells as measured by a metabolic cooperation assay. Under the same study conditions, Chol had no effect. Meyer et al. (646) observed that Chol, at a concentration of 20 μM in medium, resulted in a marked increase in gap junction particle number in Novikoff hepatoma cells. This phenomenon was dependent on the concentration of Chol, and the changes observed at 10, 30, 40, and 60 μM levels of the sterol were less than that observed at 20 μM Chol. Less junction formation, as measured by a decreased number of aggregated gap junction particles and decreased areas of plaque formation, was observed at the above concentrations of Chol than at 20 μM. The increased gap junction formation observed upon addition of Chol appeared to be dependent on protein synthesis, but not RNA synthesis, on the basis of the results of experiments with cycloheximide and actinomycin D. The effects on gap junction assembly induced by 20 μM Chol were also associated with increased intercellular communication as judged by increased junctional permeabilities as determined by dye transfer times after intracellular microinjection of Lucifer yellow. Some aspects of the role of Chol and of other lipids in gap junction formation and function have been reviewed (610).

Zwijsen et al. (1231) reported that certain oxygenated derivatives of Chol inhibit intercellular communication in human arterial smooth muscle cells as measured by a dye-transfer method. A sample of air-oxidized Chol (composition not determined) caused an inhibition of intercellular communication (with a 42% reduction at 400 μg/ml) and significant inhibition at 100 μg/ml but with no effect at 50 μg/ml. In contrast, added Chol (518 μM) was reported to cause a 30% increase. 25-OH-Chol, 5α,6β-diol-Chol, 7-keto-Chol, and 5α,6α-epoxy-Chol (in order of potency) showed significant inhibition of intercellular communication upon incubation with the arterial smooth muscle cells. All four oxysterols showed significant inhibition at 5 μM, and all but 7-keto-Chol showed significant inhibition at 1 μM. Zwijsen et al. (1230) also reported that incubation of LDL for 24 h with smooth muscle cells from human umbilical cord decreased intercellular communication as measured with a dye-transfer method (1230). This inhibition by LDL was essentially abolished by the simultaneous addition of antioxidants (either BHT, α-tocopherol, or glutathione). The mechanisms involved in the actions of oxysterols on gap junctional communication have not been established. They could be secondary to effects of the oxysterols on membrane fluidity or on calcium metabolism. Elevation of the intracellular level of Ca2+ has been stated (1231) to lead to a closure of gap junctions.

W. Oxysterols and Calcium Metabolism

In 1984, Boissonneault and Heiniger (102) reported that 25-OH-Chol (2.49 μM) caused a stimulation of the uptake of Ca2+ into P815 mastocytoma cells. This effect could be prevented by the addition of high concentrations of either Chol (130 μM) or mevalonic acid (5 mg/ml). The stimulation of the uptake of Ca2+ by 25-OH-Chol was dependent on the level of 25-OH-Chol. Mevinolin (5 μg/ml) had no effect on Ca2+ uptake (103). Inhibitors of mitochondrial respiration, which cause an efflux of Ca2+ from mitochondria, inhibited 25-OH-Chol-induced Ca2+ uptake by the cells (103). Verapamil, quindine, and diltiazem had no effect on the 25-OH-Chol-induced increase in Ca2+ uptake. Co2+ and ruthenium red suppressed in the increase induced by 25-OH-Chol.

Different oxysterols had different effects on Ca2+ influx into human erythrocytes (708). The effects of oxysterols also appear to differ with different cell types. In contrast to results presented above for P815 cells (102, 103), 25-OH-Chol has been reported to inhibit the influx of Ca2+ into human erythrocytes (708). (25S)-25-OH-Chol, 5α,6β-diol-Chol, 5α-cholestan-3β-ol, 3β,5-dihydroxy-5α-cholestan-6-one, and 3β-hydroxy-5α-cholestan-7-one caused substantial increases in Ca2+ influx into human erythrocytes (708). In contrast, 25-OH-Chol, 5β-cholesterol, and 7-keto-Chol decreased Ca2+ influx. The observed effects on Ca2+ influx were, with one exception (7β-OH-Chol), reported to be almost exclusively on nifedipine-sensitive Ca2+ flux. With 7β-OH-Chol, ~50% of both the nifedipine-sensitive and nifedipine-insensitive Ca2+ influx was inhibited. The concentrations of the oxysterols studied were high, i.e., 12.5–250 μM. Zhou et al. (1229) reported that incubation of 25-OH-Chol in egg phosphatidylcholine liposomes with bovine arterial smooth muscle cells caused an increased uptake of Ca2+ and a decreased level of activity of Ca2+-Mg2+-ATPase activity. Kawamura and Kummerow (479) noted that 25-OH-Chol (10 μM) caused a rapid (by 2 min) stimulation of the uptake of Ca2+ into human umbilical artery endothelial cells. Added Chol (10.4 μM) had no stimulatory effect on Ca2+ uptake. Zhou and Kummerow (1228) observed that (25R)-25-OH-Chol at 25 μM (but not at 12.4 μM) increased Ca2+ uptake in human smooth muscle cells. This stimulation was not affected by nifedipine. In another study (178), 25-OH-Chol (50 μM) had no effect on calcium influx through voltage-dependent channels in neuronal PC12 cells.

Holmes et al. (395) reported that 26-OH-Chol (25 μM; stereochrome at C-25 not specified), upon incubation...
with rat platelets, increased the cytoplasmic concentration of Ca\(^{2+}\). The extracellular level of Ca\(^{2+}\) was 1 mM. With vehicle (ethanol) alone the cytoplasmic Ca\(^{2+}\) level was 93 nM; with 25-OH-Chol the cytoplasmic level was 210 nM. They also noted that 25-OH-Chol increased the permeability of artificial membranes to Ca\(^{2+}\). Sevanian and Peterson (555) reported that 7-keto-Chol (25 \(\mu\)M) and 5\(\alpha\),6\(\beta\)-diOH-Chol (10 \(\mu\)M) increased Ca\(^{2+}\) influx into isolated rat hepatocytes. They also reported that 5\(\alpha\),6\(\alpha\)-epoxy-Chol (25 \(\mu\)M) increased Ca\(^{2+}\) efflux from rat hepatocytes, whereas 7-keto-Chol (25 \(\mu\)M) and 5\(\alpha\),6\(\beta\)-diOH-Chol (10 \(\mu\)M) had no effect on Ca\(^{2+}\) efflux. Experimental data on these points were not presented. Lahoua et al. (533) subsequently reported that 25-OH-Chol, 7\(\alpha\)-OH-Chol, and 7\(\beta\)-OH-Chol, at 12.4 \(\mu\)M, had no significant effect on the levels of intracellular free Ca\(^{2+}\) in normal rat kidney (NRK, fibroblastic clone 49) cells. Holmes and Yoss (396) reported that 25-OH-Chol and 25-hydroxyvitamin \(D_3\) increased the permeability of liposomes to Ca\(^{2+}\). 25-OH-Chol showed a maximum effect at \(~5\) mol\%. At a level of 10 mol\%, 25-OH-Chol stimulated Ca\(^{2+}\) uptake, whereas Chol had no effect. 25-OH-Chol (10 mol\%) also stimulated the uptake of Na\(^{+}\) by liposomes (~20 times that of Chol-containing liposomes).

Stimulation of Ca\(^{2+}\) uptake by oxysterols could contribute to the acceleration of the degradation of HMG-CoA reductase induced by oxysterols since Roitelman et al. (855) have reported results indicating that Ca\(^{2+}\) is important in the regulated degradation of HMG-CoA reductase. However, the concentrations of the oxysterols required to affect Ca\(^{2+}\) levels were, in most studies, higher than those required to lower HMG-CoA reductase activity in most cells. Changes in the levels of intracellular Ca\(^{2+}\) have been implicated in the regulation of apoptosis, a matter that has been the subject of a recent review (628). The induction of the changes of apoptosis in human aortic smooth muscle cells by 25-OH-Chol has been inhibited by two Ca\(^{2+}\) channel blockers, verapamil and nifedipine (19). 25-OH-Chol (25 \(\mu\)M) was also reported to induce oscillations in the levels of intracellular Ca\(^{2+}\) in aortic smooth muscle cells, an effect that was inhibited in incubations in Ca\(^{2+}\)-free medium (19). De Smedt et al. (249) reported that prenylation of human brain type I inositol 1,4,5-trisphosphate 5-phosphates is critical to the induction of Ca\(^{2+}\) oscillations induced in cells by ATP. If oxysterols lower the cellular levels of FPP to an extent that prenylation of this protein is suppressed, the oxysterols could suppress the ATP-induced oscillations of Ca\(^{2+}\) in cells.

Nah et al. (690) reported that ginsenoside-Rf (Fig. 13A), a trisaccharide derivative of an oxygenated triterpenoid found in ginseng, shows high potency in the inhibition of voltage-dependent Ca\(^{2+}\) channels in sensory neurons from dorsal root ganglia of rats. The IC\(_{50}\) value for the suppression of Ca\(^{2+}\) channels was comparable to that caused by morphine in the same primary sensory neurons.

McManus et al. (632) reported the identification of dehydrosoyasaponin I (Fig. 13B), a trisaccharide of an oxygenated derivative of a triterpenoid that was isolated from a medicinal herb used in Ghana. This compound was reported to be an extraordinarily potent activator of calcium-dependent potassium channels of smooth muscle cells and to represent the most potent known potassium channel opener.

Tipton et al. (1115) reported that a mixture of autoxidation products of Chol irreversibly inhibited calmodulin in a Ca\(^{2+}\)-dependent reaction. Significant inhibition was observed at 15 and 150 \(\mu\)g/ml but not at 1.5 \(\mu\)g/ml. The inhibitory effect of the sterol mixture was markedly decreased by treatment of the mixture with either NaBH\(_4\), NaBH\(_3\)CN, or NaI, a finding which was interpreted as indicating that the inhibition of calmodulin was due to one or more hydroperoxides of Chol. The chemical nature of the active species in the oxidized Chol sample was not established. The authors reported that Chol-7\(\alpha\)-hydroperoxide, prepared by chemical synthesis, was inactive, although data on this point were not presented. The authors also reported that the following compounds (all at 122 \(\mu\)M) were inactive: Chol, 5\(\alpha\),6\(\alpha\)-epoxy-Chol, cholest-4-en-3-one, 7-keto-Chol, and testosterone. Data on this experimentation were not presented. The authors stated that they had isolated 25-hydroperoxy-Chol from the oxidized Chol mixture and found it to be a potent inhibitor of calmodulin. However, no details of this experimentation were presented.

Watson et al. (1190) reported studies of certain cells, termed calcifying vascular cells, which undergo spontaneous hydroxyapatite calcification under tissue-culture conditions. Transforming growth factor-\(\beta\)1 and 25-OH-Chol stimulated these cells to form increased numbers of highly calcified nodules. Only one concentration of 25-OH-Chol (2.5 \(\mu\)M) was evaluated. Under the same conditions, a similar effect was not observed with 1,25-dihydroxyvitamin \(D_3\) (0.1 \(\mu\)M) or with the vehicle (ethanol) alone. The authors suggested a possible role of 25-OH-
Chol in the development of calcified lesions observed in arteriosclerosis (see below).

X. Oxysterols and Mitochondrial Function

One oxysterol, 3β-hydroxy-5α-cholestan-6-one (6-cholestanolo), has been reported to reverse the action of a number of uncouplers of mitochondrial oxidation and phosphorylation in isolated mitochondria (183, 1037, 1038, 1169). Chol, β-sitosterol, and stigmasterol did not show this action (1169). The concentrations of the 6-cholestanolo studied and reported to be effective were 20 μM (1038, 1169) or higher (183, 1037, 1038). The effects of the 6-cholestanolo in the mitochondria were related to reported effects of this sterol on membrane preparations (312, 986). Chávez et al. (183) ascribed the effects of 6-cholestanolo to a decrease in the membrane fluidity of the mitochondrial membranes. Suppression of HMG-CoA reductase activity by oxysterols could result in decreased ubiquinone formation, as observed with 25-OH-Chol and 7-keto-Chol (695), and impaired mitochondrial function. However, Lizard et al. (586) reported that 7-keto-Chol, at concentrations of 12.5 to 200 μM in media containing 10% FCS, had no effect on mitochondrial activity of bovine aortic endothelial cells as judged by staining of the cells with rhodamine-123. Dietary administration of another oxysterol, i.e., 3β-hydroxy-5α-cholestan-8(14)-en-15-one to rats, at a level of 0.1% in a Chol-free diet for either 8 or 11 days, has been found to have no significant effect, relative to pair-fed control animals, on succinate-dependent oxygen consumption by liver mitochondria in two separate experiments (unpublished data).

Y. Effects of Oxysterols on Other Processes

Kosykh et al. (507) reported that 7-keto-Chol inhibits the secretion of VLDL in human and rabbit hepatocytes. An inhibition of the incorporation of labeled acetate into triglycerides and Chol in VLDL of the medium was observed with 7-keto-Chol at concentrations of 12.5 and 25 μM. In the same experiments, the 7-keto-Chol (12.5 and 25 μM) inhibited the incorporation of the labeled acetate into cellular Chol but not cellular triglycerides. The 7-keto-Chol also decreased the secretion of VLDL apoB as measured by ELISA assay and the incorporation of [35S]methionine into VLDL in the medium. These findings are in contrast to the results (with another oxysterol) of others (158) obtained after preincubation of Hep G2 cells with 25-OH-Chol (5 μM) wherein the 25-hydroxycholesterol caused a stimulation (+148%) of the secretion of labeled triglycerides (derived from [3H]glycerol) and of the secretion of apoB-containing lipoproteins (+244%). Bing et al. (77) reported that 7-keto-Chol inhibited Chol uptake by carotid and femoral arteries of intact rabbits. Experimentation involved intravenous infusion of blood preincubated with 7-keto-Chol and studies of the effects on the arterial uptake of [4-14C]Chol and [1,2-3H]Chol.

Mattsson Hultén et al. (626) reported that certain oxysterols reduced the levels of mRNA for lipoprotein lipase in human monocyte-derived macrophages. 7β-OH-Chol (12.4 μM) or 25-OH-Chol (12.4 μM) caused a substantial (~70-75%) reduction of the levels of mRNA for lipoprotein lipase. In contrast, Chol (13 μM), 7α-OH-Chol (12.4 μM), 7-keto-Chol (12.5 μM), 5α,6α-epoxy-Chol (12.4 μM), 5α,6β-diOH-Chol (11.9 μM), 24-OH-Chol (12 μM), and 26-OH-Chol (12.4 μM) were reported to have little or no effect. 7β-OH-Chol (12.4 μM), 25-OH-Chol (12.4 μM), 26-OH-Chol (12.4 μM), or 5α,6β-diOH-Chol (11.9 μM) incubated with the macrophages for 24 h also resulted in a lowering of lipoprotein lipase activity in the culture medium. Under the same conditions, Chol (13.0 μM), 7-keto-Chol (12.5 μM), 5α,6α-epoxy-Chol (12.4 μM), and 24-OH-Chol (12.4 μM) had little or no effect. The authors suggested that oxysterols may be responsible for the low levels of mRNA for lipoprotein lipase observed in macrophage-derived foam cells.

Halistanol trisulfate (Fig. 14), a sulfated trihydroxy-sterol, has been isolated from natural sources (320, 464, 994) and reported to inhibit melanoma cells (1121) and to show hemolytic activity (668). Other closely related compounds were reported to have antithrombin and antifungal activities (464) and to be "cytoprotective against HIV" (76, 631). Slade et al. (994) also reported that monosulfate pp60v-src, an oncogenic protein tyrosine kinase encoded by the Rous sarcoma virus, with an IC50 value of ~4 μM. The free trihydroxysterol was inactive. Halistanol trisulfate was reported to be a competitive inhibitor of the phosphorylation of the peptide substrate [val5]angiotensin II and a mixed inhibitor with respect to ATP.

Slade et al. (994) also reported that monosulfate esters of a number of androstan, pregnane, and estrane compounds were inactive. Moni et al. (668) reported that halistanol trisulfate has a low critical micelle concentration and ascribed its hemolytic action and its inhibition of...
the growth of gram-positive (but not gram-negative) bacteria to its detergent properties.

Takei et al. (1089) reported that contignasterol (Fig. 15A), a polyoxygenated sterol isolated from a sponge, caused a dose-dependent inhibition of the release of histamine from peritoneal mast cells treated with anti-immunoglobulin E. A product of the reduction of contignasterol with sodium borohydride (Fig. 15B) resulted, at concentrations from 1 to 10 μM, in a stimulation of histamine release induced by anti-immunoglobulin E.

Shoji et al. (977) reported the isolation of two triterpenoids with a 14α-carboxyl group from an Okinawan sponge. The establishment of structures, shown in Figure 3, B and C, was largely based on NMR studies and, in the case of the 3-keto compound, on X-ray analysis and reduction to the known 3β-hydroxy compound. The two compounds showed high potency in inhibition of histamine release from rat peritoneal mast cells stimulated by anti-immunoglobulin E. The IC$_{50}$ values for the 3-keto-sterol and the 3-acetylated sterol were 1.5 and 10 μM, respectively.

In 1993, squalamine (Fig. 16A), a novel derivative of an oxygenated sterol, was isolated from the dogfish shark (Squalus acanthus) and shown to have significant antimicrobial activity (671). The new compound, a saturated 7α,24β-dihydroxysterol, had a spermidine residue at C-3 and a sulfate moiety on the C-24 hydroxyl (671, 1192). The configuration of the hydroxyl function at C-24 was subsequently established by $^{13}$C-NMR after chemical synthesis of the (24R)- and (24S)-forms of squalamine desulfate (674).

Squalamine was reported to be present (4–7 μg/g) in shark liver and gallbladder (671). It was also found in other organs: spleen and testes (~2 μg/g), stomach (1 μg/g), gills (0.5 μg/g), and intestine (0.02 μg/g). Squalamine was reported to have in vitro antimicrobial activity against Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus faecalis, Proteus vulgaris, Candida albicans, and Paramaecium caudatum (671). No activity was observed with Serratia marcescens. It also caused hemolysis of human erythrocytes but did so at concentrations higher than those required for antimicrobial action (671). Squalamine has been prepared by chemical synthesis (675, 789) and reported to have antimicrobial activity comparable to that for the compound isolated from the dogfish shark (675, 789). Another group (882) synthesized analogs of squalamine (Fig. 16, B and C), one with a spermidine moiety in an amide linkage at C-22 of the 3-sulfate ester of 3β-hydroxy-23,24-bisnorchole-5-en-22-oic acid.

The squalamine analog 1 showed antimicrobial activity against strains of E. coli (10.3 μM), P. aeruginosa (5.1 μM), and C. albicans (20.5 μM) but did not show comparable activity at >100 μg/ml (>164 μM) for S. aureus, P. vulgaris, and S. marcescens. In contrast, high activity for squalamine has been reported for S. aureus and P. vulgaris and no activity against S. marcescens. The representation of the conformation of the squalamine analogs corresponds to that presented by the authors (882) with postulated analogy of the structure of the cyclic polycylene antibiotic amphotericin B. However, no evidence was presented that such a conformation for the squalamine analogs would be significant in either aqueous or lipophilic environments.

The high activities of squalamine and analog 1 might suggest the possibility of action via its detergent properties. However, analog 2 showed no activity (>100 μg/ml) against any of the microorganisms studied. Also, it was reported that analog 1 did not lyse sheep red blood cells at concentrations as high as 100 μg/ml (164 μM). Other analogs of squalamine have also been prepared (459) including a series with a 6β-hydroxy function and no sulfate substitution (459). Very recently, Kikuchi et al. (483) described additional squalamine analogs and reported on their antimicrobial and hemolytic activities. Several analogs showed activity against a significant number of gram-negative and gram-positive bacteria and fungi. The authors suggested possible development for use as topical antimicrobial agents.

Potterat et al. (819) reported the isolation and structure determination of boophiline (Fig. 17A) from female ticks. The structure of the new compound is shown be-

---

**FIG. 15.** Structure of contignasterol (A) and the proposed structure of its reduction product with sodium borohydride (B).
low. Boophiline was reported to have antifungal and antibacterial activities.

Tachibana et al. (1086, 1087) isolated a series of compounds from the secretion of a species of sole that showed ichthyotoxicity, hemolytic activity, and shark-repellent actions. The active compounds, named pavonins, were 15α-hydroxy derivatives of (25R)-26-OH-Chol or its analogs (i.e., 3α-hydroxy, 5α-reduced, or Δ5) conjugated with N-acetylglucosamine at C-15 and acetylation of the 26-hydroxyl function. Two other pavonins were derivatives of (25R)-7α,26-dihydroxycholest-4-en-3-one conjugated with N-acetylglucosamine at C-7 and with or without acetylation of the 26-hydroxyl function (Fig. 17B). The precise mechanism of action of the pavonins in their actions was not established. However, it is interesting that the oxygenated sterols found in the pavonins correspond to (25R)-26-hydroxycholest-5-ene-3β,7α,15α,26-tetrol with or without acetylation of the 26-hydroxyl group. Other pavonins were derivatives of (25R)-cholest-4-en-3α,7α,12α,26-tetrol (or its 5β-reduced analog) with acetylation of the 26-hydroxyl function and with or without acetylation of the 6'-hydroxyl of the sugar residue.

5β-Scymnol is an interesting hexahydroxy bile alcohol, 5β-cholestane-3α,7α,12α,24,26,27-hexol (Fig. 18) that occurs as a sulfate ester in shark bile (428 and references cited therein). Ishida and co-workers (427, 428) recently isolated and characterized scymnol sulfate from the bile of the shark Rhizoprionodon acutus, with an X-ray crystal analysis of scymnol (427). This and other evidence established the structure of scymnol sulfate as (24R,25S)-3α,7α,12α,24,26-pentahydroxy-5β-cholest-27-yl sodium sulfate. Scymnol was stated to be “used in the treatment of acne where it is known to reduce oil production in sebaceous skin glands” and has been reported to reduce toxicity due to hepatotoxic agents. For example, Macrides et al. (600) reported that intraperitoneal administration of 5β-scymnol (at 20, 35, and 70 mg/kg body wt) to male mice decreased the hepatotoxicity induced by acetaminophen.

Cholesta-3,5-dien-7-one has been reported to be a fairly potent inhibitor (IC50 5–10 μM) of one isozyme (E1)
of human liver aldehyde dehydrogenase (878). 7-Keto-Chol and cholesta-4,6-dien-3-one were reported to be inactive in this respect.

Certain oxysterols have been reported to inhibit E-rosette formation with sheep erythrocytes when human lymphocytes were incubated with the oxysterols (25 μM) in a lipoprotein-deficient medium (1052). Inhibitory sterols (at 25 μM) included 20α-OH-Chol, 7α-OH-Chol, 3β,5α-dihydroxycholestan-6-one, and, to a lesser extent, 25-OH-Chol. Sterols that had no effect at a concentration of 25 μM included the following: Chol, 5α-cholestan-3β-ol, 7-ketocholestan-3β-ol, 6β-hydroxycholestanol, 22-keto-Chol, 6-ketocholestanol, 7-keto-Chol, 5α,6β-diol-Chol, 4β-OH-Chol, and the 3β-acetate derivative of 25-OH-Chol. The inhibitory effects of the active oxysterols were reported to occur without evidence of loss of cell viability. The addition of Chol (130 μM), but not mevalonic acid (10 mM), was reported to prevent the inhibitory effect of 20α-OH-Chol on E-rosette formation. It was suggested that the inhibition of E-rosette formation by the oxysterols was independent of their inhibitory effect on sterol synthesis but, most probably, was due to modification of cellular membrane sterol composition.

Other oxysterols have been shown to have high in vitro potency in inhibiting the growth of tumor cells (421, 654) or in the reversal of multidrug resistance (15) (see sect. x). Oxysterols appear to have little or no effect on IgM formation by cultured Namala cells (an IgM-producing human lymphoblastoid cell line) (737). Phelan and Mahler (799) reported that 25-OH-Chol (5 μM) addition to brain slices of the rat lateral septum affected GABA receptor-mediated inhibitory postsynaptic potentials, specifically the GABA<sub>B</sub> receptor-mediated potentials. The 25-hydroxysterol was added to the liver slices in ethanol solution. The slices were incubated in an artificial cerebrospinal fluid composed of salts and d-glucose (but no protein component). A very serious limitation of this work was the lack of statistical evaluation of the experimentation. A very short communication in 1976 (749) described the abortifacient action of several oxygenated derivatives of β-sitosterol and the 5α,8α-epiperoxide derivative of ergosterol. The compounds were administered orally to mice as a single dose (30 mg/kg body wt) in olive oil on day 1 or days 6 or 7 of pregnancy. It is important to note that β-sitosterol itself, when administered on day 1, had abortifacient action in 14 of 15 pregnant animals. No information was provided on the identity and purity of the compounds or on the amounts of olive oil administered or of its effects. The authors noted “loss of weight, lethargy, and anemia” in the treated animals. No details on these matters were presented.

**Z. Interactions of Oxysterols in the Expression of Their Actions**

Oxysterols commonly occur in nature as mixtures. As described in this review, this statement clearly applies to oxysterols in plasma, plasma lipoprotein fractions, LDL modified by oxidation, liver, atherosclerotic lesions, and food products. The occurrence of oxysterols in nature as mixtures raises the question of the possibilities of interactive effects of the oxysterols on their metabolism and on the expression of their effects in cells. Relatively little attention has been directed toward these matters. 3β-Hydroxy-5α-cholestan-8(14)-en-15-one and (25R)-26-OH-Chol, both potent regulators of sterol biosynthesis, have been shown to show synergism, albeit small in magnitude, in the reduction of the levels of HMG-CoA reductase activity in CHO-K1 cells (809). In mouse L cells in a chemically defined medium, 7-keto-Chol had no effect on the cellular uptake of [3H]25-OH-Chol, nor did 25-OH-Chol affect the uptake of [3H]7-keto-Chol (468). However, 7α-OH-Chol inhibited the cellular uptake of [3H]7-keto-Chol.

**VII. IN VIVO ACTIONS OF OXYSTEROLS**

**A. Oxysterols and Food Consumption, Body Weight, and Organ Weights**

In 1956, Steinberg and Fredrickson (1043) observed that dietary administration of cholest-4-en-3-one (1% in diet) to rats for 8 wk resulted in a substantial decrease in body weight gain. Subsequently, Suzuki (1069) reported that commercial cholest-4-en-3-one (0.5% in diet) suppressed body weight gain in male and female mice (CDF1; BALB/c × DBA). Chol (0.5% in diet) had no effect on body weight gain. Over a period of 5 mo, dietary administration of cholest-4-en-3-one was reported to have no effect on cumulative food intake. However, the practical difficulties in accurately measuring food consumption in the small animals should be recognized. Several analogs of cholest-4-en-3-one, at 0.5% in diet for 14 days, were also shown to suppress gain in body weight in the mice (1070).

[Diagram of 5β-scymnol (5β-cholestan-3α,7α,12α,24,26,27-hexanol).]
1,4,6-trien-3-one, cholesta-1,4-dien-3-one, cholest-4-ene-3,6-dione, and cholest-5-en-3-one. The δ^3^-3-ketone was more active than the parent δ^1^-3-ketone. Aramaki et al. (18) reported that administration of 5α,6β-diOH-Chol to rats at levels of 0.1, 0.25, and 0.5% in a Chol and cholate containing diet was associated with "moderate anorexia" which corresponded to dosage. No details were given. In contrast, administration of the triol (at levels of 0.2 and 0.5% in a high-Chol diet) to rabbits was reported to have no effect on food consumption or body weight. Administration of 3β-hydroxy-5α-choleste-8-en-7-one and 3β-hydroxy-5α-choleste-8-en-11-one (0.15% in a cholesterol-free test diet) to male Sprague-Dawley rats was stated (766) to be associated with a suppression of food intake and growth. However, no experimental data were presented.

Kandutsch et al. (472) reported that feeding 25-OH-Chol (0.1 and 0.25%) or 7-keto-Chol (0.25 and 0.5%) in a basal low-Chol diet suppressed gain in body weight in immature male mice. In mature mice, 25-OH-Chol (0.25%) or 7-keto-Chol (0.25 or 0.5%) decreased body weight relative to control mice fed the basal diet. The effects of the oxysterols on body weight appeared to be evident after 1 to 2 days on the diet. Addition of Chol was reported to reduce the suppressive effects of 7-keto-Chol and 25-OH-Chol on body weight gain in immature male mice. The effects of the oxysterols on body weight were attributed to reduced food consumption, which did not appear to be due to unpalatability of the oxysterol-containing diets. Limitations of this important study were the small number of animals used in some of the experiments and lack of data on quantitative determinations of the effects of the sterols on food consumption in mice.

Erickson et al. (285) observed that short-term dietary administration of 25-OH-Chol (0.1% in ground chow) to male Sprague-Dawley rats for 18 or 66 h had no effect on food consumption or body weight. The same group reported (284) that short-term feeding of 7-keto-Chol (0.05, 0.1, or 0.5% for 18 h or 0.05 and 0.1% for 66 h) had no effect on body weight of male Sprague-Dawley rats (250–300 g). However, feeding of the 7-keto-Chol at a level of 0.5% in diet for 66 h resulted in an average loss of 19 g body wt. Tamasawa et al. (1091) reported that dietary administration of 7-keto-Chol (0.1% by weight in a chow diet for 6 days) had no effect on food consumption or body weight in male Wistar rats (130–180 g) fed 15 g of food per day. However, experimental data were not presented, and the numbers of animals in each group were small (control, n = 5; treated, n = 4). Toda et al. (1117) reported that administration of 7-keto-Chol (10 mg/day for 2 wk and 20 mg/day thereafter) as an emulsion in corn oil to female chicks had no effect on growth rates. Experimental and control birds received a basal diet ad libitum. Food consumption was not measured. The same authors also studied the effects of administration of 7-keto-Chol at a high level (1% in basal diet) ad libitum to 5-day-old chicks. It was stated that "after 12 days on this regimen, chicks started dying, and survivors gained little or no weight." "Autopsy findings revealed enlarged gallbladders and intestinal atrophy." Vargas et al. (1162) observed that dietary administration of 7-keto-Chol to laying hens at a low level (0.025% in diet) had no effect on food consumption or body weight. In our own experiments (178), dietary administration of 7-keto-Chol, at levels of 0.1, 0.15, and 0.2% (n = 7 per group) in a chow diet, had no effect on food consumption and changes in body weight in male Sprague-Dawley rats except for slight initial decreases in the animals fed the sterol at a level of 0.2% in diet. However, at the end of the experiment (day 10), mean values for food consumption and body weight were not different in the control and experimental groups. Under the same conditions, administration of a synthetic F<sub>7</sub> analog of 7-keto-Chol (fluorine atoms at carbon atoms 25, 26, and 27) at a level of 0.26% in diet (the molar equivalent of 0.2% 7-keto-Chol) had essentially no effect on food consumption or body weight.

Administration of a diet containing a mixture of oxygenated Chol derivatives (at an estimated dosage of 166 mg/kg body wt<sup>−1</sup>day<sup>−1</sup>) and 2% corn oil for 11 wk to rabbits was reported (387) to have no effect on food consumption or rate of gain in body weight. However, data on these points were not presented. The percentage composition (expressed as weight %) of the oxysterol mixture was reported as follows: Chol, 0.06; 25-OH-Chol, 2.0; 7-keto-Chol, 26.0; 7α-OH-Chol, 4.5; 7β-OH-Chol, 5.3; Chol 7α-hydroperoxide, 18.2; 5α,6α-epoxy-Chol, 24.0; 5β,6β-epoxy-Chol, 17.0; and unknown, 2.9. Osada et al. (735) studied the effects of feeding a mixture of oxysterols to young (4-wk-old) and adult (8-mo-old) male Sprague-Dawley rats. The major component in the oxysterol mixture was 7-keto-Chol (26%) accompanied by a number of other oxidation products of Chol and unidentified materials. The rats were fed one of three diets for 21 days: a Chol-free diet, a Chol-containing diet (0.5% by weight), or an oxygenated sterol-containing diet (0.5% by weight). The Chol-containing diet had no effect on food consumption or on gain in body weight in either the young or adult rats. However, the diet containing the oxysterol mixture resulted in decreases in food consumption in both young (−25%) and adult (−38%) rats. The decrease in food consumption in the young rats was associated with a decrease (−47%) in weight gain relative to animals on the Chol-free diet. The adult rats fed the oxysterol mixture also showed a decrease in body weight gain relative to animals fed the Chol-free diet, with a slight (−8.4%) decrease in body weight over the 3-wk study period.

Dietary administration of 3β-hydroxy-5α-cholest-8(14)-en-15-one (0.1% in diet) caused significant decreases in food consumption and gain in body weight in short-term experiments with rats (116, 328, 651, 698, 919, 998) and in mice.
Interestingly, these effects of the 15-ketosterol appear to be rodent specific, since its oral administration to baboons (930) and monkeys (939), at rather high levels (50 or 75 mg/kg body wt), had no effect on body weight. However, the clear suppressive effects of the 15-ketosterol on food consumption and body weight in rats imposed the burden of special pair-feeding protocols, with daily measurement of food consumption in all animals, for investigations of the effects of the 15-ketosterol on a variety of parameters. The mechanisms involved in the suppression of food consumption and body weight gain have not been determined. However, these effects were not observed (328) in rats fed a synthetic (1076) F7 analog of the 15-ketosterol (with fluorine atoms at carbon atoms 25, 26, and 27). The F7 substitution was introduced to block the rapid and substantial conversion of the 15-ketosterol to polar biliary metabolites (912, 914) that appear to arise from initial oxidation in the side chain to give the 26-hydroxylated derivative of the 15-ketosterol (826, 827). The abolishment of the effects of the 15-ketosterol on food consumption and body weight by the F7 substitution suggested the possibility that these effects were due to a metabolite of the 15-ketosterol whose formation was blocked or suppressed by the F7 substitution. However, administration of synthetic (485, 983) (25R)-3β,26-dihydroxy-5α-cholesta-8(14)-en-15-one, a major metabolite of the 15-ketosterol, to rats at levels of 0.05 and 0.1% in diet for 10 days had no effect on food consumption or body weight (unpublished data). 3β-Hydroxy-25,26,26,27,27,27-heptafluoro-5α-cholestan-15-one, with no olefinic double bond, caused only a minor suppression of food consumption upon its dietary administration at 0.125% in diet. Mean values of daily food consumption showed an average reduction of 15% on days 2–10, which was very considerably less than the substantial suppression of food consumption (~48% over the same time period) caused by the parent 5α,15-dihydroxy-5α-cholesta-8(14)-15-one at an equimolar level in the diet (989). The 7α-methyl derivative of the 15-ketosterol [3β-hydroxy-7α-methyl-5α-cholesta-8(14)-en-15-one], at 0.1% in diet, had no effect on food consumption or body weight (978). Slight or moderate decreases in food consumption were observed at higher doses, i.e., 0.15 and 0.2% in diet, respectively, and only slight effects on body weight were noted at the higher dosage (0.2% in diet). The slight effects on food consumption and body weight observed at the higher dosage level are in marked contrast to the major suppression effects observed with the 15-ketosterol at a lower dosage (0.1% in diet). The F7-7α-methyl analog of the 15-ketosterol [3β-hydroxy-7α-methyl-25,26,26,27,27,27-heptafluoro-5α-cholesta-8(14)-en-15-one] had only slight effects on food consumption and body weight, effects that were not dose dependent under the conditions studied (0.05, 0.075, 0.1, 0.125, and 0.15% in diet) (1074). The 9α-fluoro analog of the 15-ketosterol [3β-hydroxy-9α-fluoro-5α-cholesta-8(14)-en-15-one], at 0.15% in diet, suppressed food consumption and body weight gain in rats (940).

Kantiengar et al. (475) reported that dietary administration of cholesta-3,5-dien-7-one to three cockerels (330 mg/bird\(^{-1}\)day\(^{-1}\) for 8 wk) resulted in little or no effect on liver weight; however, the weights of “intestines” were increased (approximately +71%) relative to three control birds. Steinberg and Fredrickson (1043) observed that dietary administration of cholest-4-en-3-one (1% in diet for 8 wk) to rats led to a marked increase in adrenal gland weight (+700%). Fredrickson et al. (315) reported that dietary administration of cholest-4-en-3-one (1% in a Chol-free diet for 43 days) to male Sprague-Dawley rats resulted in adrenal hypertrophy (~6.6 times increase in adrenal weight) relative to pair-fed control animals. Suzuki (1069) observed that, in mice, dietary administration of cholest-4-en-3-one at lower levels (up to 0.5% in diet for up to 5 mo) resulted in no adrenal hypertrophy. Experimental data on this point were not presented.

Dietary administration of 7-keto-Chol to male Sprague-Dawley rats for 10 days at a level of 0.15 and 0.2% (but not at 0.1%) resulted in significant increases in the weight of small intestine relative to either ad libitum or pair-fed control animals (164). The mean values for small intestinal weight were higher, +19% and +28% (relative to ad libitum controls), in animals fed the 7-ketosterol at 0.15 and 0.2% in diet. Very interestingly, the effect of the 7-keto-Chol on intestinal weight was abolished by F7 substitution in the side chain (fluorine atoms at carbon atoms 25, 26, and 27), a finding suggesting, but certainly not proving, that the increase in intestinal weight caused by 7-keto-Chol administration might be due to a metabolite of the 7-ketocholesterol whose formation is suppressed or eliminated by the F7 substitution.

Administration of 3β-hydroxy-5α-cholesta-8(14)-en-15-one (0.1% in diet) has been associated with no consistent alterations in the weights of liver, kidneys, spleen, adrenals, heart, or testes (328). However, a marked increase in small intestinal weight (+94%) has been found (328). The morphological changes accompanying this enlargement have been described (998). It is important to note that the changes in the morphology of small intestine, induced by the 15-ketosterol, were not observed upon treatment (50 or 75 mg/kg body wt) of a nonhuman primate (998). In rats, administration of the F7 analog of the 15-ketosterol [3β-hydroxy-25,26,26,27,27,27-heptafluoro-5α-cholesta-8(14)-en-15-one], at levels of 0.05, 0.075, 0.1, 0.125% in diet (but not at 0.025%) was associated with increased small intestinal weights; however, the magnitudes of the increases were considerably less than that of the parent 15-ketosterol. Interestingly, animals treated with the 26-hydroxy analog of the 15-ketosterol [(25R)-3β,26-dihydroxycholesta-8(14)-en-15-one], a major metabolite of the 15-ketosterol, had no effect on the weight of small intestine upon its dietary administration (0.05 and 0.1% in diet) (unpublished data). The 7α-methyl analog of the 15-ketosterol had no effect on the weights of liver, spleen,
kidneys, adrenals, heart, and testes (978). Significant, dose-dependent increases in small intestinal weight were observed (at levels of 0.1, 0.15, and 0.2% in diet). A similar dose-dependent increase in small intestinal weight was observed with the F7-7α-methyl analog of the 15-ketosterol [3β-hydroxy-7α-methyl-25,26,26,27,27,27-heptafluoro-5α-cholesta-8(14)-en-15-one] (1074). The increase in the weight of small intestine of rats treated with the 7α-methyl-15-ketosterol and the 7α-methyl-F7-15-ketosterol were considerably less than that observed with the parent 15-ketosterol (1074). Little or no effects of the F7-7α-methyl analog of the 15-ketosterol on the weights of liver, kidney, heart, adrenals, or spleen were observed. A higher value (+21%) for liver weight was observed at the highest dosage level tested (0.15% in diet).

B. Oxysterols and Serum Cholesterol Levels

3β-Hydroxy-5α-cholesta-8(14)-en-15-one represents the first oxysterol with demonstrated potency as an inhibitor of sterol synthesis and in lowering HMG-CoA reductase activity in cultured mammalian cells to be shown to have significant hypocholesterolemic action in animals (919). Dietary administration of the 15-ketosterol (0.1% in diet) consistently lowered serum Chol levels in rats (116, 328, 495, 651, 919, 998) relative to either ad libitum or pair-fed controls. Administration of the 15-ketosterol has also been shown to lower serum Chol levels in mice (919). The results in rodents prompted an extension to studies in two species of nonhuman primates, baboons (930) and Rhesus monkeys (939). Administration of the 15-ketosterol (939). Dietary administration of the 15-ketosterol (0.1% in diet) consistently lowered serum Chol levels in rats (116, 328, 495, 651, 919, 998) relative to either ad libitum or pair-fed controls. Administration of the 15-ketosterol has also been shown to lower serum Chol levels in mice (919). The results in rodents prompted an extension to studies in two species of nonhuman primates, baboons (930) and Rhesus monkeys (939). Administration of the 15-ketosterol, at a daily dose of 50 mg/kg body wt in a high-fat (41% of calories) diet for 12 days, lowered serum Chol levels in rats (940); the F7-15-ketosterol, at the low dosage of 0.025% in diet, caused a significant lowering of serum Chol levels with no effect on food consumption and body weight. Administration of the F7-15-ketosterol was accompanied by the accumulation of F7-Chol in blood and in tissues (328), a situation resulting from the enzymatic conversion of the F7-15-ketosterol to F7-Chol by the same reactions involved in the overall conversion of the parent 15-ketosterol to Chol (667). Dietary administration of the 7α-methyl analog of the 15-ketosterol [3β-hydroxy-7α-methyl-5α-cholesta-8(14)-en-15-one] showed high potency, with significant lowering of serum Chol levels at all dosages studied (0.05, 0.1, 0.125, and 0.15%) (1074). This synthetic sterol, in which both its side-chain oxidation and the conversion to F7-Chol were blocked, showed significant reduction of serum Chol levels at a dosage (0.93 μmol/g diet) far less than that required for the 7α-methyl analog (3.6 μmol/g diet). The Δ8(14) double bond of the F7-15-ketosterol was not essential for its hypocholesterolemic action in rats (1075). 3β-Hydroxy-25,26,26,27,27,27-heptafluoro-5α-cholestan-15-one was shown to cause a lowering of serum Chol levels (−47 and −43% relative to ad libitum and pair-fed controls, respectively) upon its dietary administration at a level of 0.125% (approximately the molar equivalent to 0.1% of the parent 15-ketosterol). The 26-hydroxy derivative of the parent 15-ketosterol [(25R)3β-26-dihydroxy-5α-cholesta-8(14)-en-15-one], a demonstrated metabolite of the 15-ketosterol (826, 827, 916), had no effect on serum Chol levels upon administration (0.05 and 0.1% in diet) to rats (unpublished data).

The 9α-fluoro analog of the 15-ketosterol [3β-hydroxy-9α-fluoro-5α-cholesta-8(14)-en-15-one], at 0.15% in diet for 12 days, lowered serum Chol levels in rats (940); in the same study, the corresponding 9α-hydroxy analog [3β,9α-dihydroxy-5α-cholesta-8(14)-en-15-one] was inactive. Whereas the oxime of the parent 15-ketosterol showed high potency in the lowering of HMG-CoA reductase and in the inhibition of the synthesis of DPS in mouse L cells, it had no effect on serum Chol levels upon administration (0.15% in diet) to rats (929).

Kantiengar et al. (475) reported that dietary administration of cholest-3,5-dien-7-one (330 mg·day−1·bird−1
for 8 wk) to cockerels (n = 3) did not appear to affect serum Chol levels. Administration of \(5\alpha,6\beta\)-diOH-Chol to Sprague-Dawley rats at levels of 0.1, 0.25, and 0.5% in a Chol- and cholate-containing diet had no effect on serum Chol levels (18). Intragastric administration of the triol (25 mg/day for 15 days) to rats on a semi-synthetic diet (with 75 mg Chol/day) was also reported to have no effect on serum Chol levels. In contrast, the triol (at 0.2 and 0.5%) suppressed the rise in serum Chol induced by Chol feeding in rabbits (18, 1203). Matthias et al. (625) claimed lowering of serum Chol and HDL Chol levels upon intragastric administration of \(5\alpha,6\beta\)-diOH-Chol (in olive oil) to male Wistar rats. The individual experiments involved small numbers of treated animals with, in some cases, no controls. Although body weight was reported to be decreased in the treated animals, food consumption was not monitored nor were pair-fed control animals employed. The characterization and purity of the \(5\alpha,6\beta\)-diOH-Chol were not presented. Furthermore, in one experiment, Chol administration appeared to result in lower serum Chol levels than treatment with the triol. This experimentation requires repetition in an expanded fashion. Administration of \(5\alpha,6\alpha\)-epoxy-Chol to male Fischer CD344 rats at an average dose of 40 mg/day (177 mg/kg body wt) in a liquid diet for 17 days had no effect on plasma Chol levels (831).

Kosykh et al. (508) reported remarkable differences in plasma Chol levels of rabbits receiving aged Chol containing oxysterols and animals receiving purified Chol. Rabbits received oral supplementation with 200 mg kg\(^{-1}\)day\(^{-1}\) of the Chol preparations in olive oil (0.5 ml/kg). The aged Chol preparation (5 yr at room temperature) was reported to contain \(\sim\)5% oxidized Chol derivatives consisting mainly of \(7\alpha\)-OH-Chol, \(7\beta\)-OH-Chol, \(7\alpha\)-keto-Chol, and \(5\alpha,6\beta\)-diOH-Chol. Mean plasma Chol levels of rabbits (n = 8) receiving the purified and oxidized Chol were 180 ± 30 and 900 ± 140 mg/dl, respectively, whereas control rabbits (receiving olive oil alone) had a mean level of 60 ± 8 mg/dl. It should be noted that the Chol levels were assayed using an enzymatic assay kit, and no independent analyses were made to assay the nature and levels of oxysterols present in plasma, some of which could be anticipated to act as substrates in the enzymatic assay of Chol. The increased plasma Chol concentrations in the Chol-fed rabbits were associated with marked increases in the levels of VLDL apoB-100 and apoE that were very considerably higher in the cases of the animals that received the oxidized Chol. Primary cultures of hepatocytes from the animals receiving the oxidized Chol showed very markedly increased levels of Chol esters and of the incorporation of \(\text{[}^{14}\text{C}\text{]}\)acetate into Chol esters in cells plus secreted VLDL. The authors postulated that increased plasma Chol levels observed after the administration of the oxidized Chol preparation resulted from increased Chol ester levels and a stimulation of VLDL secretion. The results with the primary cultures of the hepatocytes obtained from rabbits that received the oxidized Chol preparation differ from those in which the effects of the addition of \(7\alpha\)-keto-Chol to rabbit hepatocytes were studied. In the latter case (507), an inhibition of VLDL secretion was reported that was associated with a decrease in the incorporation of labeled acetate into cellular Chol.

Erickson et al. (285) found that short-term dietary administration of 25-OH-Chol (0.1% by weight in ground Purina Chow) for 18 or 66 h had no effect on the levels of serum Chol in male Sprague-Dawley rats (50–100 g). Erickson et al. (284) also reported that very short-term dietary administration of 7-keto-Chol (0.05, 0.10, and 0.50% by weight in ground Purina Chow) for 18 or 66 h to male Sprague-Dawley rats (250–300 g) had no effect on serum Chol levels except for the group treated at a level of 0.5% for 66 h in which “a decrease of about 50% was found.” The study did not indicate employment of a pair-fed control group that is very important since the experimental group in question showed a decrease in food consumption (magnitude not reported), an average loss of body weight of 19 g, and a decrease in liver weight of 0.72 g/100 g body wt. The levels of 7-keto-Chol in serum were not determined. Toda et al. (1117) observed that administration of 7-keto-Chol (10 mg/day for 2 wk and 20 mg/day thereafter) in an emulsion in corn oil to 5-day-old female chicks for 4 or 8 wk had no significant effect on serum Chol levels in birds fed a basal diet or a basal diet supplemented with Chol (1% in diet). Kandutsch et al. (472) studied the effect of dietary administration of 7-keto-Chol (0.25 or 0.5% in diet) and 25-OH-Chol (0.25% in diet) for varying lengths of time to immature (4 wk-old) male mice on the levels of total plasma Chol. Control animals received the basal Chol-free diet in restricted amounts. No clear indication of a hypocholesterolemic action of either of the sterols was observed. Interpretation of the results is not clear cut in view of the small number of animals used (n = 2 in all but one experiment) and the lack of a strict pair-feeding control protocol (to deal with the suppression of body weight gain, presumably by decreased food consumption, caused by the diet administration of these oxysterols under the conditions studied). In our own studies (164), no significant reduction in serum Chol levels was observed in male Sprague-Dawley rats (n = 7/group) fed the 7-keto-Chol for 10 days at levels of 0.1, 0.15, and 0.2% by weight in a chow diet (relative to either ad libitum or pair-fed control animals). Administration of the 7-ketoacetate at a level of 0.2% caused only a very slight lowering of serum Chol levels relative to ad libitum but not pair-fed control rats. Under the same experimental conditions, dietary administration of \(3\beta\)-hydroxy-\(5\alpha\)-cholest-8(14)-en-15-one at a level of 0.1% in diet resulted in a marked lowering of serum Chol levels. An \(F_7\) analog of the 7-ketosterol (with fluorine
atoms at carbon atoms 25, 26, and 27) was synthesized (164), with the goal of suppressing metabolism of the 7-ketosterol and increasing its bioavailability and its potency in lowering serum Chol levels. However, the F₇₋₇-keto-Chol, at 0.26% in diet (the molar equivalent of the 7-keto-Chol at 0.2% in diet), had no effect on serum Chol levels (164).

Dietary administration of 3β-hydroxy-5α-cholestan-6-one to either AKR/J mice or a mouse line selected for high plasma Chol had no significant effect on plasma Chol levels (187). The dosage of the 6-ketosterol was not clearly specified but presumably corresponded to the level (0.25% in diet) used in other in vivo experiments described in the same paper. The period of treatment extended to 12 days in the AKR/J mice and 7 days in the high Chol mouse line.

Osada et al. (735) reported that dietary administration of an oxysterol mixture (0.5% in diet) resulted in a lowering of the level of serum Chol in young and adult male Sprague-Dawley rats (relative to rats on a Chol-free diet). The major component in the oxysterol mixture was 7-keto-Chol (27%), which was accompanied by a number of other oxidation products of Chol and unidentified materials. An important limitation of this study was the lack of a pair-fed control group to deal with the reported decreased food consumption caused by the administration of the oxysterol mixture. Subsequently, the same laboratory (739) detected no effect of feeding of an oxysterol mixture (0.2% in basal Chol-free diet) on the level of serum Chol in young and adult rats on days 5, 10, 15, and 21 (relative to rats fed the basal Chol-free diet alone). The oxysterol mixture was similar to that described above. The same group (738) also fed the same oxysterol mixture (at a level of 0.3% by weight in diet) for 3 wk to 4-wk-old rats. No effect of the oxysterol mixture on serum Chol levels was observed. However, the liver Chol levels in the oxysterol-fed group were lower than in the animals fed the Chol-free diet and very markedly different from the very elevated hepatic Chol levels observed in the Chol-fed group (0.3% in diet). Osada et al. (740) also reported on the levels of serum Chol in 4-wk-old rats fed one of three diets: a basal Chol-free diet, the basal diet containing added Chol (0.5%), or the basal diet supplemented with Chol (0.5%) and a mixture of oxidized Chol species (0.5%). The oxysterol mixture was similar to that described above. Because dietary administration of the diet supplemented with Chol plus the oxidized Chol mixture suppressed food consumption (data not presented), the animals receiving the basal diet or that supplemented with Chol were given food only in the amount consumed by the animals receiving the diet supplemented with both Chol and the oxidized Chol mixture. No significant difference was observed in serum Chol levels between rats on the Chol-supplemented diet and those on the same diet supplemented with the oxidized Chol mixture.

3β-Hydroxy-5α-cholestan-8-en-7-one and 3β-hydroxy-5α-cholestan-8-en-11-one, found to show moderate and very low activity, respectively, in lowering HMG-CoA reductase activity in mouse L cells (765), were evaluated with respect to their effects on serum Chol levels in male Sprague-Dawley rats (766). Administration of the ketosterols was reported to suppress food consumption and growth (data were not presented). Serum Chol levels were assayed using a Chol oxidase-based enzyme kit on days 6, 13, and 21 in rats receiving the 7- and 11-ketosterols (0.15% by weight in a Chol-free test diet) and in ad libitum and pair-fed control animals. With both 7- and 11-ketosterols serum Chol levels appeared to be lower than those of either ad libitum or pair-fed control animals on day 6. However, on days 13 and 21, no differences between mean levels of serum Chol were observed between the treated rats and the control animals.

It is important to note that most studies of the effects of administration of oxysterols on serum Chol levels involve the use of Chol oxidase-based enzymatic assays. The validity of the use of this type of assay is dependent on the absence of significant amounts of sterols other than Chol in serum. Because a number of the oxysterols do, in fact, serve as efficient substrates for Chol oxidase (769, 779, 798, 924, 931, 935, 938, 997, 1108, 1109), independent determination of the levels of the concerned oxysterols must be made to establish the validity of the assay of serum Chol with Chol oxidase. This has been done in the case of a number of 15-oxygenated sterols (328, 930, 978, 1074). In addition, the absence in serum of significant levels of metabolites of the administered oxysterols that could affect the enzymatic assay of Chol must be established. This is illustrated by the case of administration of 3β-hydroxy-25,26,26,26,27,27,27-heptafluoro-5α-cholestan-8(14)-en-15-one in which significant levels of F₇₋₇-keto-Chol were demonstrated in serum and tissues by GC and GC-MS (328).

C. Effects of Oxysterols on Cholesterol Absorption

3β-Hydroxy-5α-cholestan-8(14)-en-15-one has been shown to inhibit the absorption of Chol in rats. Administration of the 15-ketosterol (0.1% in diet for 8 days) resulted in a decreased absorption of [4-¹⁴C]Chol as indicated by substantially decreased levels of ¹⁴C in various tissues and organs of the 15-ketosterol-treated rats (relative to ad libitum or pair-fed control animals) and increased levels of ¹⁴C in feces and intestinal contents at 12 and 48 h after intragastric administration of the labeled Chol (116). In another study (913), dietary administration of the 15-ketosterol (0.05% in diet for 10 days) resulted in a marked decrease (−64%) in the absorption of exogenous [4-¹⁴C]Chol in lymph duct-cannulated rats (relative to pair-fed control animals). Under the conditions stud-
ied, 15-ketosterol treatment had no effect on intestinal lymph flow. In another study (698), 15-ketosterol administration (0.1% in diet for 8 days) to rats resulted in a marked decrease (−77%) in the levels of ACAT activity in jejunal microsomes relative to pair-fed controls. Importantly, no differences were observed in mean levels of Chol (a substrate for the ACAT reaction) in jejunal microsomes of 15-ketosterol-treated or pair-fed control animals.

Osada et al. (741) reported on studies of the effect of the intragastric administration of a single dose of an oxygenated sterol mixture on the lymphatic absorption of a tracer amount of [4-14C]Chol in rats. The major component in the oxysterol mixture was 7-keto-Chol (23%) accompanied by a number of other oxidation products of Chol and unidentified materials. The oxysterol mixture (50 mg) was administered with [4-14C]Chol (1 μCi) into the stomach as an emulsion with sodium taurocholate, albumin, and triolein. Control rats received Chol (50 mg) in the same emulsion. Absorption of the [14C]Chol in thoracic duct lymph over 24 h was 33.1 ± 6.3% in the rats receiving Chol and 21.7 ± 8.6% in the rats receiving the oxysterol mixture. Under the conditions studied, administration of the oxysterol mixture was associated with a decreased flow of lymph over the 24-h study period (77.2 ± 14.7 ml/24 h in the oxysterol group vs. 125.8 ± 27.1 ml/24 h in the group receiving Chol) and an apparent decrease in the absorption of triolein. Imai et al. (424) reported that intragastric administration of 5α,6β-diOH-Chol (25 mg in a suspension with saline containing gum arabic) to Sprague-Dawley rats (~200 g) inhibited the absorption of Chol (25 mg; also administered by intragastric administration in an emulsion with taurocholate, oleic acid, and albumin) by 36%.

D. Effects of Oxysterols on Sterol Synthesis and Other Parameters in Intact Animals

Imai et al. (424) reported that intragastric administration of 5α,6β-diOH-Chol (25 mg/day for 7 days; suspended in 5% gum arabic solution) resulted in an increased incorporation of labeled acetate into Chol (either with rat liver slices or after intraperitoneal injection of [1-14C]acetate). These results were suggested to be due to the effect of the triol in inhibiting Chol absorption and/or increased excretion of sterols (424). Chen and Kandutsch (187) studied the effect of acute (18 h) dietary administration of a number of oxygenated sterols (0.25% in diet) on the incorporation of labeled acetate into sterols by liver slices. Significant inhibition was reported for 3β-hydroxy-5α-cholestan-6-one, cholest-4-ene-3,6-dione, 25-OH-Chol, and a mixture of 7α- and 7β-OH-Chol. A significant effect of sterol administration on sterol synthesis was not observed for 7-keto-Chol, 7-ketocholesterol, or 5α,6β-diOH-Chol.

Erickson et al. (285) studied the effects of short-term dietary administration of 25-OH-Chol to rats on hepatic sterol synthesis. 25-OH-Chol (0.1% by weight in rat chow) was fed to male Sprague-Dawley rats for 18 or 66 h. Significant lowering of microsomal HMG-CoA reductase activity was observed at 18 h (−62%) and at 66 h (−50%). At 18 h of feeding, the incorporation of [14C]acetate into DPS was lower in the 25-OH-Chol-fed rats. No effects of the short-term feeding of the 25-OH-Chol on body weight or food consumption were observed. Similarly, 25-OH-Chol administration for 18 or 66 h had no effect on serum Chol levels or on the levels of total Chol in liver. A slight decrease (−11%) in the level of total Chol in microsomes was observed. No effect on the level of esterified Chol in microsomes was observed, whereas a slight decrease (−8%; P < 0.07) in free Chol in microsomes was observed. Erickson et al. (285) reported that perfusion of rat liver with 25-OH-Chol (187 μM in perfusion medium; added in ethanol) lowered HMG-CoA reductase activity at 30 min (−41%) and at 120 min (−48%).

Johnson et al. (456) reported on the effect of intragastric administration of a single dose of 25-OH-Chol (suspended in 0.5 ml of distilled water by sonication for 30 min; no mention of any studies of the effects of sonication on the purity of 25-OH-Chol was provided). Control rats received only distilled water. Rats were killed at the mid-dark portion of a light-dark cycle. Liver HMG-CoA reductase activity was assayed at either 3 or 16 h after the administration of the 25-OH-Chol. The rats were maintained on a rat chow diet ad libitum. At 3 h, 25-OH-Chol (1 μg/kg) was reported to cause a 21% lowering of HMG-CoA reductase activity in an experiment involving three pairs of rats as the control and experimental animals. The 16-h experiment involved three pairs of rats for the control but only one pair of rats for each of the doses of the 25-OH-Chol. The values for the experimental animals were reported to be lower at 0.1 μg/kg (−29%), 1 μg/kg (−48%), 10 μg/kg (−10%), and 20 μg/kg (−19%). Because only pairs of livers were studied for the levels of HMG-CoA reductase, evaluation of the animal-to-animal variation and the significance of the reported changes are not possible from the data presented. Repetition of these experiments in an expanded fashion is encouraged, especially in view of the claimed lowering of hepatic HMG-CoA reductase activity at such very low dosages of the 25-OH-Chol.

Erickson et al. (284) investigated the effects of short-term dietary administration of 7-keto-Chol to rats on the levels of hepatic HMG-CoA reductase activity. 7-Keto-Chol was fed to male Sprague-Dawley rats for 18 or 66 h at levels of 0.05, 0.10, and 0.50% by weight in diet. At a level of 0.05% in diet, the 7-ketosterol had no effect on reductase levels at either 18 or 66 h. 7-Keto-Chol administration at levels of 0.10 and 0.50% in diet resulted in a 63 and 73% lowering, respectively, of reductase activity at
18 h; however, no significant changes were observed at 66 h. The lack of effect at 66 h was attributed to the development of tolerance to the 7-ketosterol that was postulated to arise from an increased metabolism to polar metabolites. Erickson et al. (284) also reported that short-term feeding (18 h) of 7-keto-Chol (0.1% in rat chow) resulted in a lowering of hepatic microsomal HMG-CoA reductase activity (−60%) and an inhibition (−69%) of the incorporation of [2-14C]acetate into DPS by a 11,500-g supernatant fraction of rat liver. The incorporation of [2-14C]mevalonate into DPS was reported to be decreased by 18%. Erickson et al. (284) also noted that perfusion of rat liver with 7-keto-Chol (188 μM in perfusion medium; added in ethanol) for 3 h resulted in a lowering of microsomal HMG-CoA reductase activity (−55%) relative to animals with liver perfusions in the absence of the added sterol.

Kandutsch et al. (472) studied the effects of dietary administration of 7-keto-Chol (0.5% in diet) and 25-OH-Chol (0.25% in diet) on the incorporation of [1-14C]acetate into DPS in small intestine and liver of 5-wk-old mice on a Chol-free basal diet at 30 min after the subcutaneous injection of the labeled acetate. Because the oxygenated sterols suppressed body weight gain (presumably by suppression of food consumption), a control group fed the basal diet at a level of 1.2 g/day was used as the basis of comparisons. The food-restricted group showed a clear suppression, relative to animals fed the basal diet ad libitum, of the incorporation of the labeled acetate into DPS in liver after 1 and 8 days. Animals fed the 7-keto-sterol showed a decreased incorporation of acetate into DPS of small intestine at 1 day but not at 8 days (relative to the food-restricted group). In contrast, animals fed the 7-ketosterol for either 1 or 8 days showed increased incorporation of the labeled acetate into DPS of liver (relative to the food-restricted group). Dietary administration of 25-OH-Chol decreased the level of acetate incorporation into DPS of the small intestine at 8 days but not 1 day. Feeding the 25-OH-Chol for either 1 or 8 days increased the incorporation of the labeled acetate into DPS of liver. Again, comparisons were made against the food-restricted group. It should be noted that these experiments do not involve a strict pair-feeding protocol but the use of a single food-restricted group.

Miller et al. (651) reported on the effects of administration of 3β-hydroxy-5α-cholestan-14(14)-en-15-one (0.1% in diet for 8 days) on the levels of activity of cytosolic acetoacetyl-CoA thiolase and HMG-CoA synthase and of microsomal HMG-CoA reductase in liver of male Sprague-Dawley rats. Significant increases in the levels of cytosolic acetoacetyl-CoA thiolase were observed (relative to either ad libitum or pair-fed controls) in each of three experiments. The magnitudes of the increases, relative to ad libitum controls, were +75, +84, and +171%, and the magnitudes of the increases, relative to pair-fed controls, were +50, +142, and +184%. Elevations of cytosolic HMG-CoA synthase were also observed, with increases, relative to ad libitum controls, of +84 and +60%, and, relative to pair-fed controls, of +44 and +41%. The levels of microsomal HMG-CoA reductase were also elevated in the 15-ketosterol-treated animals, relative to ad libitum controls, in two of three experiments (+24 and +30%), and they were increased, relative to pair-fed controls in each of three experiments (+55, +50, and +49%). The elevated levels of all three early enzymes involved in sterol biosynthesis after oral administration are clearly different from the decreases observed in cultured mammalian cells (650, 748) upon incubation with the 15-ketosterol at low concentrations. The increases in the levels of the three enzyme activities in liver after dietary administration may be due to decreased delivery of Chol to liver as a consequence of inhibition of the intestinal absorption of Chol by the 15-ketosterol. A very substantial inhibition of Chol absorption (−64%) upon dietary administration of the 15-ketosterol (0.05% in diet) has been demonstrated (913). A decrease in the intestinal absorption of Chol should result in decreased delivery of Chol to liver and an elevation of the levels of HMG-CoA reductase activity (and the 2 other enzymes). Studies with other agents that inhibit the absorption of Chol in intact animals, e.g., diosgenin (170) and β-sitosterol (1148), but which have little or no effect on sterol synthesis from labeled acetate and/or on the levels of HMG-CoA reductase activity in cultured mammalian cells (467), have been shown to cause, upon dietary administration, very marked increases in the levels of hepatic microsomal HMG-CoA reductase and/or incorporation of labeled acetate into sterols by liver preparations (170, 964). For example, administration of diosgenin (1% in diet) resulted in a substantial increase (+161%) in hepatic microsomal HMG-CoA reductase activity and a very marked increase (+2,300%) in sterol synthesis from labeled acetate in liver homogenate preparations (170). Similarly, dietary administration of β-sitosterol (2% in rat chow plus 5% corn oil) has been reported to cause a large increase (+252%) in the level of HMG-CoA reductase activity in rat liver microsomes (964). The only moderate increases (approximately +50%) in the levels of hepatic microsomal HMG-CoA reductase activity upon administration of the 15-ketosterol (0.1% in diet) are less than that anticipated from very marked inhibition of the intestinal absorption of Chol by the 15-ketosterol and is compatible with the idea that the 15-ketosterol suppresses the elevated liver HMG-CoA reductase activity that might be anticipated solely from its action in reducing the delivery of Chol to liver by virtue of its action in inhibiting the intestinal absorption of Chol. It is important to note that, despite the moderate elevations in hepatic HMG-CoA reductase activity in the 15-ketosterol-treated rats, significant de-
creases in serum Chol levels were observed in each of four experiments.

Vargas et al. (1162) reported that dietary administration of 7-keto-Chol (0.025% in diet) for 14 days to laying hens lowered (−44%) the levels of HMG-CoA reductase activity in liver. In view of the small numbers of animals (n = 3) used in the control and experimental groups in the single experiment presented, repetition of this experiment with expanded numbers of animals appears indicated.

Osada et al. (739) reported a lowering of the levels of HMG-CoA reductase activity in livers of male Sprague-Dawley rats after feeding an oxysterol mixture (0.2% in a basal Chol-free diet) for 20 days (relative to results with rats fed the Chol-free diet alone in amounts consumed by the oxysterol-fed animals). The major oxysterols in the mixture were 7-keto-Chol (26.7%), 5α,6α-epoxy-Chol (14.2%), and 7β-OH-Chol (13.3%), which were accompanied by a number of other oxidation products of Chol and unidentified material. Osada et al. (735) also studied the effects of feeding a mixture of oxysterols to young and adult male rats. The oxysterol mixture was similar to that described above. Three groups of animals were studied, i.e., those on a Chol-free diet, a Chol-containing diet (0.5%), and an oxysterol-containing diet (0.5%). After 21 days, the levels of HMG-CoA reductase activity in liver microsomes were reduced in animals fed the oxysterol mixture or Chol (to essentially the same extent). The same results were observed in young and adult rats. A limitation in this study was the lack of a pair-fed control group that appears to be necessary in view of the finding that the administration of the oxysterol mixture (0.5%) was associated with a significant suppression of food consumption in both the young and adult rats.

Oxysterols may affect chylomicron clearance. Mortimer et al. (680) reported that 7α-OH-Chol, 7β-OH-Chol, and 25-OH-Chol, incorporated into chylomicron-like emulsions of triolein, Chol oleate, sterol, and egg yolk phosphatidylcholine, decreased the clearance of Chol oleate of the emulsions from plasma and decreased the uptake of Chol oleate by liver (relative to Chol-containing emulsions). However, it should be noted that the concentrations of the oxysterols in the emulsions were only one-seventh that of the Chol-containing emulsions, and the results obtained with the oxysterol-containing emulsions were essentially the same as those observed with emulsions containing no sterol. It should also be noted that, on the basis of studies with 3β-hydroxy-5α-cholesta-8(14)-en-15-one (912), oxygenated sterols in chylomicrons in intestinal lymph in vivo would be expected to be found mostly as steryl esters and not as free sterols.

Erickson et al. (284) reported that short-term (66 h) dietary administration of 7-keto-Chol (0.1% by weight in Purina rat chow) to male Sprague-Dawley rats increased the levels of cytochrome P-450 in liver microsomes (1.20 ± 0.04 vs. 0.80 ± 0.07 nmol/mg protein for controls).

E. Effects of Oxysterols on Cholesterol 7α-Hydroxylase and Bile Acid Formation

Breuer et al. (125) reported that intravenous administration of 7-keto-Chol to rats in a commercial fat emulsion (Intralipid) resulted in increased cyp7a activity of liver microsomes. In rats fed cholestyramine (to suppress absorption of bile acids in the intestine), intravenous administration of 7-keto-Chol in the commercial fat emulsion was reported to decrease (approximately −50%) fecal bile acids. The increased level of activity of cyp7a after 7-keto-Chol administration was ascribed by the authors (125) to increased synthesis of the enzyme as a compensatory response to decreased bile acid formation induced by the 7-ketocholesterol. Tamasawa et al. (1091) found that dietary administration of 7-keto-Chol (0.1% in chow diet) to male Wistar rats for 6 days resulted in an increased level of hepatic microsomal cyp7a activity [44.7 ± 6.0 vs. 16.6 ± 2.5 (SE) pmol·min⁻¹·mg protein⁻¹]. These findings are in contrast to reports of a strong inhibitory in vitro effect of 7-keto-Chol on cyp7a activity of liver microsomes (125, 965, 1152) and the inhibitory effect of 7-keto-Chol on the binding of Chol to purified cytochrome P-450LM II (which showed high activity in the 7α-hydroxylation of Chol) from rabbit liver microsomes (108) and on the binding of Chol to a bacterially expressed rat liver cyp7a (125). In contrast, Osada et al. (739) noted a lowering of the levels of cyp7a activity in livers of male Sprague-Dawley rats after feeding an oxysterol mixture (0.2% in basal cholesterol-free diet) for 20 days (relative to results with rats fed the basal diet alone in the amounts consumed by the oxysterol-fed animals). The major oxysterol in the mixture was 7-keto-Chol (27%), which was accompanied by a number of other oxidation products of Chol and unidentified material. Mambetsiava et al. (611) reported that 3β-hydroxy-5α-cholesta-8(14)-en-15-one (0.25 μM) had no inhibitory action on the formation of bile acids by cultured rabbit hepatocytes incubated in the presence of HDL2. At this concentration, the 15-ketosterol was reported to cause a 50% inhibition of Chol synthesis from labeled acetate.

VIII. ADVERSE EFFECTS OF OXYSTEROLS AND RELATED MATTERS

A. Toxicity of Oxysterols

A number of studies have concerned the cytotoxicity of various oxygenated sterols. At present, there is frequently no clear differentiation of toxic actions due to the primary or secondary effects of the compounds and actions due to the induction of apoptotic changes that might be natural, physiological actions of some of the concerned compounds. A variety of changes have frequently

January 2000 OXYSTEROLS: MODULATORS OF CHOLESTEROL METABOLISM 501
been taken as indicative of cytotoxicity including changes in cell growth, cell viability, cell detachment, plating efficiency of cells, various aspects of morphology, transport of small molecules (i.e., 2-deoxyglucose, uridine, thymidine), MTT assay (a measure of metabolic activity in viable cells), leakage of cell enzymes (i.e., lactate dehydrogenase), protein synthesis, and DNA synthesis. The conditions of study are also of particular importance. Studies with cells incubated in chemically defined medium in the absence of added serum or serum lipoproteins provide for reduction in many variables; however, such incubation conditions cannot be considered to be "physiological." In contrast, studies with media containing serum (or even lipoproteins) present an infinitely more complex situation, including the fact that variable amounts of various oxygenated sterols may already exist in the serum (or in the lipoprotein fractions derived therefrom).

Sevian et al. (950) reported on the "cytotoxicity" of the 5α,6α- and 5β,6β-epoxides of Chol and 5α,6β-diOH-Chol in cultured rabbit aortic endothelial cells. The results indicated that the triol was more potent than the epoxides in this respect. The 5β,6β-isomer was more potent than the 5α,6α-epoxide. However, it is difficult to interpret the experimentation. The sterols were added in ethanol (0.5% by volume) to the culture medium. The authors noted that "after the 30 min preincubation in medium, a considerable amount of precipitable material was apparent, particularly when high concentrations of Chol epoxides were added."

Chisholm et al. (201) reported on the cytotoxicity of 7β-hydroperoxycholesterol-5-en-3β-ol in human fibroblasts (as measured by the release of 14C into the culture medium after incubation of the cells with [14C]adenine). The concentration of the 7β-hydroperoxide required to give a half-maximal effect was reported to be ~1.4 μM. In further studies from the same laboratory (211), 7β-hydroperoxy-Chol, under similar experimental conditions, was found to show higher cytotoxicity than 7β-OH-Chol, 7-keto-Chol, and 5α,6α-epoxy-Chol with human skin fibroblasts. The 7β-hydroperoxide was also reported to show higher cytotoxicity than 7β-OH-Chol and 7-keto-Chol with rabbit and human aortic smooth muscle cells and with bovine aortic endothelial cells. In the human fibroblasts, the IC50 values were as follows: 7β-hydroperoxycholesterol, 1.4 ± 0.8 μM; 7β-OH-Chol, 16.3 ± 5.5 μM; 7-keto-Chol, 21.6 ± 6.5 μM; 5α,6α-epoxy-Chol, 24.8 ± 6.2 μM; and 25-OH-Chol, 59 ± 4.4 μM. Oxygenated sterols used in this work were of commercial origin except for the 7β-hydroperoxide that was prepared by chemical synthesis. Chemical characterization of the product was very limited. Under the conditions studied, the 7β-hydroperoxide was reported to show higher cytotoxicity than that caused by either lysophosphatidylcholine or 4-hydroxynonenal. These findings are of importance in considerations of the cytotoxicity induced by oxidized LDL and its possible involvement in the arterial injury induced by oxidized LDL. The increased release of [14C]adenine induced by the 7β-hydroperoxycholesterol in skin fibroblasts was suppressed by the addition of known inhibitors of peroxidation of lipids, i.e., vitamin E, N,N',N'-diphenyl-1,4-phenylenediamine, and deferoxamine mesylate. It is noteworthy that none of these agents blocked the reported cytotoxicity induced by 7β-OH-Chol or 7-keto-Chol.

Very recently, Kölsch et al. (503) reported neurotoxic effects of 24-OH-Chol (C-24 stereochemistry not specified) on SH-SY5Y human neuroblastoma cells. Cell viability was not reduced at concentrations of 0.1–1 μM 24-OH-Chol, but less than half the cells survived at 10–50 μM concentration. Despite the high 24-OH-Chol levels required for toxicity, the authors concluded that "in a physiological concentration range, 24-OH-Chol damages neuronal cells."

Clare et al. (209) studied the effects of a number of oxysterols on human monocytes-macrophages maintained in a medium (RPMI 1640) containing 10% lipoprotein-deficient FCS. Cytotoxicity (increased cell permeability) was assayed following the release of 3H from cells preincubated with [3H]adenine. The specific conditions used for addition of the oxysterols (from ~1.2 to 249 μM) to the cells were not presented. The order of potency in increasing cell permeability was 26-OH-Chol > 7-oxygenated sterols (7β-OH-Chol, 7-keto-Chol, 7α-OH-Chol) > 25-OH-Chol. Simultaneous addition of Chol was reported to show significant protection against the cytotoxicity of 25-OH-Chol and 26-OH-Chol; however, Chol had little or no effect on [3H]adenine release from cells treated with 7-keto-Chol, 7α-OH-Chol, or 7β-OH-Chol. The authors indicated that the order of potency noted above does not correspond to the order of potency of the same oxysterols in lowering the levels of HMG-CoA reductase activity in mouse L cells (1103) in which 25-OH-Chol and 26-OH-Chol were considerably more potent than the 7-oxygenated sterols. It should be noted that the order of potency with regard to cytotoxicity may vary with different cell types. For example, Christ et al. (203) reported that 25-OH-Chol was more potent than 7β-OH-Chol in mouse thymocytes and in mouse lymphoma cells in reducing cell viability as measured by Trypan blue exclusion assay or MTT assay. These studies were carried out in media containing 10% heat-inactivated FCS. Liu et al. (583) observed that 25-OH-Chol at 6.2, 12.4, and 24.9 μM (but not at 2.5 μM) caused a dose-dependent increase of the leakage of lactate dehydrogenase from human monocytes incubated in RPMI medium containing delipidated FCS. In the same study a similar effect of 25-OH-Chol was not observed with human macrophages.

Guyton et al. (369) described the effects of 7-keto-Chol and 7β-OH-Chol on cultured smooth muscle cells from porcine aorta. Studies were carried out with DMEM
with lipoprotein-deficient serum (usually 0.4%) for 72 h. Remaining adherent cells were released (trypsin-EDTA) and counted. 7-Keto-Chol (added in ethanol) was studied at concentrations of 0.25 to 5 μM. 7β-OH-Chol was studied at 0.5 and 1.25 μM. Mevalonate (100 μM) did not reverse the “toxic” effects (reduced number of adherent cells) of the oxysterols. The authors noted that higher concentrations of mevalonate were not used since, at 1 and 20 μM, variable adverse effects on cell growth and even viability were observed. Mevalonate (100 μM) also had no effect on the “toxicity” (reduced cell number) caused by oxidized LDL. Lizard et al. (586) studied the effects of a number of oxygenated sterols on the growth of bovine aortic endothelial cells in media containing 10% FCS. The various oxysterols caused a dose-dependent decrease in the number of adherent cells after a 48-h incubation. The results appear to reflect the same or similar experimentation as described by the same group previously (585). Mean concentrations reported to cause a 50% reduction in the number of adherent cells at 48 h were as follows: 7β-OH-Chol, 23 μM; 7-keto-Chol, 34 μM, 19-OH-Chol, 72 μM; and 5α,6α-epoxy-Chol, 99 μM. 25-OH-Chol was considerably less potent than the above oxysterols and did not cause a 50% reduction at the highest concentration tested (199 μM). Chol was reported to have no effect at all at the concentrations tested (including 207 μM). The lowest concentrations of the oxysterols reported to give significant reduction in the number of adherent cells were as follows: 7β-OH-Chol, 12 μM; 19-OH-Chol, 25 μM; 5α,6α-epoxy-Chol, 25 μM; 25-OH-Chol, 25 μM; and 7-keto-Chol, 50 μM.

Duncan and Buckingham (268) observed that 25-OH-Chol (0.62 μM) or 20α-OH-Chol (1.24 μM), at concentrations at which very substantial inhibition of sterol synthesis from labeled acetate (−88%) was observed, showed no effect on the uptake of labeled 2-deoxy-α-glucose in HeLa cells. Marinovich et al. (616) reported 26-OH-Chol and 25-OH-Chol had little or no toxic effects in mouse epidermal cells in short-term incubations in media without added serum or lipoproteins. For example, 26-OH-Chol and 25-OH-Chol had little or no effect on cell growth (as measured by cellular protein) or on leakage of lactate dehydrogenase from the cells at concentrations of the oxysterols up to and including 200 μM (with incubation times of 2, 6, and 24 h). Similarly, 25-OH-Chol and 26-OH-Chol had little effect on protein synthesis (incorporation of [3H]leucine into cellular protein). Modest decreases were observed with 26-OH-Chol (at 100 and 200 μM) in 6-h incubations but not at 50 and 100 μM in 24-h incubations. 25-OH-Chol caused a modest decrease at 100 μM (but not at 50 μM) in 24-h incubations. In contrast to the 25- and 26-hydroxysterols, 26-aminocholesterol showed significant effects on cellular protein, protein synthesis, and the leakage of lactate dehydrogenase. The lack of effects of the oxygenated sterols could be a special feature of these mouse epidermal cells. More likely, the lack of effects of the 25- and 26-hydroxysterols is attributable to their lack of uptake by the cells under the conditions studied, i.e., direct addition of the oxysterols (in ethanol) to media containing no protein (serum, delipidated serum, lipoprotein-deficient serum). In the absence of protein in the media, it would seem unlikely that the oxysterols are present in other than a particulate form under such conditions. De Caprio et al. (245) have reported the precipitation of 26-OH-Chol upon its addition in ethanol at a level of 6 μM in DMEM. The study of Marinovich et al. (616) presents no evidence that the oxysterols entered the cells under the study conditions. For example, no direct studies of sterol uptake (or studies of effects on HMG-CoA reductase activity) were made. Marinovich et al. (616) also reported that 25-OH-Chol (20 μM) reduced leakage of lactate dehydrogenase in mouse epidermal cells caused by 26-amino-Chol or Triton X-100 in short-term (2 h) experiments. However, the results of this experimentation should be evaluated in light of the considerations presented above.

Oxysterols have been reported to have a protective effect against the toxicity induced by other agents. Duncan and Buckingham (267, 268) found that incubation of mouse L cells or HeLa cells with low levels of 25-OH-Chol (0.62 μM) or 20α-OH-Chol (1.24 μM) caused the cells to become notably resistant to the lysis of the cells induced by digitonin or by streptolysin O. The resistance induced by 25-OH-Chol was associated with a reduced level of cellular free Chol and could be reversed by the addition of Chol to the culture medium.

Ares et al. (19) reported on the cytotoxicity (as measured by MTT assay) of 25-OH-Chol (12.4 or 25 μM) in human aortic smooth muscle cells after 24 or 48 h of incubation in DMEM-F-12 medium supplemented with 5% FCS and antibiotics. Addition of the cytokines TNF-α and interferon-γ increased the toxicity of the 25-hydroxysterol.

Chang and Liu (175) studied the effects of selected oxysterols on the viability of cultured PC12 (tumor cells originating from an adrenal pheochromocytoma) and on “neuronal PC12 cells” (PC12 cells treated with nerve growth factor). In studies carried out with medium containing high levels of FCS and horse serum, decreased viability (as measured by MTT assay) was observed in the PC12 cells with each of 25-, 7β-, 22(R)-, 22(S)-, and 19-OH-Chol. 25-OH-Chol and (22R)-22-OH-Chol appeared to be most potent. The neuronal PC12 cells appeared to be less sensitive to the effects of the oxysterols on cell viability. Under the conditions studied, 25-OH-Chol did not appear to cause a decrease in the neurite outgrowth induced by nerve growth factor. Chang et al. (178) also studied the cytotoxicity induced by 25-OH-Chol in neuronal PC12 cells; vitamin E, but not ascorbic acid or aurinetricarboxylic acid, reduced the cytotoxicity caused by the 25-OH-Chol (25 μM). Chang and Liu (176) also studied the
effects of selected oxysterols on the viability (as measured by MTT assay) of cultured rat cerebellar granule cells. Oxysterols resulting in decreased viability included 7β-OH-Chol, 7-keto-Chol, 25-OH-Chol, 19-OH-Chol, and the 22R- and 22S-isomers of 22-OH-Chol. 25-OH-Chol (6.2 μM) caused a 34% decrease in viability in a 48-h experiment. 7β-OH-Chol (25 μM) caused a 50% decrease in viability and was more potent than 7-keto-Chol. The unnatural 22S-isomer of 22-OH-Chol was reported to be considerably more potent than its 22R-isomer. 19-OH-Chol showed only modest activity, with ~40% decrease in viability at 50 μM. Auranin, carboxylic acid and vitamin E, but not ascorbic acid, were partially effective in reduction of the decreased viability induced by 25-OH-Chol. Chang and Liu (177), using the same methodology, reported similar findings using rat neuroretinal cells. However, with these cells, 19-OH-Chol showed relatively high potency (~39% decrease in viability at 6.2 μM).

Ryzlak et al. (879) proposed that “... oxysterols may be the primary cause of the development of alcoholic liver diseases and damage to accessory tissues.” They observed increased levels of cholesta-3,5-dien-7-one and cholesta-4,6-dien-3-one in livers of patients with alcohol-induced fatty livers than in livers of nonalcoholic subjects. Adachi et al. (2) reported elevated levels of cholesta-3,5-dien-7-one (not detected in controls) in erythrocyte membranes from human male alcoholic subjects. In a subsequent study, Ryzlak et al. (878) observed that cholesta-3,5-dien-7-one is a fairly potent inhibitor of one isozyme (E1) of human liver aldehyde dehydrogenase, with an IC50 value of 5–10 μM. The Δ3,5,7-ketosterol was a considerably less active inhibitor of the E2 isozyme (IC50 ~ 180 μM) and showed no inhibitory activity with the E3 isozyme. The inhibition of the E1 isozyme was reported to be noncompetitive. 7-Keto-Chol and cholesta-4,6-diene-3-one were reported to show no inhibitory action; however, no data on this point were presented.

Fioriti et al. (305) reported that intraperitoneal administration of 10% 5α,6α-epoxy-Chol in monoolein showed no lethal or gross toxic effects at doses up to 2.5 g/kg. However, oral administration of the epoxide in monoolein was reported to show a 50% lethal dose (LD50) value of 1.82 g/kg. Information on the identity and purity of the administered epoxysterol was not provided. From these results it was suspected that a metabolite of the epoxysterol was a toxic species, and it was suggested that this metabolite was 5α,6β-dioOH-Chol. Nashed et al. (697) reported that 5,6β-epoxy-5β-cholest-7-en-3β-ol showed an LD50 of ~140 mg/kg body wt after intraperitoneal injection in ICR mice (body weight, ~30 g).

**B. Effects of Oxysterols on Morphology**

Hsu et al. (406) observed that incubation of a number of oxygenated sterols (25 μM; added in ethanol to a lipoprotein-depleted medium) with human erythrocytes resulted in the rapid formation of echinocytes. This process was not affected by free Chol but could be suppressed by the addition of lipoprotein-containing serum. Oxysterols were classified as to their potency in inducing the formation of echinocytes. Described as most potent were 6β-hydroxycholesterol, 5α,6β-dihydroxycholestanol, 5α-hydroxy-6-ketcholestanol, 6-ketocholesterol, and 7β-OH-Chol. Sterols of intermediate potency included 4β-OH-Chol, 7-keto-Chol, 7-ketocholesterol, and 7α-OH-Chol. Oxysterols showing very low or no activity included 20α-OH-Chol, 22-keto-Chol, and 25-OH-Chol. It is noteworthy that 20α-OH-Chol was very efficiently taken up by the erythrocytes but showed little or no activity in inducing echinocyte formation. In further studies from the same laboratory (1053), it was noted that certain oxysterols decrease the osmotic fragility of human erythrocytes, with 7β-OH-Chol, 22-keto-Chol, and 20α-OH-Chol being the most potent in the inhibition of osmotic lysis. Moreover, it was reported that 20α-OH-Chol and 22-keto-Chol (50 μM) improved in vitro osmotic fragility in red blood cells from a patient with hereditary spherocytosis. Saito et al. (884) reported that incubation of bovine erythrocytes with (22R)-22-OH-Chol (added in ethanol to Tris-ACD buffer) caused significant lysis of the red blood cells. After incubation for 30 min at 37°C, the extent of lysis of the erythrocytes caused by the 22R-oxysterol (50 μM) was reported to be 71%. This result differed markedly from that observed with the 22S-isomer of 22-OH-Chol that showed 16% hemolysis. Other oxysterols that showed significant hemolysis were (20S)-20-OH-Chol (18%), (24R)-24-OH-Chol (16%), and (24S)-24-OH-Chol (18%). The 20S- and 20R-isomers of 20-OH-Chol differed, with the 20R-isomer showing only 5.5% lysis. A number of ring B oxygenated sterols showed little or no hemolytic action (<5% lysis); these included 7-keto-Chol, 5α-cholestan-3β,6β-diol, 3β-hydroxy-5α-cholestan-6-one, 5α,6β-dioOH-Chol, 3β,5-dihydroxy-5α-cholestan-6-one, and 5α,6α-epoxy-Chol. Chol and “sitosterol” were reported to have no lytic action under the same conditions. The hemolysis induced by (22R)-22-OH-Chol was dose dependent (with significant lysis at 25 μM) and temperature dependent (with no lysis at 4 and 15°C and marked hemolysis at 25 and 37°C). The striking differences in hemolysis caused by the 22R- and 22S-isomers did not appear to be due to differences in the uptake of the two isomers since their levels in the erythrocytes were essentially the same. Examination of red blood cells incubated with (22R)-22-OH-Chol (50 μM) for 30 min at 37°C by scanning electron microscopy showed a marked change in morphology, with the appearance of “collapsed” cells.

Palladini et al. (750, 751) studied the effects of short-term (6 h) incubation of three oxysterols, i.e., 25-OH-Chol (24.9 μM), 7-keto-Chol (25.0 μM), and 5α,6β-dioOH-Chol (23.8 μM), on the morphology of a cell line (73/73) derived...
from bovine aortic endothelial cells transformed with benzpyrene. It is important to note that these incubations with relatively high concentrations of the oxysterol were carried out with media containing lipoprotein-deficient human serum (0.4%). Oxysterol treatment was associated with a disruption of the organization of actin microfilaments with a redistribution of actin filaments within the cell and with a fragmentation of F-actin. Oxysterol treatment was also associated with a loss of staining for vinculin, an actin-binding protein, in the cell body, changes in the appearance of staining for peripheral vinculin, and changes in the association between vinculin and actin microfilaments. Incubation of the cells with the oxysterols for longer periods of time was associated with cell detachment and, in the detached cells, with changes in nuclear chromatin characteristic of apoptosis.

Deckert et al. (247) observed that short-term (2 h) incubation of 7-keto-Chol (225 μM) with rabbit aortic segments had no effect on the morphology of endothelial cells or smooth muscle cells upon study by light or transmission electron microscopy. The 7-ketosterol was added in a mixture of ethanol and fatty acid-poor BSA. Lizard et al. (586) reported on the effect of commercial 7-keto-Chol (200 μM) on the morphology of bovine aortic endothelial cells grown for 48 h in medium containing 10% FCS. Studies by phase-contrast microscopy indicated that 7-keto-Chol, at this very high concentration, caused “a loss of cell connections” and the occurrence of numerous round cells. Under the same conditions, Chol (207 μM) was reported to have no effect on cell morphology.

Ares et al. (19) reported electron microscopic changes that occurred in human aortic smooth muscle cells after incubation with 25-OH-Chol (12.4 or 25 μM) in medium containing 5% FCS. At early times (4 h), increased numbers of lysosomes were observed along with the appearance of tubular membrane structure and chromatin condensation. By 8–16 h, ordered endoplasmic reticulum and Golgi membranes were decreased, and cell shrinkage was observed. At later times, mitochondrial degeneration was observed. By 48 h, most cells were reported as necrotic.

A dramatic change in the morphology of CHO-K1 cells occurs when the cells are incubated with submolar levels of 14α-ethyl-5α-cholest-7-ene-3β,15α-diol in lipid-deficient medium (430, 806). The change in morphology (elongation) was similar to that observed when CHO-K1 cells are incubated in the presence of dibutyryl cAMP (1 mM) (405, 430). The cells treated with the ethyl diol were, as studied by scanning electron microscopy, markedly elongated, and they were larger and their surfaces were covered by widely dispersed, short microvilli (430). The frequency and extent of the elongation of the cells were quantitated by measurement of the ratio of their longest to shortest cellular dimensions. The striking changes in cell morphology were associated with major changes in cellular sterol composition (and in plasma membranes of cells) induced by the ethyl diol (0.1 μM), i.e., a marked accumulation of lanosterol and 24,25-dihydrolanosterol (430). Consistent with this finding, similar changes in cellular sterol composition and morphology were also induced by incubation of the cells with 24,25-dihydrolanosterol (10 μM) or miconazole (10 μM). The elongation of the cells and the accumulation of the C₃₀ steroids induced by the ethyl diol were both reversed by transfer of the cells to fresh medium in the absence of the ethyl diol. Furthermore, the elongation of the cells and the accumulation of lanosterol and 24,25-dihydrolanosterol were prevented by exogenous Chol (at 10 or 100 μM, but not 1 μM) in the culture medium. Another oxysterol, 3β-hydroxy-5α-cholest-8(14)-en-15-one, which does not cause the accumulation of C₃₀ sterols, did not result in the morphological changes induced by the ethyl diol (430). Because the morphological changes induced by changing the sterol composition of the cells were essentially the same as those induced by dibutyryl cAMP, the involvement of cAMP was studied. Incubation of the cells with dibutyryl cAMP (1 mM) resulted in the elongation of the cells; however, no effect on cellular sterol composition was observed. Furthermore, the ethyl diol, at various concentrations (from 0.1 to 1 μM) for 24 or 48 h, had no effect on the intracellular levels of cAMP. It is interesting to note that the changes in the shape of the CHO-K1 cells induced by dibutyryl cAMP correspond to the shape changes on going from a transformed cell to a normal fibroblast-type cell. The precise mechanisms involved in the morphological changes associated with changes in the sterol composition have not been established. It is possible that changes in the sterol composition of the cells affect the interaction of the plasma membrane with cytoskeletal elements known to be involved in the control of cell shape (324). The use of 14α-ethyl-5α-cholest-7-ene-3β,15α-diol offers a very powerful probe for exploration of these matters.

C. Concerning the Mutagenic Activity of Oxysterols

Reviews of early research on the production of tumors, chiefly local fibrosarcomas, in animals after the injection of a number of oxygenated derivatives of Chol were presented by Bischoff (78, 79), and a review by Morin et al. (677) summarizes more recent research in this area. Kelsey and Pienta (481) reported that the 5α,6α-epoxide of Chol, but not Chol, was active in the transformation of hamster embryo cells, in an in vitro carcinogenesis bioassay. The authors suggested that this bioassay system may be more valuable than the Ames test in detection of potential carcinogenicity of steroids. Parsons and Goss (775) were unsuccessful in detecting trans-
formations of human fibroblasts by the $5\alpha,6\alpha$-epoxide of Chol. However, the epoxide was reported to cause chromosomal aberrations in fibroblasts, the frequency of which was increased by ultraviolet irradiation of the cells. The epoxide, but not Chol, was also reported to induce DNA repair synthesis in human fibroblasts and melanoma cells. However (and in contrast to ultraviolet-treated cells), DNA profiles from human fibroblasts and melanoma cells treated with the epoxide did not reveal breaks in DNA upon sucrose gradient ultracentrifugation. Reddy and Watanabe (838) found that intrarectal administration of the $5\alpha,6\alpha$-epoxide of Chol or $5\alpha,6\beta$-dihOH-Chol (20 mg/animal, 3 times/wk for 46 wk) to germ-free rats did not result in colon tumor formation, nor did these compounds act as tumor promoters after intrarectal administration of the known carcinogen N-methyl-N-nitro-N-nitroso-guanidine.

Blackburn et al. (99) studied the interaction of the $[4-14\text{C}]5\alpha,6\alpha$-epoxy-Chol with calf thymus DNA. The labeled complex was analyzed by cesium chloride density centrifugation and gel filtration on Sephadex G-200. It was reported that “the extent of steroid association was in excess of one molecule per hundred DNA base pairs” and that “much lower levels of physical association were observed under identical conditions for cholesterol, estradiol, and progesterone.” Prolonged incubation was reported to give extensive covalent attachment of the sterol to DNA and that “this binding survived enzymatic degradation of the DNA” giving “a principal radioactive peak on LH-20 gel chromatograms similar in elution profile to fragments resulting from the covalent binding of benzo-pyrene epoxides to DNA” observed by others.

Air-aged USP Chol, purified Chol heated at 70°C for several weeks, or purified Chol oxidized by 60°C irradiation for several days have been reported to contain mutagenic species as evaluated by the Ames test, whereas purified Chol had no mutagenic activity (14, 1009). In an attempt to identify the mutagenic species in the oxidized Chol preparations, a large number of purified oxygenated derivatives of Chol were studied and found to be non-mutagenic (14, 1009). Among the sterols tested were the $5\alpha,6\alpha$- and $5\beta,6\beta$-epoxides of Chol. It was suggested that the mutagenic species in the oxidized Chol preparations were sterols that were more extensively oxygenated than the previously established products of the autoxidation of Chol (14). These studies assayed direct mutagenicity in various $S$. typhimurium strains and did not include prior incubation of the compounds with liver enzyme preparations. El-Bayoumy et al. (275) reported that injection of either $5\alpha,6\alpha$-epoxy-Chol or $5\beta,6\beta$-epoxy-Chol (total dose, 12.3 $\mu$mol in dimethyl sulfoxide) into mammary tissue underneath thoracic and inguinal nipples of rats had no significant tumorigenic action, whereas administration of $\text{trans-3,4-dihydroxy-anti-1,2-epoxy-1,2,3,4-tetrahydrobenzo[c]phenanthrene}$ (total dose, 1.2 $\mu$mol) produced mammary tumors in each of 20 rats studied.

Sevanian and Peterson (954) reported that $5\alpha,6\alpha$-epoxy-Chol is a weak direct-acting mutagen in V79 Chinese hamster lung fibroblasts. Mutagenicity was assayed by determining the frequency of 8-azaguanine-resistant mutants. The data presented indicated that mutagenicity reported to be induced by the epoxide was not dependent on dosage or time of treatment of the cells with the epoxysterol. $5\alpha,6\beta$-dihOH-Chol was reported to be not significantly mutagenic. The authors suggested that the mutagenicity of the $5\alpha,6\alpha$-epoxide may be reduced in cells active in the conversion of the epoxide to $5\alpha,6\beta$-dihOH-Chol. Raaphorst et al. (830) also studied the mutagenicity of the $5\alpha,6\alpha$- and $5\beta,6\beta$-epoxides of Chol. Both epoxides were reported to be mutagenic in a strain of mouse embryo cells. In contrast to the study of Sevanian and Peterson (954), the frequency of transformation in the mouse cells was found to be dependent on the concentration of the epoxide and time of exposure. The $5\beta,6\beta$-epoxide was reported to result in a higher frequency of transformation than the $5\alpha,6\alpha$-epoxide. The possible mutagenicity of $5\alpha,6\alpha$-epoxy-Chol, $7\alpha$-hydroperoxy-Chol (1010), $5\alpha$-hydroperoxy-$5\alpha$-cholest-6-en-3$\beta$-ol (1010), and other sterols is deserving of further attention as well as its interaction with DNA. At the present time, the state of knowledge on these important subjects is very considerably less than that for epoxide derivatives of aflatoxin B$_1$ (457 and references cited therein) and of benzopyrene (296, 297, 635, 641, 820, 854, 1220 and references cited therein).

**D. Oxysterols and Programmed Cell Death**

Oxigenated sterols have been suggested to be involved in apoptosis, a form of programmed cell death, in cells. Interest has been especially focused on leukemic lymphocytes that appear to be very sensitive to the effects of oxysterols in the inhibition of cell growth and the lysis of the cells. These cells are known to show high rates of sterol synthesis. 25-OH-Chol has been reported to block the growth of cells in the postmitotic half of G$_1$ phase of the cell cycle (546). Bansal et al. (48) reported that 25-OH-Chol (1 $\mu$M) and mevinolin (2 $\mu$g/ml) induced apoptosis, as measured by DNA fragmentation, in human leukemia cell lines (CEM-C7, glucocorticoid sensitive; and CEM-C1, glucocorticoid resistant). Related work by Ayala-Torres et al. (39) and Thompson and Ayala-Torres (1113) concerning the effects of 25-OH-Chol on c-myc, c-Myc, and apoptosis in CEM cells is discussed in section vH.

Escargueil-Blanc et al. (289) reported on the effects of oxidized LDL on the induction of necrosis and apoptosis in human lymphoblastoid cells. Oxidized LDL was prepared by incubation of LDL in the presence of CuSO$_4$
(2 μM). The oxidized LDL was characterized only by the level of TBARS per milligram of apoB. The oxidized LDL induced a rise in the level of intracellular Ca^{2+} that was followed by activation of proteolysis and DNA fragmentation. The possibility that oxysterols in the LDL caused the observed effects of the oxidized LDL was not investigated. Actinomycin D (4 nM) and cycloheximide (1 μM) were reported to be ineffective in preventing proteolysis, DNA fragmentation, necrosis, and apoptosis caused by oxidized LDL. This differs from other forms of induced cell death such as that which occurs in developmental apoptosis (345).

Hwang (418) studied the effects of two oxygenated sterols on cell viability in two murine cancer cell lines (EL4 lymphoma and K36 leukemia) in culture media containing 5% lipoprotein-deficient newborn calf serum. With both cell lines, the 7-keto-Chol (5 μM) was reported to have “reproducibly killed more than 80–85% of the cells in culture after 48 h.” Similar results were observed with 25-OH-Chol (20–40 nM) correlated with the affinity of 25-OH-Chol for the oxysterol binding protein ($K_d \sim 31$ nM). In contrast to the results of some other studies, addition of either mevalonate or Chol was shown to inhibit the lysis of CEM-C7 lymphocytes caused by 25-OH-Chol (60 nM). 7-Keto-Chol and 20α-OH-Chol were also reported to inhibit the growth of the glucocorticoid-sensitive CEM-C7 cells with IC$_{50}$ values of ~450 and 30 nM, respectively. The combined results of this study indicated an independent action of 25-OH-Chol and glucocorticoids in their inhibition of the growth of human leukemia lymphoblasts. The reversal of the effect of 25-OH-Chol on cell death by either mevalonate or Chol in CEM-C7 cells led to the suggestion that “the lethal effects of 25-OH-Chol are linked with the inhibition of cholesterol synthesis” and were not dependent on other products of mevalonate metabolism such as dolichol or prenylated proteins. Furthermore, it was noted that the concentrations of 25-OH-Chol that occupy half of the binding site in the oxysterol binding protein correspond to the IC$_{50}$ value for cell death induced by 25-OH-Chol in the same cells.

Christ et al. (203) reported that 25-OH-Chol (10 μM) or 7β,25-diOH-Chol (10 μM) induced DNA fragmentation characteristic of apoptosis in murine lymphoma (RDM4) cells cultured in medium containing 10% heat-inactivated FCS. Under the same conditions, apoptosis was not observed in RDM4 cells in the presence of 7β-OH-Chol (or in the absence of added oxysterols). The DNA fragmentation induced by 25-OH-Chol or 7β,25-diOH-Chol was reduced by either cycloheximide or actinomycin D. DNA fragmentation was also induced in mouse thymocytes by incubation with either 25-OH-Chol (10 μM) or 7β,25-diOH-Chol (10 μM). Untreated thymocytes or thymocytes incubated with 7β-OH-Chol (10 μM) showed comparable, but significant, amounts of DNA fragmentation. Cycloheximide or actinomycin D blocked the induction of DNA fragmentation induced in thymocytes by 25-OH-Chol or 7β,25-diOH-Chol. Aupeix et al. (28) noted that 25-OH-Chol and 7β-OH-Chol, in media containing 10% heat-inactivated FCS, induced changes of apoptosis in two human monocyte cell lines (U-937 and HL-60). The effects of 7β-OH-Chol (30 μM) in U-937 cells were evident by 12 h. Inhibitory effects on the growth of U-937 cells were observed at concentrations of 10, 20, and 30 μM of 7β-OH-Chol or 25-OH-Chol. Interestingly, the effects of a combination of 7β-OH-Chol (30 μM) and 25-OH-Chol (30 μM) on the induction of apoptotic changes in U-937 cells were less than those observed with 7β-OH-Chol (30 μM) alone. The same observation was not made in the case of HL-60 cells. The potency of the 7β-OH-Chol in inducing apoptosis in the monocytic cell lines was higher than that of 25-OH-Chol. It should be noted that, in these cells, the potency of the two sterols in inducing apoptosis is quite different from their potencies in lowering HMG-CoA reductase in mouse L cells (1103) or in their affinities for the oxysterol binding protein.
Recent studies of Zhang et al. (1226) indicated that 25-OH-Chol and 26-OH-Chol induce apoptosis in mouse thymocytes. The 25-hydroxysterol was reported to be more potent in this respect. In contrast to the results of Christ et al. (203), a 7-hydroxylated derivative of 25-OH-Chol, i.e., 7α,25-diOH-Chol, was reported to show no significant activity under the conditions studied. The studies of Zhang et al. (1226) were carried out using culture medium containing BSA (0.5%) but without added serum. It was suggested that metabolism of 25-OH-Chol and 26-OH-Chol to 7α-hydroxylated species in thymus cells might represent a protective control mechanism in the regulation of the induction of apoptosis in the thymus.

Lizard et al. (585) reported on the effects of various oxysterols in causing apoptosis in bovine aortic endothelial cells in culture media containing 10% FCS. Under these conditions, various oxygenated derivatives of Chol, at rather high concentrations, induced apoptosis. After 48-h incubation in the presence of the added sterols, a variety of apoptotic changes were observed, mostly in nonadherent cells. Quantitative data on the proportion of nonadherent cells showing apoptotic changes on fluorescence microscopy (after staining with Hoechst 33342) and on the proportion of nonadherent cells with a DNA content lower than G0/G1 (designated as sub-G1) on flow cytometry analysis were presented. Oxysterols studied (in order of decreasing potency) were as follows: 7β-OH-Chol (25 μM), 7-keto-Chol (50 μM), 19-OH-Chol (75 μM), 5α,6α-epoxy-Chol (100 μM), and 25-OH-Chol (200 μM). It should be noted that the potencies of the oxysterols in inducing apoptotic changes in these cells do not correspond to their potencies in lowering the levels of HMG-CoA reductase activity in mouse L cells (1103). Under the conditions studied, Chol (207 μM) was reported to show no effects.

Other inhibitors of mevalonate formation and sterol synthesis, i.e., competitive inhibitors of HMG-CoA reductase, have been reported to induce apoptosis in human promyelocytic leukemic HL-60 cells (797). Lovastatin (10 μM) was reported to repress cell viability and to induce DNA fragmentation in HL-60 cells grown in medium containing 10% FCS. The DNA fragmentation caused by lovastatin was reported to be dependent on dosage and time of incubation. Mevalonate (100 μM) was reported to completely prevent the DNA fragmentation induced by lovastatin (10 μM). Chol (250 μM; added as a suspension after sonication with phosphatidylcholine) was “totally ineffective” in blocking the DNA damage induced by lovastatin. Dolichol (250 μM) and ubiquinone 50 (250 μM) showed a partial protection, and isopentyladenosine (250 μM) appeared to potentiate the DNA fragmentation caused by lovastatin. Lovastatin (10 μM) was reported to decrease the membrane association of lamin A and p21ras.

Palladini et al. (750) observed that incubation of a transformed line of bovine aortic endothelial cells with either of three oxysterols, i.e., 25-OH-Chol (25 μM), 7-keto-Chol (25 μM), or 5α,6β-diOH-Chol (24 μM) for 24 h in media containing lipoprotein-deficient human serum (0.4%) resulted in significant DNA fragmentation. Control cells showed low levels of DNA fragmentation (4.1 ± 1.6%), whereas cells treated with the oxysterols showed markedly higher percentages of cells with nuclear fragmentation (25-OH-Chol, 33.5 ± 6.7%; 7-keto-Chol, 35.0 ± 4.3%; 5α,6β-diOH-Chol, 39.1 ± 5.6%). In contrast to detached cells, adherent cells reportedly did not show any indication of chromatin condensation or nuclear fragmentation. The incidence of nuclear fragmentation in oxysterol-treated cells varied with the time of incubation with the oxysterol. For example, cells incubated with 5α,6β-diOH-Chol (23.8 μM) showed increasing percentages of nuclear fragmentation in detached cells (6 h, 23 ± 11%; 18 h, 42 ± 13%; 24 h, 71 ± 14%; and 36 h, 91 ± 8%).

Nishio et al. (712) reported that 7-keto-Chol induced apoptotic changes in rabbit aortic smooth muscle cells incubated in serum-free medium for 24 h. Significant apoptosis was caused by 7-keto-Chol at 10 μM (the lowest concentration studied). In other experiments, 7-keto-Chol (25 μM) or oxidized LDL (200 μg/ml) induced apoptosis in smooth muscle cells, whereas lysophosphatidylcholine (10 μM), native LDL (200 μg/ml), or acetylated LDL (200 μg/ml) had little or no effect under the conditions studied. The relevance of these findings is that both 7-keto-Chol and lysophosphatidylcholine are products of the oxidation of LDL. Thus 7-keto-Chol, a significant oxysterol found in oxidized LDL, may account for the apoptosis caused by oxidized LDL. Other investigators (373) reported the demonstration of apoptosis in smooth muscle cells in advanced atherosclerotic lesions. In another study, Nishio et al. (713) observed that another oxysterol, 25-OH-Chol, also induced apoptosis in rabbit aortic smooth muscle cells incubated in serum-free medium for 24 h. This effect was clear at concentrations of 20, 25, 30, and 40 μM. A marginal increase was observed at 10 μM. The induction of apoptosis by 25-OH-Chol (30 μM) was substantially inhibited by Chol (50 or 100 μM), whereas Chol (50 or 100 μM) had no effect on the apoptosis induced by 7-keto-Chol (30 μM). Both 7-keto-Chol (30 μM) and 25-OH-Chol (30 μM) induced a rapid marked decrease in the levels of bcl-2 protein, a species which appears to serve as an inhibitory factor in apoptosis. The decrease in bcl-2 levels caused by the oxysterols occurred very rapidly, with very major decreases observed within 60 min. Inhibitors of proteases [i.e., interleukin-1β-converting enzyme (ICE) and CPP32], which are considered to be involved in apoptosis, were studied with respect to their effect on the apoptosis induced by 7-keto-Chol and 25-OH-Chol. An ICE inhibitor suppressed the apoptosis induced by either 7-keto-Chol or 25-OH-Chol. An inhibitor of CPP32 suppressed the apoptosis induced by 7-keto-Chol, but this effect appeared to be considerably less in the case of apoptosis induced by 25-OH-Chol. Thus the
apoptosis induced by 7-keto-Chol appeared to differ in several respects from that induced by 25-OH-Chol.

Ares et al. (19) reported on the production of apoptotic changes in human aortic smooth muscle cells by 25-OH-Chol (12.4 or 25 μM) in medium containing 5% FCS. Changes induced by the 25-hydroxysterol included chromatin degradation, DNA fragmentation, and activation of the protease caspase 3. Verapamil or nifedipine, Ca2+ channel blockers, or removal of Ca2+ from the incubation medium, inhibited the apoptotic changes induced by 25-OH-Chol. The cytokines TNF-α and interferon-γ increased apoptosis induced by 25-OH-Chol as measured by terminal deoxytransferase-mediated dUTP nick end labeling assay and assays of DNA fragmentation.

The results of recent research have indicated the involvement of SREBP in programmed cell death (745, 1182, 1184). A cysteine protease, distinct from ICE, has been purified (745, 1182). Sterols (25-OH-Chol plus Chol) did not affect the apoptosis-induced cleavage of the SREBP under the conditions studied (1184).

The recent reports (224, 327, 373) that human atherosclerotic lesions contain a substantial number of cells with the characteristics of apoptosis, as well as those of primary necrosis (224), coupled with the reports of the effects of oxysterols and of oxidized LDL as inducers of apoptosis and a report that oxidized LDL (in the presence of the cytokines TNF-α and interferon-γ) induces apoptotic changes in cultured human vascular smooth muscle cells (461), will no doubt provide a powerful stimulus for further research in this area.

IX. ATHEROSCLEROSIS AND OXYGENATED DERIVATIVES OF CHOLESTEROL

Several studies have indicated that products of the oxidation of Chol may be important in the production of atherosclerotic lesions in animals. However, other studies have claimed that oxidation products of Chol are less atherogenic than Chol. Studies of this important matter are actually quite limited, both in number and experimental design. The following presents a brief review of major studies of this subject. [A careful review of this subject by Brown and Jessup (136) appeared very recently.]

In 1968, Cook and MacDougall (215) reported the production of atherosclerotic lesions in aortas of male NZW rabbits after dietary administration of 5α,6β-diOH-Chol. The sterol composition of the basal diet was not given. However, it was stated that the sterols present in the diet were “mainly phytosterols.” This frequently cited study was limited by the absence of data regarding the characterization and purity of the triol and an experimental design that involved variable lengths of administration (27–350 days) of the triol (0.1% in basal diet or ~30 mg/kg body wt−1day−1) to the individual experimental animals and a very small number of control animals (which also varied in the duration of consumption of the basal diet).

In 1976, Imai et al. (422) reported that administration of a mixture containing Chol enriched with contaminants (obtained from mother liquors derived from recrystallization of commercial Chol from methanol) was associated with electron microscopic lesions (evidence of smooth muscle injury) in aortas of rabbits (at 24 h after the administration by gavage of the sterol mixture in an aqueous gelatin suspension). The authors also carried out long-term studies of the effects of gavage administration of the above mixture as a suspension in aqueous gelatin at a frequency of three times per week. The concentrate (or purified Chol) was administered initially at a single dose of 100 mg/kg and later at 25 mg/kg. In one experiment, the total dose ranged from 700 to 1,050 mg/kg for 2 wk. In another experiment, rabbits received a total of 2,625 mg/kg over a 10-wk period. Another group of rabbits was given 7,707 mg/kg for 6 wk of either “new” or “old” USP-grade Chol. In all of these studies, serum levels of Chol (and of total protein, calcium, and phosphorus) were reported to have been within normal range with no differences between the various groups. The actual data on those matters were not presented. Administration of 1) the concentrate or purified Chol (average of 23 mg·kg−1·day−1 over 45 days), 2) either “new” or “old” USP-grade Chol (average of 179 mg·kg−1·day−1 over 43 days), or 3) the concentrate (average of 23 mg·kg−1·day−1 over 45 days or an average of 38 mg·kg−1·day−1 over 69 days) had no effect on serum levels of Chol. Rabbits receiving either the purified Chol or the old or new Chol showed no microscopic lesions in aorta. Administration of the concentrate was reported to be associated with, as detected by electron microscopy, changes in intima (chiefly fibrosis and “relatively frequent, poorly differentiated intimal cells”). Limitations of this study include the following. The chemical nature of the materials in the “concentrate” were not defined, nor was evidence presented regarding the purity (and nature and levels of impurities) in the various samples of Chol used in the study. The source and composition of the basal diet were not provided. No evidence was presented regarding the efficiency of absorption of Chol (or other sterols) when given by gavage in a suspension in aqueous gelatin. Quantitative data on the electron microscopic changes observed in the long-term studies were not presented. A subsequent publication by the same group in 1979 (1097) reviewed and extended their previous study. In this report, data relative to the sterol composition of the concentrate were presented as follows: Chol, 38.0%; 25-OH-Chol, 13.4%; 7-keto-Chol, 12.9%; 7α- and 7β-OH-Chol, 6.7%; 7 and 5 hydroperoxides of Chol, 3.8%; 5α,6β-diOH-Chol, 5.1%, and other, 21.1%. The authors (1097) made the following statements relative to the atherogeneity of Chol: “Our work strongly suggested that pure Chol (either engr-
dogenously synthesized or chemically isolated pure Chol is not atherogenic. However, when Chol becomes a mixture of Chol plus spontaneously produced toxic derivatives (which develop in pure Chol and dehydrated Chol containing foods stored in air at room temperature) it may be highly atherogenic.”

In 1980, Imai et al. (423) reported that intravenous injection (ear vein) of a concentrate of “impurities obtained from the recrystallization of impure Chol” into a Japanese strain of rabbits induced macroscopic changes in aorta. The injected material was administered at a high dosage (40 mg/kg) as a suspension in saline solution (suspended with a glass homogenizer and ultrasonication) once a day for 3 days. Lower doses of several synthetic oxysterols were administered to NZW rabbits in a similar fashion. The lower doses were employed since it was stated that, in these rabbits, purified Chol “caused immediate death” at a dosage of 10 mg/kg. Several oxysterols, prepared by chemical synthesis (but with no data on characterization or purity), were studied with regard to their effects on macroscopic morphology of minor and major branches of the pulmonary artery. 5α,6β-diOH-Chol, 25-OH-Chol, and a 24,25-epoxide derivative of lanosterol were reported to cause effects at a dosage of 5 mg·kg⁻¹·day⁻¹ for 3 days in the NZW rabbits. 5α,6α-Ep-oxo-Chol and 7-keto-Chol were reported to affect major branches of the pulmonary artery at a higher dose, i.e., 10 mg/kg. Injection of the bis-epoxide derivative of squalene was reported to affect major and minor branches at 40 mg·kg⁻¹·day⁻¹ for 3 days, whereas the squalene itself and its monoepoxide derivative were reported to have no similar effect at a dosage of 40 mg/kg for 3 days. In another highly cited paper from the same group (795), intravenous administration of 2.5 mg/kg 25-OH-Chol or 5α,6β-diOH-Chol to NZW rabbits was reported to induce changes in the luminal surface of aorta as studied by scanning or transmission electron microscopy. In view of the frequent citation of this work as evidence indicating the atherogenic nature of oxygenated sterols, consideration of some details of this experimentation is warranted. Each of the two oxygenated sterols was dissolved in ethanol (0.3 ml) and added to a serum sample (3 ml) obtained from the individual rabbits and, after shaking for 2 h (temperature not specified), the serum containing the ethanol and the oxygenated sterol was infused into an ear vein. At 24 h after administration of the sterols, the animals were prepared for electron microscopic examination of their aortas. The source and purity of the oxygenated sterols were not provided. The amounts of the sterols injected were fairly substantial. The rabbits were reported to weigh 3.0–4.0 kg. At 2.5 mg/kg, the sterol dose amounted to 7.5–10 mg. The nature of the mixture obtained after adding 10 mg of the respective sterol (in 0.3 ml of ethanol) to 3 ml of serum would be of interest and considerable potential importance in view of the limited solubilities of the oxysterols and the relatively high amounts of ethanol added to the serum. The electron microscopic findings reported were related to control animals that apparently received serum to which only ethanol was added. The major electron microscopic findings were increased frequencies of balloonlike lesions and craterlike lesions (assumed to result from rupture of the balloonlike protrusions) on the intimal surface of the aortas. The frequency of these lesions was significantly higher in the oxysterol-treated groups than in the ethanol-control group and higher in the animals treated with the triol than those treated with the 25-hydroxysterol.

Toda et al. (1117) reported that “force feeding” of 7-keto-Chol (10 mg/day for 2 wk, 20 mg/day thereafter) as an emulsion in corn oil to female chicks for 4 or 8 wk was associated with evidence of smooth muscle degeneration in the abdominal aorta. Little changes were observed in the ascending aorta. Feeding of a high-Chol (1% in diet) diet did not result in changes in the abdominal aorta similar to those in the 7-keto-Chol-treated birds. Jacobson et al. (433) reported on the effects of administration of low levels of “pure” Chol or pure Chol plus trace levels of 5α,6β-dioH-Chol to White Carneau pigeons maintained on a commercial mixed grain diet (composition not specified) supplemented with brewer’s yeast (amount and composition not specified). The Chol was of commercial origin and was reported to be “oxide-free (<0.05%) by thin-layer chromatography.” The source, identity, and purity of the cholestanetriol were not presented. The sterols, either Chol (52 mg) or Chol (52 mg) plus 5α,6β-dioH-Chol (0.16 mg), were administered in a mixture of olive oil containing ethanol (1%) by gavage three times per week. There were no differences between the two groups at 0, 5, 9, and 14 wk with regard to the levels of total plasma Chol or HDL Chol. After 3 mo, there were no differences in the levels of Chol or Chol esters in the aortas. The level of calcium in the aortas of animals receiving the triol was higher (1.16 ± 0.35 mg/g) than in aortas of animals receiving Chol alone (0.82 ± 0.27 mg/g). The percent lumen stenosis in coronary arteries was reported to be 5.23 ± 5.4% in the animals receiving the triol as compared with 1.01 ± 0.14% in the animals receiving Chol alone. The percent lumen stenosis was calculated as the percent of the area within the internal elastic lamina that was occupied by intimal lesion. Matthias et al. (625) reported that intragastric administration of 5α,6β-dioH-Chol in olive oil to male Wistar rats led to the development of microscopic changes in the aorta. Evidence for the identity and purity of the sterol was not presented. Individual experiments involved relatively small numbers of treated animals and controls (n = 3 in the only experiment with control rats). Detailed morphometric data were not presented. The dosage and duration of administration of the oxysterol varied. In some experiments, dosage was varied within a given experiment.
In contrast to the above studies, Higley et al. (387) reported that a mixture of oxidation products of Chol was markedly less atherogenic in rabbits than was Chol. Chol used in this study was commercial-reagent grade Chol that was then recrystallized twice from ethanol and acetone. The resulting sample was estimated to have a purity of >99.9% on the basis of TLC and HPLC analyses. An oxidized Chol sample was prepared by treatment of Chol in toluene with a stream of dry air at 110°C for 64 h. The crude oxygenated sterol product was subjected to preparative HPLC to remove free Chol. The oxygenated sterol mixture was reported to have the following composition (based on HPLC analysis): Chol, 0.06%; 25-OH-Chol, 2.0%; 7-keto-Chol, 26.0%; 7α-OH-Chol, 4.5%; 7β-OH-Chol, 5.3%; Chol 7α-hydroperoxide, 18.2%; 5α,6α-epoxy-Chol, 24.0%; 5β,6β-epoxy-Chol, 17.0% and unknown, 2.9%. The purified Chol and the oxygenated Chol mixture were, after heating in corn oil, added to a semipurified rabbit diet to give a diet containing 2% corn oil by weight. Control animals received the diet containing added corn oil (2%). Experimental animals received the diets corresponding to a dosage of 166 mg/kg z 1 day z 5 for either purified Chol or the oxygenated Chol mixture. No results were presented with regard to levels of Chol or oxysterols in blood. After administration of the diets for 11 wk, the animals (n = 5 in each group except for one death in the control group) were evaluated with regard to severity of atherosclerosis. The Chol-fed animals were reported to show a much larger number (5-fold) of arterial lesions than the groups receiving the oxysterol mixture. In addition, the results of microscopic examination of the arterial lesions were reported to demonstrate a significantly greater magnitude of lesions in the Chol-fed group relative to the animals treated with the oxygenated Chol mixture. Griminger and Fisher (350) studied the effects of administration of diets containing either spray-dried egg powder or fresh egg yolks on the development of atherosclerosis in young male chickens with the assumption that egg powder, but not the fresh egg yolks, contains significant amounts of oxidation products of Chol. Plasma Chol levels in the two experimental groups did not differ significantly at 30 and 43 wk. However, aortic atherosclerosis, as judged by visual and histological examination, was lower in the birds fed the spray-dried egg powder. A limitation of this study was the absence of any analysis of the levels of oxysterols in the spray-dried egg powder and the fresh egg yolks. Tipton et al. (1115) reported that the addition of a mixture of Chol oxidation products (50 mg/day) to a Chol-rich (1%) diet reduced atherosclerotic lesion formation induced by Chol feeding in rabbits. Administration of the oxidized Chol sample had no effect on the level of plasma Chol in the Chol-fed rabbits. Treatment of the oxidized Chol sample with NaI before feeding was reported to abolish the inhibitory effect of the oxygenated Chol sample on the lesion development in the Chol-fed rabbits. Administration of the oxidized Chol sample alone was reported to have "no obvious effects." This study had several limitations. The source and purity of the Chol used in the feeding experiments was not stated. The Chol (and the oxygenated sterol mixture) was added to rabbit chow pellets in ethyl ether solution, which was stated to have been allowed to evaporate under a stream of nitrogen. The conditions of storage of the diets (administered over 61 or 56 days) were not presented. No analyses of the oxysterols in the various prepared diets were presented. Finally, the experiments involved small numbers of animals (n = 3 for controls, n = 4 for experiments).

In 1998, Staprans et al. (1036) reported that oxidized Chol accelerated the development of atherosclerotic lesions in rabbits fed a Chol-enriched diet. NZW rabbits were fed (for 12 wk) either a diet containing 0.33% Chol (n = 13) or the same diet in which ~5% of the Chol was oxidized (n = 13). The Chol oxidation products were listed as containing the following: 7-keto-Chol, 42%; 7β-OH-Chol, 20%; 7α-OH-Chol, 7%; 5β,6β-epoxy-Chol, 16%; 5α,6α-epoxy-Chol, 12%; 25-OH-Chol, 3%; and unidentified, 48%. After feeding the two diets for 12 wk, the percentage lesion areas in aorta were 28.5 ± 4.9 and 57.1 ± 4.8% for the control and oxidized Chol groups, respectively. At the end of the experiments there were no significant differences in the levels of total serum Chol, β-VLDL Chol, LDL Chol, and HDL Chol in the two groups. The levels of 7α-OH-Chol, 7β-OH-Chol, and 5α,6α-epoxy-Chol (but not 5β,6β-epoxy-Chol) in fasting serum β-VLDL were reported to be significantly higher in the oxidized Chol group. No significant differences were reported for 7β-OH-Chol, 5α,6α-epoxy-Chol, 5β,6β-epoxy-Chol, and 7-keto-Chol in fasting serum LDL in the two groups. The levels of 7β-OH-Chol, 5β,6β-epoxy-Chol, and 7-keto-Chol (but not 7α-OH-Chol and 5α,6α-epoxy-Chol) in liver were reported to be significantly higher in the oxidized Chol group. It is important to note that quite high levels of the oxysterols were reported for β-VLDL, LDL, and liver from the control animals. This was interpreted by the authors as indicating their origin from endogenous production in the animals. The authors reported that the control diet “contained no detectable oxidized Chol during the feeding period.” No results were presented to preclude the possibility of artifactual generation of oxysterols from Chol during sample processing. The oxysterol levels noted above presumably reflect total oxysterol levels since the report indicates a saponification step. The cited method for oxysterol determination was that of Hughes et al. (410), in which retention time data were given for only 1% of the oxysterol mixture. The conditions of storage of the diets (administered over 61 or 56 days) were not presented. No analyses of the oxysterols in the various prepared diets were presented. Finally, the experiments involved small numbers of animals (n = 3 for controls, n = 4 for experiments).
Erosclerotic arteries is clearly warranted. Special emphasis on early stages, i.e., arteries with fatty streaks, is indicated, along with a comparison with the oxysterol composition of advanced lesions. The results of studies in humans have pointed to the presence of low levels of oxysterols in human atherosclerotic lesions. These include 26-OH-Chol, 7-keto-Chol, 7α-OH-Chol, 7β-OH-Chol, 25-OH-Chol, 4β-OH-Chol, 4α-OH-Chol, 5α,6β-diOH-Chol, and cholesta-3,5-dien-7-one (66, 84, 130, 133, 135, 160, 161, 319, 476, 1011, 1157). The latter is a known product of the decomposition of 7-keto-Chol.

26-OH-Chol has been found in normal and atherosclerotic aorta of human subjects (133, 135, 1157), and two groups (319, 1012) have reported that the levels in atherosclerotic aorta increase with severity of the disease. Using analyses of pooled samples, one group reported that the levels in normal intima of young and adult males were 13 and 17 μg/g dry wt, whereas fatty streaks and atheroma of adult males contained 301 and 494 μg/g tissue (133, 135, 1157). The latter is a known product of the decomposition of 7-keto-Chol.

In 1971, Brooks et al. (133) reported the presence of the following oxysterols (in a sterol ester fraction) in human atherosclerotic plaques obtained “within 24 h postmortem”: 7α-OH-Chol, 7β-OH-Chol, 24-OH-Chol, and 26-OH-Chol. Mass spectra of the TMS ether derivatives of the latter three oxysterols were presented. GC retention time data were presented for the TMS ether derivatives of the four oxysterols on SE-30, OV-17, and QF-1 columns. No quantitative data were presented. The oxysterols were suggested to be present as monoesters. In 1975 Teng and Smith (1107) reported the presence of 24-OH-Chol in human aortic intima. These workers were unable to detect the presence of the free sterol by GC at the level of 1 μg/g tissue. However, significant levels of 24-OH-Chol (albeit considerably lower than that of free 26-OH-Chol or its fatty acid esters) were recovered after saponification of sterol ester fractions obtained by column chromatography and TLC. The 24-OH-Chol was characterized by GC of the free sterol and its TMS derivative and by IR (not presented). The levels of the 24-OH-Chol present in aortae from normal rabbits and rabbits with atherosclerotic arteries induced by cholesterol feeding, nor- epinephrine administration, or the administration of nor- epinephrine plus thyroxine. The authors noted that “both 7-keto-Chol and 25-OH-Chol may be artifacts of autoxidation during sample preparation.” It is also noteworthy that while quite high levels of 26-OH-Chol were observed in human atherosclerotic arteries, Smith and Pandya (1007) reported in 1973 that analysis of other human organs (heart, kidney, liver, lung, muscle, pancreas, spleen, and adipose tissue) did not show detectable levels of 26-OH-Chol. However, it should be noted that the sensitivity of the methods used was considerably less than that used in subsequent studies by others.

In 1971, Brooks et al. (133) reported the presence of...
consistently high values for 26-OH-Chol and its reported increase with increasing severity of atherosclerosis. 26-OH-Chol is generally considered not to originate by autoxidation of Chol. In the limited studies reported to date (see sect. IV) 26-OH-Chol was not detected in samples of oxidized LDL or in samples of MM-LDL. The oxysterols, reported to date, in modified LDL or MM-LDL, include only those sterols known to arise from autoxidation of Chol and do not include 26-OH-Chol, the predominant oxysterol found in plasma of normal human subjects. Again, if oxidized LDL or MM-LDL is important in the pathogenesis of atherosclerosis and if certain oxysterols are important components of oxidized LDL and MM-LDL, detailed studies of oxysterols in normal and atherosclerotic lesions are clearly indicated. In addition, the results from the previous studies of atherosclerotic lesions indicate a different type of oxysterol than that reported so far in oxidized LDL, a situation which supports further studies of all oxysterols, including 26-OH-Chol and 24-OH-Chol. Moreover, these studies should concentrate on comparisons of normal artery with arteries in the early stages of atherosclerosis. Separate analyses of intima and media are indicated.

Salonen et al. (890) recently reported on the levels of 7β-OH-Chol in serum in human subjects (none with a history of myocardial infarction) classified as with either “fast progression” or “slow or no progression” of carotid artery atherosclerosis. Levels of 7β-OH-Chol in the two groups were reported as 0.079 ± 0.005 μM (n = 20) and 0.070 ± 0.001 μM (n = 20), respectively. Whereas these values did not differ significantly (P = 0.087), the levels of 7β-OH-Chol in serum were reported to be significantly associated with progression of carotid atherosclerosis. The mean levels of 7β-OH-Chol in the serum samples of both groups were higher than those reported previously from the same laboratory for normal human subjects, i.e., 0.008 ± 0.012 μM (n = 31) (273) and <0.0007 μM (n = 8) (121). In a closely related study, van Poppel et al. (1160) reported on the levels of a number of unesterified oxysterols in the plasma of subjects with severe coronary atherosclerosis (“>80% stenosis in one of the major coronary vessels”; n = 80) and controls (“no or minor stenosis”; “<50% stenosis in all three major coronary vessels”; n = 79). The mean levels (± SD) of the unesterified oxysterols in plasma from the controls were reported as follows: 7α-OH-Chol, 0.032 ± 0.017 μM; 7β-OH-Chol, 0.0035 ± 0.015 μM; 7-keto-Chol, 0.0033 ± 0.018 μM; 5α,6β-epoxy-Chol, 0.0037 ± 0.0017 μM; 5α,6α-epoxy-Chol, 0.0033 ± 0.0033 μM; and 25-OH-Chol, 0.0015 ± 0.0007 μM. The mean values for the subjects with severe coronary atherosclerosis were essentially the same as those for the controls with exception of 7α-OH-Chol, where the mean value for the severely atherosclerotic subjects (0.038 ± 0.023 μM) was reported to be significantly (P < 0.05) higher than the value for controls (0.032 ± 0.017 μM). No significant association between the levels of any oxysterol and the degree of coronary stenosis was observed.

Among a large number of studies, reviewed elsewhere by others (156, 252, 372, 1041, 1042, 1044), in support of the hypothesis that oxidative modification of LDL may be important in the pathogenesis of atherosclerosis are studies indicating antiatherosclerotic effects of the administration of antioxidants to animals. Several groups have reported that probucol inhibits the development of atherosclerosis in WHHL rabbits (119, 157, 240, 496, 689) and that the administration of probucol (242) or BHT (91) to Chol-fed NZW rabbits suppresses the development of atherosclerosis. Of particular relevance was the report in the latter study (91) that administration of BHT resulted in decreased levels of unesterified 7-keto-Chol and unesterified 5α,6α-epoxy-Chol in plasma. However, this study did not include other important oxysterols, nor did it include free plus esterified oxysterols, nor did it involve controls to detect and quantitate artifactual generation of oxysterols during sample processing and analysis. Probucol has also been reported to inhibit the formation of atherosclerotic lesions in a nonhuman primate fed a high-Chol high-fat diet (896) and to reverse xanthomases in FH subjects (1210). Administration of probucol has been reported to result in significant prevention of restenosis after coronary angioplasty (1096). However, in one study (1040), probucol did not inhibit the development of atherosclerosis in Chol-fed rabbits when compared with controls with comparable levels of plasma Chol. In one clinical trial (1176), probucol treatment for 3 years was reported to be without effect on femoral artery atherosclerosis as judged by quantitative arteriography. In addition, the results of a very recent study (1227) of apoE-deficient mice indicated that treatment with probucol, while lowering plasma Chol levels, was associated with an enhancement of the development of atherosclerosis. Unexpectedly, more rapid development of aortic atherosclerotic lesions occurred in apoE−/− mice treated with probucol relative to untreated apoE−/− controls. The lesions in the probucol-treated animals were considerably larger and more mature. Furthermore, in another recent study (685), no beneficial effect of either vitamin E acetate or BHT on aortic fatty streak formation was observed in C57BL/6 mice fed a diet containing 1% Chol and 0.5% cholic acid for 15 wk. In fact, the animals fed BHT developed a significantly larger area of aortic fatty streak lesions than the control animals. However, a very recent study with LDL receptor−/− mice found that administration of the combination of vitamin E (0.1%), β-carotene (0.05%), and ascorbic acid (0.05%) inhibited the development of fatty streaks in animals fed a high-fat, high-Chol diet (220).
currence and levels of individual oxysterols in aortas of control and Chol-fed (1% in diet for 6 wk) NZW rabbits. Extremely high levels of oxysterols were reported for both control and Chol-fed animals, with mean values of "total cholesterol oxides" of 239 and 600 µg/g tissue (presumably wet weight), respectively. The mean level of the total oxides of Chol in the aortas of control animals was ~12% of the mean level of Chol. The same individual oxysterols (all known autoxidation products of Chol) were also found in this study at comparable levels in plasma (see sect. IV.A). As noted in section IV.A, the results in both the control and Chol-fed animals must be considered extremely cautiously in view of the methodology applied. In another study (388), the same laboratory reported lower levels of Chol and of individual oxysterols (7a-OH-Chol, 7b-OH-Chol, 5a,6β-epoxy-Chol, 5β,6β-epoxy-Chol, and 5α,6β-diOH-Chol) in aortic samples from Chol-fed NZW rabbits treated with probucol.

Crisby et al. (225) studied the levels of 26-OH-Chol in atherosclerotic aortas obtained from male NZW rabbits (3.0 kg) fed 1% Chol in laboratory chow diet for an unspecified period of time. 26-OH-Chol was reported to represent 5.5 ± 0.6% (SE; n = 6) of the oxysterols detected. The mean level of 26-OH-Chol in the atherosclerotic aortas was reported as 5 ng/mg tissue or 0.1 ng/mg Chol. No expression of variation was presented. The mean value for 26-OH-Chol in aortas of control rabbits was reported to be 0.2 ng/mg tissue or 0.1 ng/mg Chol. In 1997, some of the same workers (316) reported on the levels of 2 classes of oxygenated sterols in atheromas from male NZW rabbits (3.0 kg) fed a Chol-rich (1%) diet for 10 wk. Analyses of the oxysterols were made by GC-MS (273). Results were presented as "7 oxygenated sterols" (combination of 7α-OH-Chol, 7β-OH-Chol, and 7-keto-Chol) and "5,6 oxygenated sterols" (5α,6α-epoxy-Chol, 5β,6β-epoxy-Chol, and 5α,6β-diOH-Chol). The levels of 7 oxygenated sterols and 5,6 oxygenated sterols were reported to be 0.09 ± 0.05 and 0.64 ± 0.15 µg/mg Chol, respectively. Ascorbic acid administration (500 mg/day in drinking water) was reported to have no effect on the atheroma or on the levels of oxysterols in atheroma.

Chisholm et al. (201) reported the presence of 7β-hydroperoxycholest-5-en-3β-ol in freshly excised atherosclerotic lesions obtained from human subjects undergoing carotid endarterectomy. The 7β-hydroperoxide was characterized only by TLC and/or HPLC. Although an internal standard of labeled Chol was not employed to detect or quantitate oxidation of Chol during storage or sample processing, the authors reported no formation of Chol autoxidation products in a separate experiment in which [14C]Chol was injected into the lesion before sample processing. In the same study, Chisholm et al. (201) reported that 7β-hydroperoxycholest-5-en-3β-ol was the major cytotoxic oxysterol present in LDL subjected to oxidation with Cu²⁺.

Carpenter et al. (160) noted significant levels of 26-OH-Chol in severe human aortic atheroma. They studied the oxysterols of human aortic atheroma (the "necrotic gruel from the interior of advanced atherosclerotic plaques") and of lesion-free intima plus inner media of human normal aortas. In contrast to some previous human studies, the analyses were based on individual samples and not on pooled material. There was a significant time between time of death and necropsy (8–127 h for cases with atheroma and 5–96 h for normal aortas). The samples were transported to the laboratory within 1 h, where they were stored at -20°C under nitrogen until analysis. The frozen samples were thawed, weighed (typically 5–10 mg), and chopped with a scalpel. "Care was taken to minimize exposure of the samples to air." After the addition of internal standards (heptadecanoic acid, coprostone, and cholestanol along with 50 µg BHT), the samples were extracted with 2:1 CHCl₃-methanol with vigorous vortexing and sonication (without mention of concern about autoxidation). The material in the organic extract was processed according to previously described procedures from the same laboratory (159) that involved borohydride reduction, saponification, and formation of TMS ether derivatives. "Care was taken throughout to minimise exposure of samples to air, and sodium borohydride reduction, saponification, derivatisation and storage of samples were all under nitrogen." Standards for GC and GC-MS were apparently limited to 26-OH-Chol, 25-OH-Chol, and cholestan-3β,5α-diol. Three oxysterols, 26-OH-Chol, 7β-OH-Chol, and cholestan-3β,5α-diol, were reported to be present in all of the atheroma samples, and at notable levels (i.e., 1.41, 0.27, and 5.16 mg/g tissue, respectively). Identifications were based on retention times of the TMS ether derivatives and GC-MS results (which were not presented). The authors regarded the cholestan-3β,5α-diol to be an artifact; however, determination of its origin was not demonstrated. The same three oxysterols were reported to be present in advanced aortic atheroma at even higher levels. The analyses described were limited to 26-OH-Chol and 7β-OH-Chol (some of which was probably formed from 7-keto-Chol or from the 7-hydroperoxide upon reduction with borohydride). The cholestan-3β,5α-diol (and another component believed to be a cholestanediol) possibly resulted from hydride reduction of the 5,6-epoxide of Chol. The lack of fresh samples also presents a limitation in this study. In 1995, Carpenter et al. (161) extended their studies on oxysterols in atherosclerotic lesions. Methodologies were essentially the same as those reported previously (160) [i.e., "Bligh and Dyer extraction (with sonication), sodium borohydride reduction, saponification, and conversion to TMS ethers]. However, data were presented only for two oxysterols, i.e., 7β-OH-Chol and 26-OH-Chol (which were determined by capillary GC with, in some cases, confirmation of identity by GC-MS). The study involved analysis of
individual arterial samples from 94 subjects. Samples were taken at various times after death (from 11 to 138 h). This study involved analyses of sterols from normal arterial tissue samples and from individual lesions which, in this study, were classified into subgroups, e.g., macrophage rich and fibrous. In normal arterial tissue samples, very low or nondetectable levels of 7\(\beta\)-OH-Chol and 26-OH-Chol were reported. The concentrations of the oxysterols (and of Chol) varied with the severity of the lesions, with higher levels in macrophage-rich lesions than in fibrous lesions.

Björkhem et al. (84) reported the presence of 26-OH-Chol in five specimens of human atherosclerotic femoral arteries obtained at surgery and frozen in liquid nitrogen. The frozen material was extracted, saponified (15 h at room temperature under argon), extracted, and subjected to solid-phase extraction followed by GC-MF using deuterium-labeled internal standards. Total oxysterols were reported as 6.6 ± 30 ng/\text{µg} tissue, with a Chol content of 5.4 ± 1.4 \text{µg/µg} tissue. Thus total oxysterols represented 0.12% of total sterol. 26-OH-Chol and 7-keto-Chol were the major oxysterols. The mean values for the various oxysterols were as follows (expressed as a percentage of total oxysterols): 26-OH-Chol, 28; 25-OH-Chol, 2; 24-OH-Chol, 1; 7\(\alpha\)-OH-Chol, 12; 7\(\beta\)-OH-Chol, 14; 7-keto-Chol, 24; 5\(\alpha\),6\(\alpha\)-epoxy-Chol, 5; 5\(\beta\),6\(\beta\)-epoxy-Chol, 5; and 5\(\alpha\),6\(\beta\)-diOH-Chol, 5.

Suarna et al. (1059) reported the presence of 7-keto-Chol (free and esterified) in advanced human atherosclerotic lesions in arteries removed surgically. The 7-keto-Chol was not detected in normal artery samples. The free-7-keto-Chol was reported to be present at a level of 0.6 ± 0.4 (SD) mmol 7-keto-Chol/mol free Chol (\(n\) = 11). The identification and quantification of the 7-keto-Chol (and its esters) was based solely on reverse-phase HPLC. The presence of other oxysterols was not mentioned. It should be noted that with the reverse-phase HPLC method employed (516), 7-keto-Chol elutes quite quickly from the column along with other oxysterols (516). Breuer et al. (124) reported the occurrence of 4\(\beta\)-OH-Chol and 4\(\alpha\)-OH-Chol in atherosclerotic tissue from five human subjects. The levels of the two 4-OH-Chols were about the same, i.e., from \(-1\) to 2 \text{µg/g tissue} (or \(-2.5\)–5 \text{nmol/g tissue}). It was stated that the 4-hydroxysterols “made up between 1.0 and 1.8% of total oxysterols in the plaques.” The 4-OH-Chols were also reported to be present in normal human vessel walls, but at \(-10\%\) of the levels found in atherosclerotic tissue. The methodology described would measure only the sterols in the unesterified form. Crisby et al. (225) reported on the levels of oxysterols in atherosclerotic plaques from carotid arteries of human subjects. Their results, using GC-MS methodology, also included values for control vessels. Higher levels (\text{µg/g tissue}) in the atherosclerotic lesions were reported for each of the following oxysterols: 7\(\alpha\)-OH-Chol, 7\(\beta\)-OH-Chol, 7-keto-Chol, 5\(\alpha\),6\(\alpha\)-epoxy-Chol, 5\(\beta\),6\(\beta\)-epoxy-Chol, 5\(\alpha\),6\(\beta\)-diOH-Chol, 24-OH-Chol, 25-OH-Chol, and 26-OH-Chol. Very high levels of 26-OH-Chol (273 \text{µg/g tissue vs. 0.09 µg/g tissue in control vessels}) were observed.

Mattsson Hultén et al. (626) presented very interesting results on the oxysterols present in arterial macrophage-derived foam cells from Chol-fed NZW rabbits. The rabbits were fed a high-Chol diet (1% in rabbit chow for 12 wk). The source and purity of the Chol were not provided, nor was the manner of addition of the Chol to the chow diet. The rabbit macrophages were isolated after incubation with “rabbit antiphotocytolytic antibody.” The cells “were kept in PBS with BHT and stored at \(-20^\circ\text{C}\) until analysis of the oxysterols.” The oxysterols were analyzed, after mild saponification, by GC-MS methods described previously by the same laboratory (122). The results from the rabbit arterial macrophage-derived foam cells showed notable levels of certain oxysterols, i.e., 7\(\alpha\)-OH-Chol, 7\(\beta\)-OH-Chol, 7-keto-Chol, 5\(\alpha\),6\(\beta\)-epoxy-Chol, and 5\(\alpha\),6\(\alpha\)-epoxy-Chol. Considerably lower levels were observed for 5\(\alpha\),6\(\beta\)-diOH-Chol and 25-OH-Chol. None or negligible levels of 26-OH-Chol and 24-OH-Chol were observed. The same authors (626) also presented results of analyses of the oxysterols present in human arterial CD14\(^+\) macrophages from atherosclerotic samples obtained at the time of surgery. The macrophages were isolated after incubation with an antibody that detects the CD14 antigen. A “total foam cell preparation” was isolated by density gradient centrifugation. The oxysterol levels in the human macrophages and foam cells were markedly different from those observed in the rabbit macrophages. 26-OH-Chol was present in high amounts in the human cells. The major oxysterol in the human cells was 26-OH-Chol (in 2 of 3 preparations), which was accompanied by lower levels of 7-oxygenated and 5,6-oxygenated sterols.

Reiss et al. (842) reported the formation of 26-OH-Chol with bovine aortic endothelial cells. Incubation of the aortic cells showed a time-dependent increase in the levels of 26-OH-Chol in the medium at 3, 6, and 24 h. Identification of the product was based on GC-MS analysis of the diacetate derivative. The configuration at C-25 was not studied. In addition to the 26-OH-Chol, 3\(\beta\)-hydroxycholest-5-en-26-oic acid was also detected in the media from incubations of the bovine aortic cells. The C-27 acid was characterized by GC-MS of the methyl ester of its 3-acetate derivative. The presence of the 26-hydroxylation in the mitochondria of the bovine aortic endothelial cells was detected by Western blot analysis. Björkhem et al. (84) also reported evidence indicating the formation of 26-OH-Chol and 3\(\beta\)-hydroxycholest-5-en-26-oic acid in human alveolar macrophages and in human endothelial cells from umbilical veins. The endothelial cells were reported to be considerably less active in this respect than the pulmonary macrophages. Crisby et al. (225), using antibodies to the 26-hydroxylation, studied the immunoreactive cells of hu-
man carotid artery atherosclerotic plaques. Most of the immunoreactive cells of the lesions were reported to be macrophages. Immunoreactivity was more prominent in macrophage-rich lesions than in fibrous plaques. Most endothelial cells were reported to be positive for 26-hydroxylase immunoreactivity.

As noted previously, 26-OH-Chol has been reported to be present in atherosclerotic lesions and to increase with the severity of the lesions. Cyclosporin, an inhibitor of the conversion of Chol to 26-OH-Chol, is used for immunosuppression in kidney and heart transplantation. Whether or not the levels of 26-OH-Chol increase with the severity of the lesions in transplantation atherosclerosis, as observed in atherosclerotic lesions of subjects without immunosuppression therapy, is not known. The development of transplant arteriosclerosis is a major complication after heart transplantation. Cyclosporin treatment has been shown to have at least one potentially adverse effect with respect to the development of atherosclerosis. Ballantyne et al. (47), in a study in patients with amyotrophic lateral sclerosis, have shown that treatment with cyclosporin resulted in significant increases in plasma total Chol and LDL Chol. However, the results of a recent study (9) have indicated a significant inhibition of the development of transplant arteriosclerosis by cyclosporin in aorta-allografted rabbits with careful control of plasma Chol levels. Moreover, another study (8) indicated that cyclosporin administration to aorta-transplanted rabbits fed a diet without Chol supplementation suppressed the markedly increased permeability of the transplanted aorta to LDL. One study (398) has indicated an increased level of oxidized LDL in plasma in patients with transplant-associated atherosclerosis. Studies of oxysterol levels in these patients were not made. Administration of another immunosuppressant, FK506, has been found to increase the development of atherosclerotic lesions in Chol-fed NZW rabbits without affecting plasma lipid levels (624).

Liu et al. (580) recently reported that incubation of rabbit aortic smooth muscle cells with Chol oxidase led to the formation of cholest-4-en-3-one and 20α-OH-Chol, which appeared to be dependent on the concentration of Chol oxidase and time of incubation of the cells with the enzyme. Identification of the two sterols was limited to HPLC analysis for which full details were not presented. The identification of 20α-OH-Chol is particularly worthy of further study, since its formation from Chol under the conditions studied is unanticipated and the data presented were not adequate for assignment of structure. A subsequent publication (836) from the same laboratory presented an HPLC analysis that was interpreted as indicating the formation of 20α-OH-Chol and 25-OH-Chol upon incubation of rat liver nuclei with Chol oxidase. The chromatogram presented provides no basis of assignment of structure to the oxysterols. In fact, the methodology employed does not provide a basis for establishing that the compounds were sterol in nature.

A recent study by Devaraj et al. (250) suggested a possible role of remnantlike particles in the pathogenesis of atherosclerosis. They found that the levels of remnantlike particle Chol were significantly higher in men with coronary artery disease than in controls and that normolipidemic men with coronary artery disease have increased levels of remnantlike particle Chol that would not be detected by conventional lipid screening. In another recent study (824), the intimal retention of fluorescent-labeled chylomicron remnants was demonstrated after perfusion of rabbit carotid artery segments. If chylomicrons and/or chylomicron remnants are indeed atherogenic and the results of studies of the absorption, transport, and metabolism of 3β-hydroxy-5α-cholestone-8(14)-en-15-one (912) apply also to other oxysterols, then the possible high levels of oxysterols in chylomicrons (and chylomicron remnants) after ingestion of the oxysterols could conceivably be of relevance to the pathogenesis of atherosclerosis. Furthermore, smoking could affect the early metabolism of oxygenated sterols after their absorption in the small intestine, since Axelsen et al. (29) and Mero et al. (644) reported that smokers show postprandial elevations of triglycerides and triglyceride-rich particles in plasma. It is conceivable that smoking might delay the clearance of the oxysterol-rich chylomicrons after the intestinal absorption of the oxysterols.

As noted previously, Watson et al. (1190) have presented evidence suggesting that 25-OH-Chol might play a role in the development of calcified lesions observed in arteriosclerosis. With the introduction of new or improved imaging techniques, the demonstration of calcium deposition in arterial lesions has assumed increasing importance in medicine. These techniques have indicated mineral deposits in 90% of patients with coronary artery disease (4, 399) and even in very young subjects with FH (391). The presence of significant calcification may be indicative of an unfavorable prognosis due to increased tendency for plaque rupture. Recently, a special subpopulation of arterial cells that resemble osteoblasts has been identified (107, 1190). These cells, designated as calcifying vascular cells, showed high levels of markers associated with bone formation, e.g., alkaline phosphatase (AP) and collagen I, expression of osteonectin and osteopontin, and the production of bone-specific hydroxyapatite and osteocalcin (1190). Watson et al. (1190) reported that 25-OH-Chol (2.5 μM) stimulated the osteoblast-like cells to calcify. 25-OH-Chol markedly stimulated calcified nodule formation in six of six clones studied (1190). However, it should be noted that other oxysterols might be of much higher physiological relevance in this regard than 25-OH-Chol. For example, as noted previously in this review, 25-OH-Chol is not a major oxysterol present in plasma (or LDL) of human subjects (Tables 1 and 2) or in atheroscle-
rotic lesions. As noted above, high levels of 26-OH-Chol in atherosclerotic lesions have been found in numerous studies, and the levels of this oxysterol in the lesions have been reported to increase with the severity of the disease. Significant levels of other oxysterols (7-keto-Chol, 7β-OH-Chol, 7α-OH-Chol, 5α,6α-epoxy-Chol, 5β,6β-epoxy-Chol, and 5α,6β-diOH-Chol) have been reported in oxidized LDL and in human atherosclerotic lesions; however, their occurrence due to artificial generation from Chol cannot, in many cases, be excluded. The results of preliminary studies with Drs. Linda Demer and Simon Jackson (unpublished data) involving 40 oxygenated sterols of defined structure and purity, have indicated that several side chain-oxygenated sterols (including 26-OH-Chol) induced high levels of AP in the calcifying vascular cells. In contrast, oxysterols related to oxidized LDL or peroxidative metabolism of Chol were considerably different. For example, 7α-OH-Chol, 5β,6β-epoxy-Chol, 5α,6a-epoxy-Chol, 5α,6β-diOH-Chol, and 4β-OH-Chol showed little or no induction of AP, and 7-keto-Chol and 7β-OH-Chol caused only moderate increases in AP. Preliminary studies of Ca\(^{2+}\) uptake into the vascular cells have indicated very marked stimulation by (25R)-26-OH-Chol and essentially no effect with 7-keto-Chol and 7α-OH-Chol. The high potency of 26-OH-Chol in AP induction and Ca\(^{2+}\) uptake correlates with the high levels of this oxysterol in atherosclerotic lesions (and the severity of the lesions). The low potencies of oxysterols of the type reported for oxidized LDL are notable.

Daugherty et al. (241) reported findings of potential importance with regard to the formation of oxygenated sterols in arteries and in the pathogenesis of atherosclerosis. These authors reported the presence of myeloperoxidase in human atherosclerotic arteries. Immunoreactive myeloperoxidase was observed in detergent extracts of 14 of 14 atherosclerotic arteries through the use of a rabbit polyclonal monoclonal antibody for the enzyme. Moreover, the immunoreactive species showed similar behavior as myeloperoxidase on lectin column chromatography and on high-resolution nondenaturing size exclusion chromatography. In other experiments, peroxidase activity was reported to be present in six of nine atherosclerotic lesions and absent in three grossly normal arteries. The peroxidase recovered after lectin chromatography was shown to generate HOCl. The authors also reported on the localization of myeloperoxidase in frozen sections of atherosclerotic lesions. Intense perinuclear immunoreactivity was observed in macrophage-rich regions. Furthermore, the localization of myeloperoxidase in macrophages was indicated by the close distribution of immunostaining for myeloperoxidase with that for macrophages. As noted previously, Heinecke et al. (380) have reported the in vitro formation of halohydrins and epoxides upon incubation of Chol, incorporated into multilammar vesicles containing phosphatidylcholine, with myeloperoxidase. At the present time, no information is available on the occurrence of the sterol chlorohydrins in atherosclerotic lesions or in LDL modified by oxidation.

Myeloperoxidase may prove to be important in the pathogenesis of atherosclerosis or in defensive responses. The sterol halohydrins could provide reactive species that may play roles in these matters. It should be recognized that HOCl formation can induce major changes not only in sterols but also in fatty acids and proteins. Two recent studies in 1997 (1212, 1213) concerned the extensive oxidative modifications of the proteins of LDL by in vitro incubation with hypochlorite. In addition, Hazen and Heinecke (379) have recently reported the chlorination of tyrosine residues of human LDL upon its incubation for 1 h at 37°C with myeloperoxidase in the presence of NaCl (100 mM) and \(H_2O_2\) (100 \(\mu M\)) or with HOCl (100 \(\mu M\)) (in the absence of myeloperoxidase). Under the conditions studied, the extent of formation of 3-chlorotyrosine (\(\mu mol/mol\) l-tyrosine) was 230 under the former condition and 310 under the latter condition. Further analyses of LDL isolated from normal and atherosclerotic articular tissue from human subjects indicated an increased level of 3-chlorotyrosine in the atherosclerotic tissue, with a ratio of 3-chlorotyrosine to tyrosine (\(\mu mol/mol\)) of 424 ± 26 for the atherosclerotic tissue and 76.6 ± 6.6 for the normal tissue. The level of 3-chlorotyrosine in LDL of atherosclerotic tissue was ~30 times higher than that of LDL of plasma. The combined findings were interpreted as providing further evidence suggesting a possible important role of myeloperoxidase in the oxidation of LDL and the pathogenesis of atherosclerosis. In this connection it is interesting to note a very recent report of Santanam et al. (894) which indicated that the in vitro oxidation of LDL by myeloperoxidase was increased by an extract of cigarette smoke. The effects of the extract on the formation of oxygenated or halogenated sterols were not studied.

A number of studies (812, 895, 1095) have demonstrated that oxidized LDL causes a contraction of arteries and inhibits the endothelium-dependent relaxation of smooth muscles of coronary arteries and aorta. Several studies (567, 812, 1095, 1214) have indicated that the production of NO by endothelial cells is reduced by oxidized LDL, and multiple mechanisms appear to be involved (566, 567). The inhibition, by oxidized LDL, of the endothelium-dependent relaxation, presumably caused by NO, is not caused by lysolecithin (812, 1095) but appears to be due to some other lipid present in the oxidized LDL (200). Liu et al. (582) found that oxidized LDL caused an inhibition of NO production in macrophages. No inhibitory action was exhibited by the protein component of oxidized LDL; however, the lipid component of oxidized LDL caused a substantial inhibition of NO production. Cu\(^{2+}\) oxidation of a mixture of linoleic acid plus Chol or Chol alone resulted in materials that inhibited NO production in macrophages. The authors suggested that oxy-
Sterols present in oxidized LDL might be the inhibitory species present in oxidized LDL responsible for the inhibition of NO production in macrophages. In another study (381), oxidized LDL, but not native LDL, decreased NO synthase mRNA expression in bovine aortic endothelial cells. Deckert et al. (246) reported that 7-keto-Chol (at 75, 150, and 300 \( \mu \)M) and 7b-OH-Chol (149 \( \mu \)M) caused an inhibition of histamine-induced release of NO from human umbilical vein endothelial cells. Chol (155 \( \mu \)M), 5a,6a-epoxy-Chol (149 \( \mu \)M), and 19-OH-Chol (149 \( \mu \)M) had no significant effect. Under the conditions studied (120-min incubation), 7-keto-Chol (150 \( \mu \)M) had no effect on cell morphology or viability.

Liu et al. (583) reported that 25-OH-Chol stimulated the production of the cytokine IL-8 by human monocytes and macrophages isolated from human atherosclerotic plaques. Other oxysterols, i.e., 5a,6\( \beta \)-diOH-Chol, 7\( \beta \)-OH-Chol, and 24-OH-Chol, were also active in this respect but were less potent than the 25-hydroxysterol. 7-Keto-Chol and 5a,6a-epoxy-Chol showed stimulatory action in the macrophages but not in the monocytes. The various oxysterols were tested at 5 \( \mu \)g/ml (or 12.4 \( \mu \)M for the mono-hydroxysterols, 12.5 \( \mu \)M for 7-keto-Chol, and 11.9 \( \mu \)M for the triol) in RPMI 1640 medium containing delipidated FCS. The stimulation of IL-8 production in human monocytes by 25-OH-Chol was concentration dependent, and apparently significant stimulation was observed at a concentration of 2.5 \( \mu \)M. The same findings were made with a human leukemia cell line (THP-1) except that clear stimulation was observed at a lower concentration (1.24 \( \mu \)M) of the 25-hydroxysterol. The stimulation of the production of IL-8 by 25-OH-Chol (6.2 \( \mu \)M) was blocked by cycloheximide. It was suggested that the increased production of IL-8, induced by the oxysterols, may act by the recruitment of T lymphocytes and smooth muscle cells into the subendothelial space as part of the development of atherosclerotic lesions.

Oxysterols have also been studied with regard to the possible monoclonal origin of atherosclerotic lesions. In 1973, Benditt and Benditt (57) reported findings suggesting the monoclonal origin of fibrous atherosclerotic plaques. This claim was based on studies of the enzyme glucose-6-phosphate dehydrogenase (G-6-PD), a polymorphic, X-linked gene product and specifically of the frequency of G-6-PD isoenzymes in fibrous plaques in aortas of black females. These results were confirmed and extended by Pearson and co-workers (782–784). In fibrous plaques, almost all contained only one isoenzyme (57, 784), whereas normal aortic tissue contained more than one isoenzyme. Fatty streaks showed an intermediate frequency of lesions with only one isoenzyme (783, 784). The monoclonal nature of the fibrous plaques is suggestive of a mutational event and/or a selective resistance of one cell type to a toxic agent, conceivably an oxygenated sterol. In 1981, Lee et al. (549) reported crossing of two species of hare to give female hybrid hares showing G-6-PD mosaicism. 25-OH-Chol (source and purity not provided) was reported to cause a shift in the ratios of two types of G-6-PD (designated as types T and E) in 8 of 11 cultures from the heterozygous hares (688). The shift to predominantly E type was reported to be dependent on the concentration of 25-OH-Chol, which was varied from reported values of 2.5 to 62 or 124 \( \mu \)M. It was suggested that the shift to the E type was due to a higher resistance of this cell type to the cytotoxic action of 25-OH-Chol (438). Further studies of this matter were pursued in the intact hares fed a high-Chol (~1.1 or 1.0%) diet or the same high-Chol diet supplemented with 25-OH-Chol (439, 549, 550) or 5a,6\( \beta \)-diOH-Chol (439, 550). It should be noted that the levels of the added oxysterols in the diet were very low. In one study, the concentration of the 25-hydroxysterol was not provided (549); in two other studies, the levels of the 25-hydroxysterol were reported as 0.00027% (439) and 0.00025% (550), and the level of the 5a,6\( \beta \)-diOH-Chol in the two studies (439, 550) was reported as 0.00013%. The animal experimentation described in the two studies (439, 550) appears to involve the same animals. The source and purity of the oxysterols and of the Chol used in these studies were not provided. The method of preparation of the sterol-containing diets and the conditions of its presumed storage were not described. These matters become important, since comparisons between results obtained with the Chol-containing diet and the same diet supplemented with such very low levels of 25-OH-Chol or 5a,6\( \beta \)-diOH-Chol become almost meaningless if one considers the possibility of the presence of oxygenated sterols in the Chol used in the experiments or the formation of oxygenated derivatives of Chol by autoxidation during the preparation and/or storage of the Chol-containing diet. For example, the presence of only 0.03% of oxysterol in the Chol used in these experiments or formation by autoxidation of the Chol during the preparation and/or storage of the diet would lead to a dietary oxysterol level of 0.0003%, or higher than that reported for the exogenously administered oxysterols. Interpretation of the results is also complicated by variable periods of dietary treatment and the use of “usually intermittent” administration of the Chol (nature not specified) and alternation of the Chol-containing diet "on a weekly basis with a pellet-only diet." Despite these limitations and deficiencies, administration of the oxysterols was reported to be associated with the development of monotypic foci in atherosclerotic lesions and/or normal aortic tissue in some of the animals (439) and with slight increases in the percentage of T-type cells in arterial media and lesions (550). It should be noted that the predominance of the E-type fibroblasts in the tissue culture experiments associated with 25-OH-Chol addition (at substantial levels) contrasts with the claimed predominance of T-type cells in the in vivo experiments in which
25-OH-Chol was administered in diet at very low levels. The latter findings should be viewed with caution in view of the limitations and deficiencies noted above.

X. OXSTEROLS AS POTENTIAL CANCER CHEMOTHERAPEUTIC AGENTS

Oxygenated sterols can be considered as potential chemotherapeutic agents for the control of cellular growth of both normal and cancer cells. Such an effect could be by simple inhibition of sterol synthesis, thereby reducing the availability of Chol for the new membrane formation required for cellular replication. Inhibition of the formation of other mevalonate-derived products such as dolichol, ubiquinones, or isopentyl-substituted adenine moieties in tRNA, or the modified farnesyl group in hemoglobin of cytochrome oxidase (343, 353, 909) can also be envisioned as affecting tumor cell growth. Furthermore, inhibition of the formation of mevalonic acid could also affect tumor cell growth by inhibition of the formation of isoprenoid pyrophosphates required for the prenylation modifications of ras and other key regulatory proteins. However, it is interesting to note that the potential utility of regulators of HMG-CoA reductase activity (competitive inhibitors or oxysterols) as anticancer agents has been questioned (988). Mevinolin, at concentrations that produce ~50% inhibition of sterol synthesis, had no effect on prenylation of lamin A in CHO-K1 cells. The authors considered it unlikely that competitive inhibitors of HMG-CoA reductase would affect prenylation of proteins under the conditions in which they are used for treatment of atherosclerosis. Furthermore, the extent of inhibition of prenylation of p21ras540 and lamin A by mevinolin appeared to be identical. Whereas inhibition of prenylation of p21ras540 might constitute a rational approach for anticancer therapy, simultaneous inhibition of prenylation of other key proteins may be undesirable. At this time, considerably more effort appears to be directed toward inhibition of enzymes catalyzing the prenylation of key regulatory proteins (see Refs. 556 and 559 for recent reviews) than in effecting prenylation by lowering the levels of prenylpyrophosphates in cells. Oxygenated sterols could also have a favorable action in chemotherapy by increasing the sensitivity of tumor cells to the antineoplastic action of other agents. This increased sensitivity could occur by the action of the oxysterols in lowering the levels of Chol in the cells or by some other mechanisms. In 1997, Lenz et al. (554) reported that reduction of the Chol levels of cultured neuroblastoma cells by ~50% resulted in a marked increase in the sensitivity of the cells to merocyanine 540, a light-sensitive fluorescent dye which generates toxic species upon irradiation. Gaffney et al. (321) reported increased sensitivity of leukemia cells (L1210 cells) to merocyanine 540 after incubation of the cells with 25-OH-Chol (37 and 75 μM). The authors ascribed the increased susceptibility of the cells to merocyanine-sensitized irradiation by 25-OH-Chol to a reduction in Chol content that was associated with an increased uptake of the dye.

In 1974, Chen et al. (188) first described the inhibitory action of three oxygenated sterols (25-OH-Chol, 20α-OH-Chol, and 7-keto-Chol) on the growth of cultured mammalian cells. In studies with mouse L cells, grown in chemically defined sterol-free medium, the 25-hydroxy-, 20α-hydroxy-, and 7-ketosterols inhibited cell growth by >90% at concentrations of 2.5, 6.2, and 25 μM, respectively. The inhibitory action of these oxygenated sterols was reported to be reversed by the addition of desmosterol (65 μM), the major sterol of L cells, or by mevalonic acid (~1 mg/ml). 7α-OH-Chol, at 25 μM, showed no inhibitory action on cell growth; however, at a much higher concentration (124 μM), the 7α-hydroxysterol was reported to show 50% inhibition of cell growth. A large number of oxygenated sterols have been shown to suppress the growth of both normal and transformed cells in culture. It has been reported that 7β-OH-Chol exhibits some degree of selectivity in its effects on tumor cells relative to normal cells (196, 386 and references cited therein, 523).

In 1993, Iguchi et al. (421) reported the isolation of an oxygenated sterol, aragusterol A (Fig. 19A), from a marine sponge that was very active in the inhibition of growth of KB cells (IC50 ~0.092 nM) and showed significant in vivo antitumor activity against L1210 leukemia cells and P388 cells in mice. The chemical synthesis of its 5β-isomer, 5-epiaragusterol A (Fig. 19B), was recently reported (657), and the compound was shown to be highly active in the inhibition of growth of KB cells (IC50 ~0.090 nM). The isolation of other related analogs of aragusterol A from the Okinawan sponge has recently been reported (661).

Mimaki et al. (654) observed the extraordinarily high potency of glycosides of an oxygenated sterol (3β,16β,17α-trihydroxycholest-5-en-22-one), isolated from bulbs of Ornithogalum saundersiae, in the inhibition of cell growth of HL-60 leukemia cells. The structure of one of the most potent of these compounds (IC50 = 0.25 nM) is shown in Figure 20.
At a concentration of 115 μM, the compound showed no hemolytic action with human red blood cells. The sterol was reported to be more potent in the HL-60 cells than other anticancer agents including mitomycin C, cisplatin, camptothecin, and taxol. Removal of the acetyl and p-methoxybenzoyl moieties was reported to decrease potency. The sterol glycoside also showed high potency in inhibiting the growth of other tumor cells in culture including some cell lines that were resistant to other anticancer agents. Melanoma cell lines were reported to be "particularly sensitive" to the compound. The compound was also reported to show favorable in vivo effects against a mouse P388 tumor.

Iverson et al. (429) reported the results of a single experiment studying the effect of an oxysterol mixture on chemically induced mammary carcinomas in animals. A 9:1 mixture of (22R)-7β,22-diOH-Chol and (22R)-7α,22-diOH-Chol was administered to female Sprague-Dawley rats, which at 55 days of age, received an intravenous injection of 7,12-dimethylbenz(a)-anthracene (4 mg) in vehicle. Iverson et al. (429) reported that this dose "usually produces mammary carcinomas in 90% of the animals within 12 wk." Characterization of the two trihydroxy-sterols was not given. The mixture of the two sterols "was added to the drinking water as an alcoholic solution in a quantity that gave a sterol dose of 250 μg per animal per day at a 3.6% concentration of alcohol in the drinking water." Three groups of rats were studied. Group A (n = 29) was given the sterols from the time of injection of the carcinogen for 35 wk. Group B (n = 28) was given the sterols for only the first 12 wk. Group C (n = 30) received no sterols during the first 12 wk; from week 13 they received sterols for the remaining 23 wk. No control groups (water alone or water plus ethanol) were included in the study. By 35 wk, Group A showed a very high survival (low mortality), i.e., ~95% (from graph). Group C showed low survival (~40%). Group B was intermediate. All of the groups showed ~90 ± 5% incidence of tumors by the end of the experiment. However, administration of the sterols during the first 12 wk (or throughout the study) had an inhibitory effect on the rate of development of the tumors. The authors cautiously noted the "great biological variations in carcinogenesis experiments" and that "the results presented here must therefore be regarded as interesting, but preliminary and will need to be confirmed in further studies." The estimated dose of 250 μg/day is very low, i.e., ~0.6 μmol/day (or ~1.25 mg/kg⁻¹·day⁻¹ for a 200-g rat). It is not at all clear how the stated dosage was estimated or maintained throughout the study. In addition, the level of the ethanol in the drinking water was high, and its effects were not studied.

Rong et al. (857) reported that the sodium salts of the bis-hemisuccinate esters of 7α-OH-Chol and 7β-OH-Chol showed, upon intraperitoneal injection, significant antitumor activity in mice bearing ascites tumors (Krebs-II murine carcinomas). Ji et al. (451) prepared two monophosphonic acid diesters of 7β-OH-Chol and of pyrimidine nucleosides to provide water-soluble derivatives of 7β-OH-Chol that could be used in in vivo studies with animals. The compounds were esters at C-3 of the sterol with nucleosides (5-fluoro-2′-deoxyuridine or 2′-deoxyuridine). Both compounds were reported to show high solubility in water (>30 g/100 ml) and high antitumor activity upon intraperitoneal administration to mice with ascites tumors. The dosage used was 80 μmol·kg⁻¹·day⁻¹, which was given for 2 or 3 days after administration of the ascites cells. Ji et al. (450) reported that the 3β-deoxyxyridine monophosphate esters of 7β-OH-Chol and 7β,25-diOH-Chol showed antiproliferative action against murine and human tumor cells. However, the new derivatives were less cytotoxic to the cells than were the parent 7β-hydroxy- and 7β,25-dihydroxysterols. Christ et al. (202) studied the effects of two water-soluble phosphoric acid diesters, 3β-hydroxy conjugates of 7β-OH-Chol with 2-deoxyuridine (JB69) or thymidine (XA29). The latter two derivatives of 7β-OH-Chol were reported to be less potent than 7β-OH-Chol itself in an in vitro assay of antiproliferative activity with cultured mastocytoma cells (P815) (202). Christ et al. (202) reported that daily intraperitoneal administration of XA29 (at 20 mg·kg⁻¹·day⁻¹) and JB69 (at 20 or 40 mg·kg⁻¹·day⁻¹) to mice, in which mastocytoma cells (P815) were implanted subcutaneously, showed longer survival times than controls (saline vehicle alone). The results were not analyzed statistically. XA29 (0.5 mg/day; ~20 mg·kg⁻¹·day⁻¹ for 15 days) was reported to cause a modest decrease in body weight. At doses of the analogs “exceeding 40 mg·kg⁻¹·day⁻¹, mice began to lose weight, become weak, and died if we prolonged the treatment.” At doses between 20 and 40 mg·kg⁻¹·day⁻¹, “in all the cases, injection of the phosphodiester induced abdominal cramps.”

Very recently, Aoki et al. (15) reported that a polyhydroxylated sterol, agosterol A, from a marine sponge reverses the multidrug resistance of human carcinoma cells. The structure of the basic sterol was shown to be 5α-cholest-7-ene-3β,4β,6α,11α,22R-pentol, which occurs...
in the sponge as acetate esters of the 3-, 4-, and 6-hydroxyl functions.

XI. CONCLUDING REMARKS

I have attempted to critically review a wide variety of areas of investigation on oxysterols, a class of compounds of increasing interest and importance in biology and medicine. A large number of important areas can be regarded as in only preliminary stages and can be anticipated to expand very substantially and rapidly. Despite important advances in the understanding of the mechanisms of action of some of the oxysterols, current information points to the challenging complexities of these matters and the existence of multiple mechanisms. Increased effort in the chemical synthesis of oxysterols and their analogs can be anticipated to provide critical materials leading to expansion of studies of the activities and mechanisms of actions of oxysterols not only in cultured cells but also in whole animals. Oxysterols isolated from natural sources have provided novel compounds with unanticipated actions and exceptionally high potency. Some areas of research, most notably studies of the levels of oxysterols in blood plasma and tissues, and other biological materials, are in need of not only increased attention and effort but also of much higher standards of research. Valid information on these matters, coupled with an expansion of knowledge on the biosynthesis and metabolism of the various oxysterols, is critical to a continuing assessment of the physiological significance of the actions of the various oxysterols.

Oxysterols are extraordinarily potent regulators of Chol metabolism. In addition, they have important actions on a variety of other processes. Major advances in research on the activities, mechanisms of actions, and potential applications in medicine can be anticipated in the coming decade. I hope that this review is helpful not only in stimulating research on these matters but also in affecting the directions of future investigations in this important field.

Studies in the author’s laboratory have been funded by grants from the National Heart, Lung, and Blood Institute; the Robert A. Welch Foundation; the Texas Advanced Technology Program; the American Cyanamid Company; and the March of Dimes Birth Defects Foundation.


Address for reprint requests and other correspondence: W. K. Wilson, Biochemistry Department, MS 140, Rice University, 6100 Main St., Houston, TX 77005 (E-mail: billw@bioc.rice.edu).

REFERENCES

18. ARAMAKI, Y., T. KOBAYASHI, Y. IMAI, S. KIKUCHI, T. MAT-


30. AYAN, M., B. ELIASSON, AND O. LARSSON. Low density lipoprotein (LDL) cholesterol can be converted to 7α-hydroxycholesterol in human fibroblasts. Evidence that an increase in hepatic cholesterol 7α-hydroxylase mRNA levels is not a sole cause for reduced cholesterol esterification in young hypercholesterolemic patients treated with cholestyramine. Biochem. Biophys. Acta 1044: 357–360, 1990.


transferase in mammalian cells as studied with specific antibodies.


ing of the human cholesterol 7α-hydroxylase gene (CYP7) and localization to chromosome 8q11–q22. Genomics 14: 153–161, 1992.


348. GUPTA, A. K., AND J. BOATELLA. Plasma membrane sphingomyelin


365. GUSTAFSSON, J. Biosynthesis of cholic acid in rat liver: formation of cholic acid from 3α,7a,12α-trihydroxy- and 3α,7a,24-tetrahydroxy-5β-cholestanolic acids. *Lipids* 15: 119–121, 1980.


392. HERNÁNDEZ-PERERA, O., D. PEREZ-SALA, J. NAVARRO-ANTO-


420. HYLEMON, P. B., E. C. GURLEY, R. T. STRAVITZ, J. S. LITZ, W. M. PANDAK, J. Y. L. CHIANG, AND Z. R. VLAVECIC. Hormonal regulation of cholesterol 7α-hydroxylase mRNA levels and trans-


441. JI, Y.-H., C. MOOG, J.-P. BECK, P. BISCHOFF, AND B. LUU. Anti-proliferative effects of two hydrosoluble derivatives of oxysterols in cultured mouse cells by cholesterol derivatives oxygenated at the nuclear receptor LXR


KRAAIPOEL, R. J., J. H. DEGENHART, V. VAN BEEK, H. DE...


682. MORI, T. A., K. D. CROFT, I. B. PUDDEY, AND L. J. BEILIN. Analysis of native and oxidized low-density lipoprotein oxysterols


715. OKUDA, K., and N. TAKIGAWA. Separation of 5β-cholestane-3α,7α,12α,26-tetrol oxidoreductase, ethanol-NAD oxidoreductase, 5β-cholestane-3α,7α,12α-triol-26-al oxidoreductase, and aldehyde-


743. OSTLUND FORRANTS, A.-K., A. NILSSON, AND J. I. PEDERSEN. Human hepatoblastoma cells (HepG2) and rat hepatoma cells are defective in important enzyme activities in the oxidation of the C7 steroid side chain in bile acid formation. J. Lipid Res. 34: 2041–2050, 1993.


542 GEORGE J. SCHROEPFER, JR.


January 2000

OXESTERS: MODULATORS OF CHOLESTEROL METABOLISM

543


GEORGE J. SCHROEPFER, JR. Volume 80


OXYSTEROLS: MODULATORS OF CHOLESTEROL METABOLISM


1147. USUI, E., M. NOSHIRO, Y. OHYAMA, AND K. OKUDA. Unique property of liver mitochondrial p450 to catalyze the two physiologically important reactions involved in both cholesterol catabolism and vitamin D activation. FEBS Lett. 274: 175–177, 1990.


1151. VAN CANTFORT, J. Contro ˆle par les glucocorticoste ´roides de l'activite ´ circadienne de la choleste ´rol-7-


1158. VAN LIER, J. E., AND L. L. SMITH. Sterol metabolism. I. 26-

1159. VAN LIER, J. E., AND L. L. SMITH. Sterol metabolism. III. Chro-

1160. VAN LIER, J. E., AND L. L. SMITH. Sterol metabolism. XIII. Chro-

1161. VAN LIER, J. E., AND L. L. SMITH. Sterol metabolism. XII. Chro-


