Ischemic Cell Death in Brain Neurons

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I. INTRODUCTION

A. Defining Cell Death

1. Cell death is an active process

This review is geared toward those working in the field of ischemic tissue damage and toward students and others who want to develop a critical overview of the area. The text carefully examines the evidence that certain chemical and ionic changes, protein and enzyme changes, and changes in key functions are involved in ischemic cell death. The long-term goal of this review is to make as cogent a model of ischemic cell death as is possible at present, which is done in the final section, and to highlight what is not known to excite rigorous, causally directed, experimental work. The latter is done throughout the text.

The ischemic cell death that is clinically relevant, and that is largely considered in this review, is different from the cell death that would eventually ensue, as a consequence of the second law of thermodynamics, from complete and permanent inhibition of energy metabolism. That would occur because of the inability to couple oxidative metabolism to endergonic processes and the re-
sulting inability to reduce the entropy of the cell at the expense of exergonic reactions. Cell entropy would increase; ion gradients would dissipate, macromolecular synthesis would cease, and cell structure would be lost. This would be considered a “dissipative” cell death. The phenomenon considered in this review, and by the vast majority of studies, ensues from relatively short and/or relatively mild insults which, themselves, do not lead to dissipative cell death; rather, they cause acute ionic and chemical changes that initiate sets of highly interactive ionic and biochemical changes that cause cell death.

2. A working definition of cell death

To study what causes cell death, cell death has to be recognized. However, there is no well-accepted definition of the point at which a cell dies (706, 1137). The most satisfactory definition of cell death, and the one that would be the most valuable to know therapeutically, is the point at which the cell becomes unable to recover its normal morphology and function even if all processes leading to dissolution are stopped pharmacologically (the point of no return). This would be analogous to cessation of breathing or cardiac function for death of the organism. At this stage, however, we do not know enough about the ischemic death process of a cell to be able to identify what the point is. Thus the only unequivocal definition of cell death is a morphological one, the elimination of the cell, which can arise either by cell disintegration or phagocytosis. These are both very clear end points, and they are often used in studying mechanisms of cell death. Before this there are certain metastable morphological states toward which dying cells tend that are almost certainly precursors of disintegration or phagocytosis (121, 814) and that are also commonly used as end points. In this review these are termed the end stages of the cell death process and are generally considered to represent dead cells, undergoing necrosis, autophagocytosis, or the end stages of apoptosis (Fig. 1, column 5). As defined in this way, cell death is an anatomical event that represents a state from which the cell cannot recover to even near normalcy. The state reflects major macromolecular change, and the study of cell death, at this stage, is the study of what leads to these drastic macromolecular changes.

Ultimately, it will be important to identify the point of cell death, defined above as the point of no return, because some agents might block the major morphological change but not block the irreversible damage to the cell. It seems reasonable that prolonged damage to the plasma membrane, mitochondria, cytoskeleton, or protein synthesis could each be a point of cell death defined as above, and section IV is devoted to evaluating their roles in ischemic cell death.

B. Major Features of Ischemic Cell Death

1. Three major modes of cell death

There appear to be at least three distinct modes of cell death that participate in ischemic cell death (149, 205, 706). Two of these, apoptosis and autophagocytotic cell death, clearly involve ordered physiological processes in that new structures are formed that are integral to the process, and the progress toward cell elimination is stereotyped. Furthermore, they are a part of normal cell function, during development and other times (205). There is some conflict as to whether autophagocytosis can be profound enough to cause cell death (149, 205, 835), but there are quite persuasive arguments by Clarke (205) that it is. These include the high percentage of neuronal area that is covered by autophagic vacuoles during some developmental cell death (205), as well as the ability of 3-methyladenine, a phosphatidylinositol kinase blocker (101) which prevents autophagocytosis, to completely prevent cell death in at least one case (149).

A third category of cell death, which has been termed necrosis or necrotic cell death, is fundamentally different from apoptosis and autophagocytotic cell death in that it only occurs after an exogenous insult; furthermore, no new structures are elaborated as part of the process. Thus it does not appear to be a programmed, or a normal, physiological process. Because the process is not yet well characterized, it is best defined now as cell death that is neither apoptotic nor autophagocytotic.

There are at least two quite different forms of this cell death; by far the most common is ischemic/homogenizing cell change, where the cell first shrinks dramatically and becomes very electron dense. Less common is edematous cell change, where the cell swells greatly and organelles lose their form. There is no satisfactory term for these modes of cell death. It is widely termed necrosis or necrotic cell death, but necrosis is really the “decay” process after the point of cell death and occurs in all forms of cell death (1137). However, for the sake of continuity, necrotic cell death and necrosis will be used in this review. At this stage, it is not completely known why a particular mode of death occurs in a given cell as a result of a particular ischemic insult. However, it is apparent that one insult can spawn more than one mode of death in the same population of cells and also that in many cases one cell will manifest signs of more than one mode of cell death (205, 716). Thus neurons have the potential for exhibiting all modes of death in response to an ischemic insult. The pathway taken must be a function of the nature of the insult, the cell type, age, and very probably the state of the cell at the time of the insult (716). This issue is considered in section III.
FIG. 1. Pathways of ischemic cell death. This is an overview of processes involved in ischemic cell death that is consistent with organization of review. It illustrates major events that are hypothesized to contribute to cell death and also the extremely complex interactions between these events. This figure is not meant to be complete, but does include processes known or thought to be important at this stage. Column 5 lists 5 principal morphological forms taken by dying or dead cells after an ischemic insult. Determining how these end stages are reached is the ultimate goal of research on ischemic cell death. Column 4 lists 6 long-term functional or structural changes, all of which, except changes in membrane permeability, are known to occur as a result of ischemia. It is hypothesized that one or more of these account for one or more forms of ischemic cell death. As indicated by lack of 1:1 connections in figure, no direct causal sequence has been established between any of functional changes in this column and particular end stages shown in column 5. Potential relationships are discussed extensively in text (see sects. III and IV). Column 3 lists actions that are likely to cause long-term functional changes described in column 4. These are termed "perpetrators" because they are considered to be key damaging events in ischemic cell death. No direct effects of perpetrators on critical functional changes are insinuated in figure because none has been completely established. Many such effects are, however, reasonably well established and are discussed in text. Columns 2 and 1 show changes in many variables, initiated by original inhibition of electron transport, whose most important end result is considered to be activation of perpetrators, but which may also have more direct effects on cell damage, that are not shown. Major outputs of these changes, lumped together as initiators and activators, are changes shown in figure because none has been completely established. These causal interactions are indicated by including changes within same toned horizontal band, as shown for columns 2 and 3. For example, both increased calcium and gene activation contribute to formation of increased nitric oxide. This way of describing events indicates the very high level of interaction between different changes caused by ischemia, involving many positive-feedback loops. Interpreting the figure: as indicated above, colored boxes encompass different changes that cause change in variable represented by that color. For example, increased nitric oxide (peach color) results from increased calcium and gene activation. Colored horizontal arrowheads represent all events within box of that color. For example, red arrowhead (for ATP) represents loss of oxygen and increase in sodium, which are enclosed within red box associated with ATP (near bottom of figure). Using this notation allows the very large connectivity of system to be represented in a manageable way. For example, changes putatively contributing to increase in sodium, enclosed within green box at top of figure, are extremely numerous when "contents" of each arrowhead are considered. In addition, changes contributing to increase in cytosolic calcium, and hence calpain activity, are all those included in boxes and arrowheads in top gray band. Large number of positive-feedback cycles is readily seen. Depol, depolarization; pHi, intracellular pH; Na, intracellular Na⁺; Ca, intracellular Ca²⁺; FFA, free fatty acids; PAF, platelet-activating factor; e⁻ Transport, electron transport.
2. Ischemic cell death is usually delayed

In addition to its multimodal nature, ischemic cell death is also characterized by a long delay between the insult and manifestation of major cell damage. This delay varies greatly, depending on the nature of the insult and the brain region being affected. In some cases it is as long as several days or even weeks (276, 582), whereas in others it is a few hours or less (742). It is clear that, all other factors being equal, the greater the energy deprivation, the less time it takes for damage to develop (579). The very prolonged delay is quite an extraordinary phenomenon; it shows that very short-lived, albeit profound, derangements of metabolic patterns can initiate the development of damage and cell death that takes place, between a day and 3 wk later. Remarkably, normal homeostatic mechanisms are unable to prevent this process. Thus lethal ischemic insults are those that move cells far enough from their steady state to eliminate the possibility of reattaining that steady state; sublethal insults produce early damage, but this is able to be recouped by the cell. One very important and unresolved issue is what is the basis for this “destabilization” of the cell. It is discussed theoretically in section IX.

C. Overview of Ischemic Cell Death

1. Stages of ischemic cell death

Ischemic cell death is initiated by changes that result directly from inhibition of oxidative phosphorylation and create an early maelstrom of activity. These changes include decreased pH, decreased ATP, initiation of free radical production by the mitochondrial chain, increased cell Na⁺ and membrane depolarization as a result of the loss of ATP substrate for the Na⁺-K⁺ pump. These lead to secondary changes in ion and chemical concentrations which, in concert with the initiating changes, result in activation of damaging processes. These damaging processes are termed “perpetrators” in this review and are characterized by their abilities to produce long-term changes in macromolecules. Activation of one or more of the perpetrators is considered the key step in necrotic aspects of ischemic cell death. The perpetrators include proteases, phospholipases, and free radical actions. The macromolecular changes they cause, and the ensuing functional changes, are considered to be the proximal causes of cell death. At present, the way that ischemic apoptosis is activated is not well enough understood to include the same perpetrators in that process with any certainty, although caspases certainly are involved. Thus the process of cell death has at least three major stages: an early development of ionic and chemical changes, a resulting activation of perpetrators, and a subsequent change in critical functions and structures that denote or lead to cell death by one of three different routes.

The overall process is extremely complex due to the large number of interactions between the variables, illustrated by Figure 1. Although incomplete, Figure 1 provides a good working description of the probable flow of damage and the very complex interactions between different changes.

2. Limitations in current knowledge

Two very important limitations in our understanding are not apparent from Figure 1. The first is the degree of certainty that the different changes shown are actually involved in ischemic cell death. It is fairly certain that most, or all, of the activators and perpetrators in Figure 1 are actually involved in ischemic cell death, although, as will be seen, experimental evidence is often surprisingly weak. Much less is known about how these changes lead to cell death (Fig. 1, columns 4 and 5). As will be seen, the identities of the critical functional changes, how they actually might lead to cell death, and the end stages are little known for any of the three modes of cell death.

The temporal aspect of cell death is also missing from Figure 1. End stages or cell disintegration develop anywhere from 6 h to several days after the ischemic insult. The basis for these time delays is not yet known. Gene activation and subsequent synthesis of damaging proteins such as cytokines, inducible cyclooxygenase-2 (COX-2), inducible nitric oxide (NO) synthase (iNOS) may be important. However, it is also likely that there are long-term low-level activations of perpetrators when the insult is relatively mild so that major changes in cell function occur only after prolonged periods. The factors that determine the temporal aspects of ischemic death are poorly understood at the moment.

D. Organization and Methodology of the Review

Figure 1 provides a framework for the organization of the review. After the different model systems used for studying ischemic damage are considered (see sect. II), the main body of the review begins from the right-hand side of Figure 1 and essentially walks backwards; for example, section III describes the end stages of ischemic cell death. Knowing the downstream events makes it easier to evaluate the role of a particular change. For example, understanding that mitochondrial dysfunction may be a mediator of cell death (see sect. IV) provides a focus for examining the targets of free radical action. Despite the connectivity of the different major parts, each section is designed to be usefully read independently of its order of appearance and to fully examine the possible contribution of a different variable to damage.

The achievable goal of the review is to critically evaluate the involvement of different variables in ischemic cell death. This entails describing the changes in the
variable that occur during and after ischemia, determining how they are likely to occur, critically assessing pharmacological and gene manipulation studies done to show that the change contributes to cell death, and then trying to understand the mechanism by which the change is contributing to cell death.

In section IX, a still speculative attempt is made to synthesize the different sections and describe probable sets of events that lead to cell death in the different experimental models. It is hoped that this will provide useful directions for future studies.

II. SYSTEMS USED TO STUDY ISCHEMIA

Many experimental models are used to study ischemic damage. Mechanisms of cell damage are determined by testing effects of different manipulations on the extent of cell death in a model. To meaningfully compare results between models, the essential differences between the ischemic insults and the way damage is assessed in these models need to be appreciated. The factors influencing development of damage in the different models need to be known, and problems in interpreting measurements of cell death have to be recognized.

The three main classes of in vivo rodent models are global ischemia, focal ischemia, and hypoxia/ischemia. In the latter, vessel occlusion is combined with breathing an hypoxic mixture; this modified Levine preparation is now almost exclusively used in young animals. Larger mammals have been used to study ischemia (534), as have nonmammalian vertebrates (453), but this review will focus on what has been learned from rodent models. In vitro models that include exposure of neuronal, neuronal/glial or organotypic slice cultures, or the freshly isolated hippocampal brain slice, to anoxia or to anoxia in the absence of glucose (in vitro ischemia) have provided important information and are discussed when they provide insight into events in vivo. Other single-cell or synaptic models have been used very effectively (307, 340) but will not be considered because the metabolic states of these preparations may be quite different from in vivo (307).

A. Insult Variables That Determine Damage

The degree to which blood flow is reduced directly determines the changes in three variables that are almost undoubtedly the genesis of all future changes. Two of these, inhibition of electron transport and decreased ATP, appear to be directly related to the extent of flow reduction (see Table 1). The third variable, free radical production, is likely to be a more complex function of flow rate because it is activated both by electron transport inhibition and also by oxygen so that it will be maximal at blood flow values greater than zero. (The precise relationship between flow rate and free radical production has not been determined.) Other damaging changes such as decreased pH and leukocyte accumulation are more severe in the presence of residual blood flow. Thus the amount of damage need not be a direct function of the decrease in blood flow. Elevated blood glucose enhances damage in many cases despite maintaining ATP levels so that the fall in ATP is not a reliable indicator of the efficacy of an
insult either. As a further complication, the nature of damage (e.g., extents of apoptosis vs. necrosis) may be a complex function of the amount of the ATP/electron transport decrease and free radical increase and thus may be a complex function of the flow rate during ischemia (194, 870). Thus, except for increased duration of an insult, which always enhances and accelerates damage, it is difficult to estimate, a priori, the potential impact of an insult based simply on the degree of ischemia and fall in ATP.

B. Differential Vulnerabilities of Cell Populations

In vivo, distinct neuronal populations have very different vulnerabilities to ischemia, and these have been reviewed relatively recently (169). For example, 5-min global ischemia caused delayed death in almost all CA1 pyramidal cell neurons with no effects on other populations, whereas 20 min caused cell death in CA3 neurons but had almost no effect on dentate granule cells in the hippocampus (579, 583) or on interneurons in CA1 (322). The basis for differential vulnerability has not been determined, although, as will be seen, there are many differences between vulnerable and less vulnerable cells that might well account for it, as described in Table 5. In addition to these, differences in presynaptic events may be responsible for apparent selective vulnerabilities of postsynaptic neurons as will be seen in section VI. Further, as described in Table 5, although, as will be seen, there are many differences between vulnerable and less vulnerable cells that might well account for it, as described in Table 5.

The phenomenon has been used to provide insight into mechanisms of damage and is discussed in that context throughout the review.

C. In Vivo Models: Introduction

The Levine model, in which hypoxia is combined with unilateral carotid occlusion (644), adapted in the mid-1960s in landmark studies by Brown, Brierly, and co-workers (121, 129, 742), established the basis for systematic study of brain cell damage in rodents. Most in vivo models now rely on vessel occlusions that predominantly affect the forebrain, developed between the late 1970s and early 1980s (331, 366, 461). They are generally divided into two categories, global and focal ischemia, but a modified Levine preparation (hypoxia/ischemia) has some properties of both models.

D. Global Ischemia

Global ischemic insults are most commonly produced by vessel occlusions, and less commonly by complete brain circulatory arrest. Although the former are not actually global, a large portion of the forebrain is quite uniformly affected.

1. Anatomy: effects on flow and electrophysiology

The three most widely used global ischemia models are four-vessel occlusion (4-VO) and two-vessel occlusion (2-VO) combined with hypotension in the rat, and two-vessel occlusion in the gerbil. The importance of transgenic mice has spawned the use of the 2-VO in the mouse also, and the latter is now being systematically studied (588, 1074). Results to date are similar to those with rat and gerbil (588).

a) 4-VO in Rat. The 4-VO in rat involves permanent coagulation of the vertebral arteries (which has no deleterious effects) and temporary ligation of the two common carotids. Ligation of temporal muscles reduces flow more reliably (926, 928). In Wistar rats, which show loss of righting reflex within 15 s, blood flow is reliably <3% of control values in hippocampus, striatum, and neocortex (931). The electroencephalogram (EEG) generally becomes isoelectric within 30–40 s (935), and spontaneous cortical activity is abolished within 1 min when skull temperature is maintained at 37°C (1233). No anesthetic is required during ischemia, and usually none is used.

b) 2-VO in Rat. This involves ligation of the common carotid arteries only, along with a blood pressure reduction to ~50 mmHg, and has slightly more profound effects than 4-VO. Blood flows fall to ~1% in hippocampus, neocortex, and striatum (1055, 1056), and the EEG generally becomes isoelectric within 15–25 s. However, in the hands of one group, blood flow is only reduced to 15% of control levels (1089). This less-intense insult is more easily protected against, for example, by N-methyl-D-aspartate (NMDA) receptor blockade, and this has led to some confusion in the literature that will be dealt with in section VI. Anesthetic is used during ischemia.

c) 2-VO in Gerbil. This is induced by temporarily ligating the carotid arteries, with no reduction in blood pressure. Because there are no posterior communicating arteries in gerbils, this produces profound forebrain ischemia (583). Changes are similar to those in the rat models; blood flow in cortex is <1% and in hippocampus is ~4% of control values (548), whereas EEG failure occurs within 20 s (1088). Damage has been successfully dissociated from the occurrence of postischemic seizures that also characterize the insult in gerbils (493). Anesthetic is not used during ischemia.

d) Complete versus Incomplete Global Ischemia. Finally, there is complete global ischemia, generally achieved by neck-cuff (259, 297, 682), cardiac arrest (228, 294), or by ligating or compressing all arteries stemming from the heart (560, 915). Blood flow to the whole brain is zero or <1% in these models (297).

Vessel occlusion is often termed “incomplete isch-
3. Development and measurement of damage

Tissue metabolic rate is generally depressed by 50% or normal for most of the reperfusion period (162, 929). Ops in the face of ATP levels that are at least 70 – 80% of well after global ischemia so that delayed damage develops. However, there are many differences between the two species, both in terms of metabolic and genetic changes during and after ischemia, and the abilities of different agents to protect against damage. Thus the processes of cell death may be different; results from one species cannot necessarily be generalized.

2. Changes in energy metabolism

Global insults are generally short, between 3 and 30 min, with 5–20 min being the usual exposure times. Longer insults become lethal, and these shorter insults are quite good models for reversible cardiac arrest pathology. Levels of ATP fall quite rapidly during this period (Table 1). By 2 min, levels are 15% or lower in gerbil, and also in rat during complete ischemia. Unfortunately, there are no good kinetic studies for the rat vessel occlusion models; by 10 min, ATP is 10% or less of normal values (Table 1), but the rate of decay, which may be important in determining extent of damage, is not known.

Levels of ATP in vulnerable cell regions recover quite well after global ischemia so that delayed damage develops in the face of ATP levels that are at least 70–80% of normal for most of the reperfusion period (162, 929). Tissue metabolic rate is generally depressed by 50% or more, for at least 6 h after the ischemia (291, 306, 606, 760). The basis for this is not known, and its consequences have not been determined, but they are likely to be important.

3. Development and measurement of damage

A) Development of damage. A cardinal feature of global ischemic insults is a substantial delay between the end of the short insult and cell death. This is generally between 12 h and several days. The delay depends on the cell population and the duration of the insult. This “delayed neuronal death” was first noted and documented in rat by Pulsinelli and Brierly (926, 927) and in gerbil by Kirino (580). It is most widely studied in the CA1 region of the hippocampus, but the stratum, parts of the CA4 region of hippocampus, and layers 2 and 5 in cerebral cortex also show the phenomenon in response to short insults.

Overall, gerbil hippocampus is slightly more sensitive than rat. With body temperature at 36°C, but brain temperature falling normally, 5 min of ischemia caused massive cell death in the hilus of the dentate gyrus (CA4) and the striatum within 24 h and complete cell loss in the CA1 region of the gerbil hippocampus within 3–4 days (226). This damage is certainly as severe as the incomplete global ischemia models (see sect. I.D.3).

Overall, gerbil vessel occlusion is the most studied model because the operation is simpler than the rat. However, there are many differences between the two species, both in terms of metabolic and genetic changes during and after ischemia, and the abilities of different agents to protect against damage. Thus the processes of cell death may be different; results from one species cannot necessarily be generalized.

When rat brain was maintained at 36–37°C, 6–10 min of 4-VO caused near-complete CA1 pyramidal cell death in 3 days (647, 681), and 10 min of 2-VO caused almost complete cell death in 7 days (210, 519, 819, 921). Ischemia for 5 min caused 50% loss (519) compared with complete cell loss in gerbil, and 2-min ischemia caused a (very minor) neuronal loss in CA1 and CA4 (1055). As with the gerbil, hilar and striatal neurons die much more rapidly than CA1 pyramidal cells (896, 997), although, interestingly, a greater insult duration was necessary to initiate the cell death in striatal neurons than in hippocampal neurons (997). This shows a dissociation between factors that initiate the damage and those involved in its development.

Damage from successive insults can be cumulative. When spaced 1 h apart, three insults that normally gave no damage individually caused major damage (543).

B) Morphological Changes during Delayed Neuronal Death. There are four major sets of changes during development of the delayed death in gerbil. Most notable is a massive proliferation of stacks of endoplasmic reticulum (ER) that peaks after ~2 days; ER fragments accumulate, with lysosomes, around the nucleus (579, 581). After ~3 days, there is a regression of the proliferated ER and the formation of a large number of autophagosomes with a very large increase in the lysosomal proteases, cathepsins B, H, and L (894). The autophagic vacuoles may arise from the expanded ER as the two have been associated during developmental cell death (205). Thus a long-term program for autophagocytosis may have been activated by 5 min of ischemia.

There is also a gradual increase in the numbers of small dense bodies ~1 μm or less in diameter in both somata and dendrites, which largely localize under the plasmalemma. Throughout the 3 days, the dendrites show progressive swelling, microvacuolization, and disappearance of microtubules, beginning immediately after ischemia (1236). After early swelling, mitochondria remain normal until ischemic cell change (ICC) or edematous cell change (ECC) become apparent.

The delayed death in the rat appears very different, although it develops at about the same rate. The extent of ER proliferation is far less (251, 581, 897) and, perhaps relatedly, no autophagosomes have been explicitly identified. The small dense bodies are somewhat more prevalent than in the gerbil and may be undegraded proteins
that participate in damage (251, 582), although there is no explicit evidence.

As the duration of the insult increases, and so presumably the extent of the biochemical changes, so does the rate of cell death increase (579, 897). The nature of the process may ultimately change; ER proliferation, for example, is absent in gerbils after a 30-min insult that causes cell death in 12 h (897).

At a certain point, these rather subtle changes are transformed into quite abrupt major changes in cell structure associated with the end stages of cell death. These are discussed in section III. The mechanisms of this dramatic change are not known; presumably accumulated changes become great enough to cause what is essentially a “phase change,” as discussed by Gallyas et al. (350).

4. Summary

There are profound reductions in ATP levels for very short periods during ischemia, possibly for as little as 1–2 min. Nevertheless, there is massive cell death in vulnerable regions. There is a significant delay between the ischemia and obvious morphological signs of cell death. This delay can be as much as 4 days and as little as 12 h depending on the length of the insult and the cell population; for any one region, the longer the insult, the shorter the delay. Understanding why this delayed cell death arises from such short insults is a major goal of research in the field.

E. Focal Ischemia: Characteristics of Different Models

Almost all focal ischemic models, whether larger mammal such as cat (1077) or nonhuman primate (1092) or rodent, primarily involve occlusion of one middle cerebral artery (366). In some cases, the carotid artery is also ligated. The insult is thus differentiated from global ischemia in two very important ways. First, even at the core of the lesion, the blood flow is almost always higher than during global ischemia so that longer insults are required to get damage. Secondly, there is a significant gradation of ischemia from the core of the lesion to its outermost boundary, and hence there are different metabolic conditions within the affected site. Because of its duration, and heterogeneity, the insult is much more complex than global ischemia, but it is an invaluable model for stroke and is thus widely studied.

In permanent focal ischemia, the arterial blockage is maintained throughout an experiment, usually for 1 to several days, whereas in temporary focal models, vessels are blocked for up to 3 h, followed by prolonged reperfusion. Despite the big difference in insults, maximal lesion sizes are comparable in the two cases, and the progression of damage, in terms of the numbers of damaged neurons and the extent of that damage over 6–72 h, is remarkably similar (1283). Nevertheless, there are clear differences illustrated by effects of different protectants so that the mechanisms of cell death may well be different. As with global ischemia, mouse models have now been introduced (434, 588) but will not be discussed explicitly.

There are variations within each of the two basic classes of insult: temporary and permanent ischemia. Several different rat strains are used (one is a spontaneously hypertensive strain that has a less extensive collateral circulation during ischemia and is more easily damaged; Refs. 137, 222, 391). Different strains show minor quantitative differences in lesion locations and sizes but qualitative differences in responses to different protectants.

Also, different sites and techniques of artery occlusion are used, to try and obtain reproducibility of flow reduction and lesion size (462, 860). There are two principal occlusion sites. In proximal occlusion, the middle cerebral artery (MCA) is occluded close to its branching from the internal carotid, before the origin of the lenticulostriate arteries (860, 1104, 1105). Appropriate care can produce lesion sizes with coefficients of variation that are <20% (860). A newer and now widely used approach to proximal MCA occlusion is the insertion of a nylon suture into the carotid artery past the point at which the MCA branches so that the latter is occluded at its origin (79, 598, 655, 798). Used optimally, the coefficient of variation in lesion size is ~8% and of blood flow is 35% (79). A similarly small coefficient of variation in lesion size, along with a large lesion of ~250 mm³ at 24 h, was obtained with the silicone-coated suture in Fischer 344 rats (44).

There are some potentially important artifacts with this widely used method. Flow following temporary ischemia is somewhat compromised by the partial occlusion of the carotid arteries with the filament (79). Also, even a temporary occlusion leads to a long-term increase in core temperature at ~1.5°C due to decreased circulation and damage to the hypothalamus, at least in Wistar rats (1241, 1297). Finally, there is quite extensive damage to small arteries in the ischemic field, with loss of endothelium and muscle damage (832). This occurs after normal insults (e.g., 6-h ischemia or 2-h ischemia and several hours of reperfusion). It has been suggested that this damage may affect subsequent neuronal cell death, for example, by exacerbating the leukocyte response in the period after ischemia (435). This is not at all proven but needs to be considered.

In distal MCA occlusion, flow to the basal ganglia is not blocked so that damage is only cortical. The occlusion can be made surgically, in which case the lesion is readily reversible (137) or, less invasively, by creating a (permanent) thrombus by laser irradiation when the artery is perfused with Rose Bengal (714). Distal MCA occlusion has to be combined with occlusion of the ipsilateral carotid artery to get adequate flow reduction (122, 189).
F. Focal Ischemia: Core and Penumbra

1. Blood flows

In both proximal and distal MCA occlusions, there are core ischemic regions, served primarily by the occluded MCA, where blood flow is reduced to <15%, and there are penumbral regions where flow is <40%. There are also extrapenumbra cortical regions in which flow is >40%. These approximate flow-based definitions of core (284, 816, 977, 1104) and of penumbra (48, 369, 462) as well as the extrapenumbral (or peri-infarct) region are generally agreed upon, although there is still much discussion. Using flow is a simple and physiologically meaningful way to differentiate the regions, which suffer fundamentally different insults as indicated by efficacies of protectants, and major morphological changes. Given a long enough temporary (or permanent) insult, both core and penumbra become infarcted (1299), whereas the extra penumbral zone only shows death of isolated neurons. The density of cell death is not great enough to cause infarct.

In proximal MCA occlusion, the core is in the lateral portion of the caudate putamen and overlying parietal/somatosensory cortex (106, 751, 798, 1104). Most other regions of neocortex and entorhinal cortex, as well as the medial caudate-putamen, constitute the penumbra (79, 106, 751, 798). In the distal MCA occlusion/carotid artery occlusion, the striatum is spared, and core and penumbra span the parietal cortex, with penumbra occupying the more inferior regions. There is significant variation in the extent of the different regions among Sprague-Dawley rats, spontaneously hypertensive rats, and Wistar rats (48, 122, 137, 714).

With development of on-line measurements of flow using positive emission tomography (PET) scans, and the use of more detailed time course studies using iodoantipyrine, there is emergent evidence that blood flow varies throughout the penumbral region and also decays with time, although the extent of decay varies widely in different models (79, 438, 798, 1297). The implications of this decay are currently being considered. Clearly, it intensifies the insult in that region over time, and so may be important in the development of penumbral damage (325). It is considered in greater depth in the above-referenced papers dedicated to flow measurements and analysis.

2. Ionic and metabolic changes in the core and penumbra

As indicated, there are major differences in physiology and biochemistry between the core and penumbra and, indeed, these represent another way in which the regions are differentiated. Furthermore, differences in metabolism and in susceptibilities to protectants strongly suggest that different mechanisms cause the cell death in the two regions.

The core undergoes rapid anoxic depolarization within 1–3 min with a concomitant rise in extracellular K+ to ~70 mM, which may show some short intermittent returns to baseline (359, 818). There is a large decrease in extracellular Ca2+ within 1–2 min at flow levels that are present in the core (428, 429), signifying entry into the tissue. During the reperfusion period, after 2 h temporary focal ischemia, extracellular K+ returns to control levels for 6 h before rising slightly (to 5 mM) for the remainder of a 24-h recirculation period. Despite this return to near normalcy, the insult produces severe damage with a large infarct including all of the core (359).

Events in the penumbra are less drastic, although they still lead to infarct. There is EEG silence (1077) and very much reduced evoked transmission (977), but there is no permanent anoxic depolarization with concomitant ion changes during the ischemia. Presumably ATP levels, which are maintained at 50–70% of normal, do not fall enough to allow the anoxic depolarization. However, there are sporadic transient depolarizations (369, 759) that are here termed “intraschismic depolarizations” (462, 818). They arise differently from anoxic depolarizations. Anoxic depolarizations are independent of NMDA-type glutamate receptors (394, 611, 635, 934, 1231) and only very slightly (1231) or not at all (635) dependent on non-NMDA glutamate receptors. Anoxic depolarizations may actually originate from glutamate release in the infarct core. Nitric oxide synthase knockout mice show reduced frequencies along with less release of glutamate in the core of the lesion (1031). The depolarizations occur very abruptly, last between 3 and 5 min, and have the characteristic form of spreading depressions. They do not occur during reperfusion (818). As discussed in section nH, they may well enhance damage.

3. Energy metabolism in core and penumbra

Levels of ATP fall to ~25% of basal values in the core and remain there between 5 min and 4 h of ischemia (328, 329, 1084, 1201) (Table 1). Later measurements have not been reported. After damaging durations of temporary ischemia, core ATP levels return to about two-thirds normal, for at least 4 h (329), although damage is as great as in permanent ischemia. Oxygen levels return to normal (801), showing that compromised flow is not the basis for the damage that occurs during reoxygenation.

Levels of ATP in the penumbra average ~50–70% of normal during ischemia (Table 1). Glucose utilization is actually elevated in the penumbral region (48, 816, 1252) at early times but falls to ~50% normal, equal to the core, by 3.5 h (1252), suggesting significant compromise of cell
function at this point. Unfortunately, there are no reports of oxygen utilization during the insult, which would allow a more complete assessment of the rate of energy metabolism. Although penumbral blood flow declines during the insult, it remains far higher than in the core; infarct develops despite this flow difference.

Levels of ATP during the reperfusion period after temporary ischemia do not seem to recover much from their ischemic values (489, 590), although further measurements are needed to confirm this conclusion. Glucose utilization in some penumbral tissue is reduced to well below normal during early reperfusion, and this is considered a reliable indicator of the tissue that will later become part of the infarct (79, 1299). Presumably, the low glucose metabolism indicates severe cell damage of some kind at this early stage.

Thus, as with global ischemia, the core of the focal lesion undergoes massive ion and metabolite changes that recover quite strongly after a temporary insult. Despite this recovery, it is almost impossible to prevent damage by any intervention during the reperfusion period. It has also proven extremely difficult to prevent core damage by any single intervention, even before the onset of ischemia, when ischemia lasts for 2–3 h. The penumbra can be rescued by many interventions. These differences suggest different mechanisms of damage in the two regions. As with global ischemia, posts ischemic energy metabolism appears to be compromised in both core and penumbra.

4. Free radical changes in core and penumbra

In contrast to ion and metabolite changes, a recent study demonstrated very little free radical change in the core as compared with penumbra (1060). Free radicals, as measured by hydroxylation of salicylate in microdialysates, increased early during ischemia in the penumbra, remained elevated throughout 3 h, and then became further elevated with the onset of reperfusion. In marked contrast, there was no increase in the core during 3 h of ischemia. There was an increase in the core after ~4 h of reperfusion. Thus the exposure of the core to free radicals during the main period of damage development must be considered minimal. The opposite is true of the penumbra.

G. Focal Ischemia: Development and Measurement of Damage

Unlike global ischemia, which leads to neuronal cell death in isolated regions, focal ischemia produces a contiguous mass of damaged brain tissue termed the infarct. Rather than measuring damage by counting dead cells, as in global ischemia, damage is generally expressed as the volume of the infarct, for which there are standard measurement techniques (e.g., Ref. 860). This is a rough measurement in that the appearance of infarct is predicated on a large fraction of the cells in the region being damaged; otherwise, individual cell damage is noted.

1. Development of the infarct over time

The gold standard of infarct delineation is a pallid region when tissue is stained with hematoxylin and eosin or pure Nissl stains (860). The pallor results from vacuolization of the neuropil, including swelling of glia, presynaptic elements and dendritic elements, and the loss of Nissl staining in the neuropil (355). So identified, it signifies cell damage but not necessarily cell death. This is exemplified by a delayed reduction in infarct size in animals in which iNOS is blocked (799).

In permanent ischemia, the infarct is first seen after 3–12 h (76, 137, 276, 355, 359, 814, 860). The wide time range appears to reflect strain differences, with the early infarct delineation seen in Sprague-Dawley rats (860) and the later delineation seen in Wistar rats (355), possibly because the lesion is less uniform in the latter (351, 355). The infarct begins in the core but reaches close to its maximal size, which includes core and penumbra, 6–24 h after the onset of ischemia. The infarct continues to grow after 1 day, although at much slower rate, so that between 24 and 72 h it grows by ~30% in Wistar rats (355). Thus damage continues to increase in penumbral regions.

Different durations of temporary ischemia lead to graded involvement of different regions in the infarct as measured after 1–2 days reperfusion (752). Ten to twenty minutes produces scattered dead neurons in the core (653, 752), whereas 1 h leads to infarct in the core (354, 526, 752). Infarct in the penumbra develops fully when the temporary occlusion is 2–3 h (354, 526, 752). In this case, the size of the infarct is the same as it is in permanent ischemia (e.g., Refs. 752, 1283), although it develops slightly more slowly (1283). After ~7 days, there is almost complete loss of cellular elements in the infarcted region; this is termed pan-necrosis. As little as 30 min of temporary ischemia also leads to an infarct in the core region, but this only first appears after 3 days and takes 2–3 wk to mature to its full size (276).

In summary, development of the infarct is quite rapid, but it matures over several days, indicating a significant delayed component of damage in tissue where the insult intensity is relatively small. As indicated above, expansion of the infarct indicates expansion of the region in which the large preponderance of cells are damaged. The relationship of infarct to cell death is considered in section 16G.

2. Specific cell changes during development of the infarct

Other changes associated with the infarct provide more specific insight into the nature of the progression of damage and provide other ways of delineating the lesion.

Diffusion weighted magnetic resonance imaging
(DWMRI) delineates the core of the (future) infarct as early as 1 h after the onset of ischemia, and at later times, DWMRI quite faithfully defines the developing infarct (151, 362). It is very likely that the altered signal intensity represents regions of tissue in which there is intracellular edema (nicely discussed in Ref. 362).

Staining frozen sections with 2,3,5-tetraphenyltetrazolium chloride (TTC) (359), which is colored red by electron transport in active mitochondria, defines a region in which mitochondrial function is severely compromised. Damage is not necessarily irreversible. The region is slightly smaller than the infarct size defined by hematoxylin and eosin (H and E) at early times but overlaps the H and E staining at 24 h (955).

Finally, the loss of mitogen-activated protein (MAP) 2 immunostaining, which is almost certainly a reflection of increased cytosolic Ca\(^{2+}\) and activation of calpain (Y. Zhang and P. Lipton, unpublished data) quite faithfully follows the development of the infarct as defined by H and E staining (233). Thus several markers of cell dysfunction parallel the expansion of the infarct as measured by loss of Nissl stain, indicating that the cellular elements that are poorly stained are metabolically compromised.

3. Individual cell death within the infarct

It is critical to relate the infarct to cell death. Individual cell damage within the infarct develops slowly, but more rapidly than delayed cell death in global ischemia, suggesting that the prolonged focal insults have more impact than the shorter, more intense global insults. The best descriptions of this cell damage have come from Garcia and co-workers (351, 355, 1283). Cell death, defined as eosinophilic shrunken neurons (advanced ischemic cell change) or neurons showing autophagocytotic morphology (178), and also neurons showing apoptotic morphology (178, 652) (see Fig. 1), first becomes significant at \(\sim 6–12\ h\) in the core of the lesion, with a major increase between these two time points. At 12 and 24 h, \(\sim 80\%\) of the neurons have suffered cell death. A similar although less extensively described time course is seen in spontaneously hypertensive rats after 80 min of ligation (202). Although development of cell death in the penumbra appears to lag development in the core, it is hard to be sure, from the data provided. A less-detailed time course study found that 2 days after 2-h focal ischemia, 55% of the cells in the core and penumbral infarcts were dead, whereas 30% were damaged but did not show signs of death; 15% appeared healthy. These may represent different cell types, but that was not discussed (656).

At least for the first 72 h, the rate and extent of developments of cell death caused by 2-h temporary ischemia are the same as they are for permanent ischemia (1283). Furthermore, the number of apoptotic cells in the penumbra is the same in the two models throughout this 72-h period (785).

4. Evaluation of protective treatments in focal ischemia

Inferred mechanisms of focal ischemic damage are almost always based on the assumption that when a protective treatment reduces infarct size it is blocking the process of ischemic cell death in neurons.

For the early infarct (before pan-necrosis), on which most measurements are made, the implicit assumption is thus that the uniform pallor and other indicators within the infarct largely result from neurons so severely damaged that they will die or have already died. Overall, this is justified in that a large percentage of neurons within the infarct die after 24–72 h, at least in Wistar rats. Thus, if a treatment reduces the radius of the early infarct, it is very likely to be slowing down cell death in penumbral neurons. On the other hand, in at least one case, infarct size was reduced after 48 h when delayed protective treatments were applied (1278), indicating that the majority of cells at the borders of the infarct are not yet dead after 48 h (1278). Overall, it is most satisfactory to measure infarct size after 72 h, but this is often difficult to do.

In general, there may not be a precise 1:1 correspondence between infarct size and the amount of cell death. Infarct requires a certain percentage of the cells to die (as witnessed by the peri-infarct area that has cell death but no infarct), indicating that the infarct in a certain region may be abolished but that significant cell death may well remain. It would be much more useful for determining cell death mechanisms if cell counting as well as infarct size were used to assess cell death whenever possible. This being said, the infarct size provides a reliable semi-quantitative measure of the extent of neuronal death and is far easier to measure than individual cell death.

5. Full development of the infarct into pan-necrosis

The final stage of infarct development in focal ischemia is pan-necrosis, in which the neuronal death is accompanied by glial and vascular cell death and loss of cellular elements. This does not occur in most global models that lead to widespread neuronal death, although it does occur in extremely insulting conditions, such as low pH (522) or a prolonged very intense insult (259). It occurs about 1 wk after most focal insults. The basis for pan-necrosis is not clear, but for some reason, both the endothelial and glial cells become major targets, suffering early changes and eventual death (914). In hyperglycemic global ischemia, which leads to infarct, glial pH falls to 4.3 (607), a level that can kill these cells (608). No measurements were made of endothelial cell pH. It would be very useful to measure focal ischemic effects on glial (or endothelial cell) pH to see whether drastic lowerings might explain development of the infarcts in those conditions.

During prolonged hypoxia/ischemia, which also leads to infarct, there was an early activation of pinocytic transport across the endothelial layer, which was quite
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specific to conditions in which infarct was developing (895, 898). This might be important, but it is not known whether it occurs in focal ischemia. Direct exposure of brain to free radical-generating systems leads to infarction-like changes (174), suggesting that prolonged production of free radicals may be leading to pan-necrosis, but there is no explicit evidence. Thus there are reasonable lines of investigation of the pan-necrotic mechanism. It does not seem to relate to neuronal death because it occurs so late, but it might.

H. Role of Intraischemic Depolarizations in Focal Ischemic Damage

The bases of the intraischemic depolarizations or spreading depression-like depolarizations (818) discussed previously are not completely known; they may well be the spread of increased extracellular K\(^+\) and glutamate from the core of the lesion (463, 818). Their frequencies are halved by either NMDA or \(\text{DL-}\)\(-\)\(\alpha\)-amino-3-hydroxy-5-methylisoxazole-propionic acid (AMPA) antagonists, indicating that glutamate-mediated depolarization contributes markedly. The evidence linking the development of damage in the penumbra to these depolarizations is extensive but is largely correlative (47, 151, 462, 463, 818). For example, factors that reduce the frequency of the intraischemic depolarizations, such as glutamate antagonists and hypothermia (462, 818), as well as blockade of iNOS (1031), reduce the size of the infarct that develops in the penumbral region. The most persuasive direct evidence that they are damaging is that artificially increasing the numbers of depolarizations during ischemia, by about twofold, using either KCl (151) or electrical stimulation (47), increased the size of infarct, and peri-infarct damage, by 30–100%, respectively. In one case, this was measured 24 h after distal MCA occlusion and in the other 2 h after the onset of proximal MCA occlusion, using DWMRI (255). Thus the artificial depolarizations do appear to be damaging in ischemic conditions, and this suggests that the endogenous depolarizations are also damaging. Unfortunately, it is very difficult to specifically target the latter with a drug, to more directly establish their importance.

The basis for the putative damaging effect of the depolarizations is not known; similar magnitude and duration depolarizations are not damaging in nonischemic tissues (818), although in those cases there is an increased blood flow that may help to maintain energy levels. One possibility is that they lower ATP levels because blood flow is reduced and that this is damaging. There is evidence, from an abstract published several years ago (1101), that the depolarizations are responsible for the reduction in ATP in the penumbra. However, ATP only falls to \(~70\)%, and it is not clear at all that this would be damaging during the 5-min depolarizations. It would be very useful to measure the effects of the artifically increased frequencies of intraischemic depolarizations on ATP levels to see if there were a correlation between the latter and the increased damage.

I. Role of Leukocytes in Focal Ischemic Damage

One of the most intriguing lines of study to develop over the past several years has been three lines of evidence that white blood cells contribute to focal ischemic damage after temporary insults (41, 204, 248, 431, 592). These are 1) the appearance of neutrophils in ischemic tissue at an appropriate time; 2) the protection afforded against damage by agents that should prevent the accumulation or activation of neutrophils and 3) known damaging actions of these cells when they accumulate in tissue (1200). Effects of various manipulations suggest that the neutrophils are important in temporary, but not permanent, focal ischemia (203, 775, 1285). The evidence is generally strong and is bolstered by the probable involvement of neutrophils in ischemic injury in other tissues (399, 933). However, there are important conflicting reports and alternate interpretations of data that need to be considered. Furthermore, it remains to be determined whether damage results from effects on blood flow or from direct toxic effects on the vasculature or the neurons.

I. Accumulation of neutrophils in ischemic tissue

There is an accumulation of neutrophils in infarcted tissue 24 h after permanent or temporary ischemia (63, 120, 542, 1244). At this time, neutrophils are present in the vasculature and have, in most cases, infiltrated the parenchyma (63, 353). A very notable exception to the latter is human stroke where neutrophils accumulate early in severe cases (9) but, in the one relevant study, were almost exclusively confined to the vessels even more than a day after the stroke (665).

If it helps cause neuronal damage, neutrophil accumulation should be present well before 24 h after ischemia, and this does seem to be the case. Polymorphonuclear leukocytes accumulate in blood vessels within 4 h of the onset of permanent (353, 592), or temporary (563) ischemia (the latter measurements made in baboons). Perhaps the most striking evidence correlating neutrophil accumulation with damage emerges from studies using PET scans on humans. Accumulation begins within 6 h of the stroke, and there is a striking positive correlation between the amount and duration of leukocyte accumulation and both the size of the infarct and the severity of neurological outcome (9).

An important issue is whether the neutrophils act after infiltration through the blood vessels. Significant infiltration into the parenchyma only begins 12 h after the onset of permanent occlusion, by which time there is
major cell necrosis (353, 1283), but it is more rapid following temporary ischemia, the insult for which there is evidence that neutrophils are damaging. There was significant accumulation 6 h after 1- to 2-h occlusion in two different studies (202, 542, 1283), which increased by 50% at 12 h (1283). This time course is consistent with parenchymal neutrophils causing cell damage. However, in one of the studies, the location of the early accumulation was not described (202) and in the other the accumulation did not reach significance in cortex at 6 or 12 h (1283); cortex is the region in which damage is prevented by antileukocyte treatment. Thus there is no well-documented evidence that accumulation in the parenchymal tissue is likely to be causing damage.

In contrast to the large number of results showing timely accumulation of neutrophils in at least the vasculature, Bartus and co-workers (435) found that there was no significant neutrophil accumulation until 21 h after 2-h distal MCA occlusion (1283). This time course is consistent with parenchymal neutrophils causing cell damage. Two very careful sets of studies on spontaneously hypertensive rats, using essentially identical methodologies, showed either a large neutrophil accumulation 6–12 h after 80 min of MCA occlusion in the penumbra (63, 202) or no neutrophil accumulation except in severely damaged tissue after 60 min of MCA occlusion (642). In the latter, there was a major activation of microglia/macrophages ~6 h after the occlusion. Thus as yet unidentified factors may determine the nature of the inflammatory response to an insult.

It has been suggested that artifactual mechanical effects of the insult may actually cause the neutrophil accumulation (for example, filament occlusion or laser irradiation) (435), and this has not been ruled out by careful studies. However, neutrophils do accumulate in the vasculature after human stroke (9, 665), where there is clearly no mechanical artifact. At present, there is no simple explanation for the conflicting data on neutrophil accumulation in rodents. Certainly they suggest that these white blood cells may not always be important in damage.

2. Mechanism of neutrophil accumulation

Neutrophil accumulation in vessels requires interactions between several adhesion molecules. These include intracellular adhesion molecule (ICAM)-1 and E- and P-selectins on the endothelial cells, fibronectin and laminin in the extracellular matrix, and integrins and E-selectins on the white blood cells. There are excellent short reviews of the process (204, 964, 1216) and, indeed, antibodies and peptides that bind to these molecules profoundly inhibit the accumulation of neutrophils after transient or permanent ischemia (197, 1243, 1245, 1285, 1287).

Ischemia activates this adhesion system. On endothelial cells, ICAM is increased 2 h after 2-h MCA occlusion (542, 1286) and also after human stroke (665). E-selectin is upregulated some time between 4 and 24 h (427, 1287). On the neutrophils, the integrin complex CD11/CD18 may also be upregulated, although this is less certain (63).

There is reasonable but very incomplete evidence that one or both cytokines, interleukin (IL)-1β and tumor necrosis factor-α (TNF-α), which do upregulate ICAM (965), are mediators of this response. It seems likely that there is a cascade involving free radical generation, increased TNF-α and IL-1β, and the activation of the nuclear transcription factor NFκB (1186). The NFκB upregulates more proximally than the cytokines and is probably a critical intermediary step. Other factors are also probably necessary (56). There may be important positive feedback between the cytokines and NFκB and free radicals as they all are able to activate each other (1186), and it is not clear which of these is the initiating step.

Tumor necrosis factor-α is increased within 30 min of the onset of focal ischemia in the circulation and in brain tissue (154, 636), as is IL-1β (1295). The only study directly assessing the importance of the cytokines showed that treatment with the IL-1 receptor antagonist reduced accumulation of neutrophils during permanent MCA occlusion (352). Less directly, but still significant, blockade of TNF-α action with a recombinant receptor greatly enhanced the number of perfused microvessels at the end of 4-h permanent MCA occlusion (412).

The postulated interaction is supported by studies in cultured endothelial cells (1035). Hypoxia induced the formation of IL-1, the subsequent expression of ICAM and E-selectin, and increased adherence of leukocytes. Antagonizing IL-1 action attenuated leukocyte adherence by ~60% (unfortunately adhesion molecule changes were not explicitly measured). A similar effect was observed in microvascular cells from human brain (1068). Further studies on these cells show that activation of the transcription factor NFκB is very probably critical to the expression of ICAM. Hypoxia and reoxygenation caused activation of NFκB and, later, ICAM (464). Both these responses were blunted by the proteasome inhibitor n-tosyl-Phe-chloromethylketone (464), which blocks the activation of NFκB. The ICAM upregulation was also blocked by the free radical scavenger pyrrolidine dithiocarbamate, and cerebral endothelial cells in culture produce abundant free radicals under these conditions (1226).

Although evidence for protective effects of blocking cytokine action is now abundant (see sect. vmlE), this cannot be taken to show that leukocyte accumulation is damaging; blockade of cytokines reduces tissue damage even in permanent ischemia (352, 946), against which adhesion antagonists are ineffective (203, 775, 1285).
3. Effects of preventing leukocyte accumulation

A very large number of studies show that conditions that reduce or completely block leukocyte accumulation greatly reduce infarct size. Infarct volume 24 h after 45- to 60-min ischemia was reduced by about two-thirds when circulating neutrophils in mice (215) or in rats (729) were completely depleted with an antineutrophil antibody. Inhibiting the activity of the endothelial adhesion molecule ICAM, either by creating null mice (215, 1062), or using monoclonal antibodies in rats (197, 730, 1285), strongly reduced neutrophil accumulation and reduced infarction size by 50% (rats) to 75% (mice). Slightly smaller reductions, ~33%, were produced in rats by antibodies to the neutrophil integrins CD10/CD18 (197, 730) and by peptides that interfere with fibronectin binding (1243, 1244) or laminin binding (1245). A peptide that binds to selectin (775) decreased infarct volume by >50% in spontaneously hypertensive rats. Many of these studies involved 45-min to 1-h exposures to ischemia, but in some, ischemia was 2 or 3 h so the protective effects are not restricted to short insults. These effects are quite dramatic; there is essentially no further development of infarct once reperfusion begins.

Importantly, leukocytes do not appear to be involved in damage during permanent focal ischemia, despite their accumulation in the tissue vasculature (120, 542). Several interventions against this insult, which are protective against temporary ischemic damage, were ineffective in preventing damage (203, 775, 1285).

Although these studies taken together are impressive indictments of neutrophils in ischemic damage, there is at least one exception, which has not been explained away. In the study where no neutrophil accumulation was measured, neutropenia had no protective effect (435). Because of this, it is important to consider alternate explanations for the effects of the antileukocyte accumulation studies.

Neutrophil depletion may be protective as a result of lowering blood levels of damaging cytokines, TNF-\(\alpha\) and IL-1\(\alpha\), that can be produced by these cells (280). Measurements of the effects of neutropenia, or other protective treatments, on cytokine levels during ischemia would help interpret results. The adhesion molecules and basal laminar proteins may also allow critical damage to vasculature or neurons in ways other than via leukocyte accumulation. Indeed, there is a marked depletion of lamin and fibronectin that is thought to contribute to microvascular damage (416). Perhaps the laminin and fibronectin peptides that attenuate damage (1243, 1245) are acting by maintaining vascular integrity. Although these explanations are not particularly likely, it is important to ensure that they, or others, are not correct. This has not been done.

4. Mechanisms of damage

Leukocytes might induce damage by causing local vascular occlusion, or they might initiate toxic reactions, including free radical production by NADH oxidase, production of hypochlorous acid, or protease activation (504, 728, 1200). The latter two require prior free radical production.

A) CHEMICAL TOXICITY. Efforts to determine whether free radical production is important have not been conclusive. The one study in which effect of neutrophils on free radicals was actually measured, carried out by Kogure and co-workers (728), showed that neutropenia completely abolished the generation of free radicals in cortex after 60-min MCA occlusion. This very dramatic effect was seen by measuring formation of ascorbyl free radicals (728), a reliable semiquantitative method (143). Unfortunately, this is the only such report, and the only study in which this method has been used to measure free radical formation. Further experimental support for the conclusion is very necessary as it seems, a priori, unlikely that neurons and glia themselves do not produce free radicals during and after ischemia. If it is correct, it ascribes a very important role to neutrophils in damage, as free radical attenuation produces major reductions in infarct size. However, at this stage, it cannot be considered likely.

Neutrophils produce free radicals via activation of NADPH oxidase. Walder et al. (1185) studied transgenic mice lacking NADPH oxidase. Infarct size was reduced by 50%, suggesting that leukocyte NADPH oxidase mediated the damaging effect of the neutrophils. However, Walder et al. (1185) then found that selective elimination of either leukocyte or parenchymal NADPH oxidase did not reduce damage; both had to be eliminated. Thus elimination of neutrophil NADPH oxidase does not account for the protective effects of eliminating neutrophil accumulation; if it did, then eliminating the enzyme selectively in neutrophils would have prevented damage.

An hypothesis that is consistent with both sets of studies is that neutrophils are necessary for the generation of toxic levels of free radicals but themselves are not the major source of the free radicals. Thus large-scale free radical production would be dependent on leukocytes, as noted by Kogure and co-workers (728), but could be initiated by NADH oxidase in parenchymal tissue or in the leukocytes. The way in which leukocytes might lead to free radical generation is unknown. It might be by producing cytokines (280, 349) which then activate free radical and NO production by parenchymal tissue or by leukocytes themselves (177, 671). Certainly, the data of Walder et al. (1185) suggest a central role for NADH oxidase. Unfortunately this has not been explored further.

B) INDUCIBLE NOS. The inducible form of the enzyme, iNOS, begins to appear in neutrophils that have invaded the ischemic tissue between 1 and 2 days after the onset of permanent focal ischemia (480). Any role for
this iNOS is very questionable because blocking neutrophil ischemic accumulation has no effect on permanent focal ischemic damage (1285). Thus the fact that aminoguanidine (AG), which inhibits iNOS, attenuated the increase in infarct size that occurred between 24 and 72 h (481) may well be due to inhibition of the iNOS induced in endothelial cells (479, 836). Alternatively, AG may be protective because it is inhibiting another enzyme, for example, polyamine oxidase, which is very damaging (208, 498). Although protection was eliminated by the NO precursor arginine (481), this does not necessarily show that inhibition of NOS by the AG was giving the protection. The excess NO production due to arginine might have overwhelmed the effects of inhibiting another damaging reaction.

Alternatives to free radical-mediated damage are damage mediated by hypochlorous acid produced by myeloperoxidase, or protease activation; they have not been explicitly tested.

C) BLOOD FLOW REDUCTION. The alternative to chemically mediated damage is that leukocytes block blood flow (no reflow). White cell accumulation does appear to attenuate blood flow in the early postischemic period (63, 775). However, evidence that this is damaging is not at all compelling because, as discussed in section IX, there is no evidence that postischemic penumbral flow is limiting. One hour after 3-h ischemia, 40% of the small (<6 μm) capillaries appear to be plugged by leukocytes (247) but at many later time points after 2-h ischemia there was much less vessel plugging (~10%) in core or penumbral regions (1283). Indeed, plugging was far less than during permanent ischemia, where it was ~50% (353, 1283). Thus, if vessel plugging is important, it is surprising that antileukocyte adhesion paradigms do not reduce infarct size in permanent ischemia.

Hemispheric blood flow 24 h after ischemia was improved by antileukocyte treatment (e.g., Ref. 215). However, this may well reflect the attenuation of infarct size and hence more actively metabolizing tissue in the protected animals.

5. Role of leukocytes and microglia in global ischemic damage

There is no evidence that leukocytes enhance damage after normal short global ischemic episodes. There was a delayed accumulation of leukocytes 3 h after a global insult, but only if ischemia was extended to 40 min or longer, far longer than the 10 min necessary to get major damage (20). When neutropenic rats were challenged with 15-min global ischemia, there was elevation of blood flow in the postischemic period relative to control rats, but there was no amelioration of damage to CA1 or CA3 neurons (1007).

On the other hand, there is proliferation of microglia in damaged regions within a day after short-term global ischemia, which is prolonged in regions that will go on to suffer damage (512). Intriguingly, when proliferation of the microglia is prevented by tetracycline derivatives, minocycline, or doxycycline, damage after 5-min ischemia in gerbil is attenuated by 75% (1265). Although this is consistent with an important role for microglia in damage, it does not prove it. Nevertheless, the fact that microglia are major sources of caspase-1 (ICE) by 2 days after ischemia (94) and so may contribute to IL-1 production and iNOS production (1265), indicate that these cells may play an important role in damage (512).

6. Summary

The large number of cases in which agents that prevent leukocyte accumulation also attenuate damage in the penumbra is strong evidence that this accumulation somehow makes a major contribution to damage after temporary focal ischemia. The activation of accumulation is probably initiated by ischemia-induced cytokine synthesis in endothelial cells or glia, although the evidence here is not extensive. The trigger for the very early cytokine synthesis is not known.

There are two caveats to the conclusion. The first is the existence of one very well-documented case and one somewhat less well-documented case in which there is no neutrophil accumulation, and in which neutropenia had no protective effect. This indicates that accumulation does not always occur and that it may depend on the nature of the insult in ways that have not yet been determined. A systematic study of neutrophil accumulation and damage size following different methods of occlusion would be very helpful. Although artifact may contribute to accumulation, the accumulation in (nonartifactual) human stroke certainly shows that the phenomenon is a real one in important conditions.

The second uncertainty stems from the difficulty in pinpointing a mechanism of damage. This results firstly from the lack of a definitive answer as to whether there is a timely invasion of the parenchyma so that it is not known where the leukocytes are acting. Second, it has not been determined whether free radical (or other biochemical) action, or effects on flow are the damaging principles, or whether blood vessels or neurons are the targets of any biochemical action.

A simple model that reconciles some difficulties is that leukocyte accumulation acts as a “catalyst.” In this model, activated leukocytes initiate damaging processes in parenchymal cells or blood vessels by early production of cytokines or other agents, rather than by producing free radicals or by blocking vessels. In this case, actions on flow and on free radical production might mediate damage. Microglia/macrophages may play the damaging role that neutrophils play in some focal models and also in global ischemic damage, but there is no direct evidence for this.
J. Hypoxia/Ischemia

Unilateral carotid occlusion is combined with hypoxia. In adults, the oxygen is lowered to ~3% and leads to eventual infarction in the MCA territory. It is essentially a focal insult that is now used infrequently (895) because adult animals do not survive the prolonged hypoxia reliably enough (948). However, the technique has been very successfully adapted to neonates that can reliably survive for days after insults of up to 3.5 h, and it is considered a very good model for major forms of neonatal metabolic brain damage (1164). Oxygen is generally lowered to 8% (for review, see Refs. 948, 959, 1141). This is almost a pure hypoxic insult, because the hypoxic vasodilation brings blood flow up to near control levels on the ligated side (982), although lateral cortex and caudoputamen still show blood flow reductions (368).

Fifteen to thirty minutes of hypoxemia (3%) causes early ischemic cell change and delayed neuronal death in CA1-CA3, striatum, and layer 5 of cortex in adults, much like global ischemia (980). In young rats, exposures of 60 min or longer to 8% hypoxia cause delayed development of infarct providing ATP levels fall to between 50 and 70% of control values (1213), much like their values in focal ischemia.

K. Postischemic Blood Flow and Damage

I. Global ischemia

There are major changes in brain blood flow after global ischemia. It is important to know if they exacerbate damage because drugs used to study damage mechanisms often affect blood flow also.

There is a rapid hyperemia following vessel occlusion or complete ischemia, which lasts ~15 min, where blood flow may be as much as a two- to threefold higher than control levels (228, 515, 931). There is then almost (1180) a marked hypoperfusion that lasts between 6 and 24 h, with blood flow between 30 and 50% of normal (228, 291, 297, 515, 632, 760, 931, 1070).

The literature strongly suggests that this postischemic hypoperfusion does not cause damage; rather, it appears to reflect a reduced metabolic rate in the postischemic tissue. The latter may reflect mitochondrial damage or reductions in ATP-utilizing processes. Whatever its basis, there is an excellent correlation between the lowered glucose utilization and the hypoperfusion after global ischemia in dog (291, 756) and rat (806, 931). pH, ATP, and lactate levels are about normal (228). Neutrophil depletion had no effect on the marked hypoperfusion in hippocampus and striatum, two very vulnerable structures (396), further indicating that the hypoperfusion in these vulnerable regions is metabolically driven. Neutrophil depletion did increase flow somewhat in nonvulnerable structures (396).

In contrast to the above studies, when ischemia is prolonged beyond the time required to cause major neuronal death there is a larger reduction in blood flow than in glucose metabolism or high-energy phosphate utilization, in tissues which will become damaged (367, 645, 646). In one of these cases, the region that suffered damage, the striatum, was the region with the largest dissociation between flow and metabolic rate. Although the mismatch may contribute to damage in extreme cases, shorter ischemic exposures are lethal in the absence of any mismatch between flow and metabolism.

2. Focal ischemia and hypoxia/ischemia

There is no compelling evidence that reduced postischemic flow following temporary focal ischemia contributes to development of the infarct. Core regions have normal blood flow levels (137, 798), whereas flow in the penumbra is generally ~40–50% of normal during the 12 h after temporary focal ischemia, when damage is developing (184, 798). However, as with global ischemia, there is no evidence for a mismatch between flow and metabolism. In penumbral regions destined for infarct, glucose utilization is decreased by 50% or more in the first hour after ischemia, and recent detailed studies show a strong correlation between the decrease in blood flow and in glucose metabolism in the postischemic period (79, 1299). In the few measurements that have been made, ATP levels during this time are between 70 and 100% of their preischemic values, only a small reduction. One study showed a reasonable correlation between increased postischemic blood flow and reduction of early infarct size when prostaglandin synthesis was inhibited (184). However, there are many other potentially beneficial effects of blocking prostaglandin metabolism aside from vasodilation, for example, preventing free radical production. Thus the experiments do not establish the importance of postischemic blood flow, and the general preservation of metabolism-to-flow ratios argues against reduced flow as a cause of damage. This was also discussed in section IV.

3. Summary

At this stage, there is no evidence that damage is increased by reduced blood flow, after either global or focal ischemia. The evidence is quite consistent with reduced postischemic metabolism driving the decreased blood flow. Thus increasing blood flow in the postischemic period should not, in and of itself, be protective.

This conclusion should not obscure the potentially critical sensitivity of damage to blood flow during the insult, particularly in focal ischemia where the penumbra has flow rates that range from very damaging to barely damaging. Effects of drugs on flow rates during ischemia may be critical to damage.
1. Effects of Ischemia on the Vasculature

The role of vascular change, and resulting edema, will not be considered at any length in this review because it really is its own topic. Nevertheless, it is useful to have a general idea of what contribution vascular changes may make to damage. Certainly capillary endothelial cells are quite vulnerable to short periods of anoxia and reperfusion. Cultured brain endothelial cells are damaged in a free radical-dependent manner after 20 min of anoxia, as assessed by a large increase in lactate dehydrogenase (LDH) leakage (1226).

1. Global ischemia

The blood-brain barrier becomes severalfold more permeable to poorly permeant small solutes such as inulin and sucrose 6 h after global ischemia but then returns to normal by ~24 h (922). There is little evidence of major uptake of protein into the brain during much of the post-ischemic period, except immediately after the insult. However, starting ~12–24 h before delayed cell death, there is a quite massive uptake of horseradish peroxidase or protein-bound Evans blue into the parenchyma (370). This may well result from activation of pinocytosis in the endothelial cells because there is no major extracellular edema and there are no reports of major cell damage to the endothelium. There is no indication as to whether the increased blood-brain barrier transport contributes to damage.

2. Focal ischemia

Focal ischemia is quite different. There is a major extracellular edema that develops early during the focal ischemic insult, largely due to activation of Na⁺ transport across the blood-brain barrier (91). Reperfusion and very prolonged permanent ischemia both cause much more dramatic changes. There is damage to the endothelial cell layer, along with extravasation of protein and protein-bound Evans blue, as early as 30 min after recirculation following 60-min ischemia. This does not occur during at least 6 h of permanent ischemia (527, 800, 1247). The dependence of this vascular damage on reperfusion is striking; it may result from enhanced free radical production (849), but this has not been proven. This endothelial transport change very probably contributes to edema also (1247).

The relationship of the vascular damage and extracellular edema to cell death has not been well studied. Some evidence indicates that at least early neuronal death occurs in the absence of vascular changes. In a study specifically directed at this question, there was significant ischemic cell change 6 h after 1-h focal ischemia in caudate putamen, but there was no evidence for extravasation of albumin, or leakage of the smaller molecule, amniobutyric acid from the circulation into the parenchyma of the caudate-putamen at that time (10).

On the other hand, recent studies suggest that movement of molecules from blood to brain across the “damaged” endothelium are important in the general development of neuronal damage. A matrix metalloproteinase, MMP-9, was upregulated in endothelial cells during the first 24 h of permanent ischemia, probably by cytokines, and when an antibody to this enzyme was administered systemically, it reduced infarct size after 24 h, by 30% (958). Both MMP-9 and other matrix metalloproteins act on the extracellular matrix associated with the vascular basement membrane, and it is reasonable to assume that protection by the antibody results from inhibition of basement membrane breakdown and maintenance of vascular integrity. Further studies are needed to show that vascular integrity is indeed maintained when the enzyme is inhibited. It would be of much interest to make a similar study in temporary ischemia, where vascular integrity seems to be most compromised.

Although there is no established causal relationship between edema and neuronal damage, there is a very strong correlation between infarct size and the amount of hemispheric edema when protective, or damaging, agents are used to alter cell death (e.g., Ref. 527). Indeed, edema is often used as a measure of damage. Effects of edema on blood flow might be important; for example, it may partially account for the progressive decrease in blood flow in the penumbra during occlusion, discussed above. However, at this stage there is no evidence that edema contributes directly to cell damage. Increased edema may simply reflect local blood-brain barrier damage caused by the damaged neurons, glia, and endothelial cells.

M. Effects of Temperature on Damage

1. Artificial maintenance of temperature

There is little spontaneous change in brain or core temperature during focal ischemia except in the filament occlusion model discussed above, where temperature rises.

However, global ischemia is very different. Brain temperature in gerbils and rats drops spontaneously by 3–5°C (152, 762, 1241). Core temperature drops by about the same amount in rats, but actually rises somewhat in gerbils (211, 584, 628). Temperatures recover within ~1 min of reperfusion (152, 762), although in gerbils there is a short period of hyperthermia beginning ~15 min after ischemia and lasting ~45 min. It is 1.5°C in anesthetized and 2.5°C in unanesthetized animals (768). Because of its strong effect on damage, brain temperature should be known or regulated during and for at least 24 h after an insult (see below). This means maintaining the temperature of the brain at a known level during the insult, with a lamp or other device, and it means maintaining or
knowing body temperature after the insult. These precautions eliminate the possibility that treatments will affect brain temperature during the insult via changes in flow or other variables and eliminate the possibility that they will affect core temperature, and hence brain temperature, after the insult. Many agents do the latter when the animal is not regulated. Brain temperature is most faithfully monitored in the temporalis muscle (152). However, when it is warmed by a lamp, the skull faithfully remains \(\sim 1-2^\circ\text{C}\) below brain temperature (211, 762) and is easier to monitor. Although brain temperature is now generally well regulated during an insult (152, 211, 762), there is quite a lot of variability in the duration for which core temperature is maintained after an insult, and this seriously compromises some studies. These cases are pointed out in the review.

2. Effects of brain temperature on damage

Table 2 shows the dramatic effects of different brain temperatures during or after global ischemia on the degree of postischemic damage.

Not shown in Table 2, damage is actually exacerbated when brain temperature is maintained at 39°C (258).

Effects of temperature are manifested for long periods after an insult. Hypothermia is protective when it occurs up to 12 h after 2-VO, and if temperature is elevated to 39°C even 24 h after mild (5–7 min) 2-VO, global ischemia damage is strongly enhanced (49). Thus even the delayed processes involved in cell death are remarkably temperature sensitive. Temperature differences during the insult do not just influence the rate of appearance of damage; they affect the final outcome. For example, hypothermic protection lasts for at least 1 mo in adults and 6 mo in young gerbils (219). Temperature thus strongly determines the damaging potential of insults.

Temperature is also important in focal ischemic damage. Mild hypothermia reduced infarct size by 35% during 12-h permanent MCA occlusion (53). Reducing temperature to 30°C for 1 h after 2-h temporary focal ischemia reduced infarct size, measured after a week, by 50% (1284). Hypothermia (34°C) was very protective against 3-h hypoxia/ischemia in young rats (1235).

These results show that at least one of the major processes involved in ischemic damage is extremely sensitive to temperature. Furthermore, the very long-lasting sensitivity discussed above indicates that a process occurring at least 24 h after the insult is also temperature sensitive. Unfortunately, as is discussed in several of the ensuing sections, a great many potential contributors to ischemic damage are very sensitive to temperature so that no particular mechanistic information is provided by the dependence (see Ref. 62 for a good discussion of this).

<table>
<thead>
<tr>
<th>Protocol</th>
<th>37°C</th>
<th>35°C</th>
<th>33°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 min of 2-VO in rat (763)</td>
<td>95</td>
<td>70</td>
<td>20</td>
</tr>
<tr>
<td>20 min of 4-VO in rat (152)</td>
<td>93</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>5 min of 2-VO in Gerbil (228, 1241)</td>
<td>80</td>
<td>60</td>
<td>0</td>
</tr>
<tr>
<td>After 20 min of 2-VO in rat (180, 210)*</td>
<td>95</td>
<td>45</td>
<td></td>
</tr>
</tbody>
</table>

Unless noted otherwise, temperatures are intracranial, during ischemia. * In this study core temperature was reduced, between 12 and 17 h after ischemic episode. Damage was measured as loss of CA1 pyramidal cells 3–7 days after ischemia. Reference numbers are given in parentheses.

N. In Vitro Models: Brain Slice

1. Nature of insult

Brain slices, and particularly the hippocampal slice, have become widely used models for studying anoxic or ischemic damage (531, 673, 686, 695, 1008). In this insult the bathing solution is rapidly changed from O_2/CO_2 equilibrated to N_2/CO_2 equilibrated. When glucose is maintained in the anoxic buffer, the insult is termed anoxia (or hypoxia), and when glucose is omitted, the insult is termed in vitro ischemia or oxygen/glucose deprivation. Adenosine 5'-triphosphate falls less completely during in vitro ischemia than it does during global ischemia and falls more slowly in the presence of glucose (Table 1). Lowering temperature reduces the degree of damage (949).

2. Nature of damage: comparison with damage in vivo

Generally 5–7 min of in vitro ischemia at 36–37°C, or a period of ischemia extending 2–3 min beyond the anoxic depolarization, leads to rapid damage to various properties of CA1 pyramidal cells which lasts for the 8- to 14-h life of the slice. Somewhat longer exposures are necessary for damage to dentate granule cells so that slices do show the same ranking of selective vulnerability as in vivo tissue. However, differences are not nearly as dramatic because acute damage, rather than prolonged damage, is being measured (532), and acute damages are very similar in different hippocampal regions in vivo (348).

Properties that are damaged include synaptic transmission (531, 1008), protein synthesis (164, 936), maintenance of ATP levels (916), cytoskeletal integrity (1291), and neuronal morphology (936). Damage to all these occur within the first 30 min and persist throughout the reperfusion period (286, 348, 916). Changes in protein synthesis are similar to those observed in vivo, but the profound loss in (evoked) synaptic transmission and the very intense morphological damage and disruption of the cytoskeleton are generally not observed as early or as strongly in vivo, indicating that slices are more sensitive to ischemic damage than are cells in situ. Nevertheless, slices fulfill a very important role by facilitat-
ing analysis of mechanisms of early changes. Although it is always possible that these are different from in vivo, rates of change of ions, metabolites, and protein synthesis that have been measured so far are quite similar to those measured in vivo, suggesting that mechanisms are the same.

Shorter insults, which are stopped just when the anoxic depolarization occurs, lead to a more slowly developing damage, requiring ~12 h to be manifested. This is closer to the delayed neuronal death seen in vivo. To date, only damage to synaptic transmission has been studied in this way (1188), but the approach holds much promise for more sophisticated comparisons between slice and in vivo conditions.

O. In Vitro Models: Cell Cultures

1. Different models

Primary neuronal/glial cultures from cortex (237, 376), hippocampus (704, 1199), cerebellum, and hypothalamus (791) of embryo or perinatal rats and mice have been used extensively to study anoxic or ischemic damage since 1983 (966). Organotypic hippocampal slice cultures from perinatal rats were first used in about 1995 and are being used increasingly (1072, 1073). They promise to become more valuable models, particularly as they show delayed death (923, 1072, 1073). However, although they do show some selective vulnerability of CA1 versus CA3 versus dentate gyrus if the in vitro ischemia is kept relatively short (30 min) (65, 1072), it is not very dramatic. There is twice as much cell death in CA1 as CA3. Longer durations (60 min) produce larger and equal amounts of cell death in all hippocampal regions (947, 1072). Like the cultured cells, this system also requires prolonged in vitro ischemia to produce cell damage (1072).

In most studies the insult is in vitro ischemia, although there are several studies of “chemical” anoxia or “chemical” ischemia in which oxygen is maintained but mitochondrial inhibitors are used (1199). The latter is a poor model, and its results will not be considered here. Free radical generation is almost undoubtedly much higher than during anoxic conditions. Indeed, in liver cells, such damage is often markedly decreased by concurrent anoxia (115, 234). A major difference from damage in vivo is the very long duration of oxygen and glucose deprivation that is generally required to induce cell death. This is true for both primary cultures and organotypic cultures (376, 1072). Although 30- to 60-min deprivation is not long for focal insults, the degree of oxygen and glucose deprivation that cultures are exposed to is similar to that in global ischemia, where 5–10 min at 36°C produces profound delayed damage. There are several possible reasons for the difference. In the widely used model developed by Goldberg and Choi (376), ATP does not fall nearly as much as it does in vivo (Table 1) and, probably relatedly, the release of glutamate is very delayed (376). The small fall in ATP would certainly explain the requirement for the prolonged insult, because it approximates that in the focal ischemic penumbra (Table 1). Why the energy disturbance is less is not apparent, but an intriguing possibility is that the cells in culture may adopt protective mechanisms that reduce ATP turnover during hypoxia as has been nicely discussed for the general case by Hochachka et al. (453). This possibility is given added credibility by the fact that the ATP fall during chemical hypoxia is far greater than during actual anoxia (Table 1).

Another significant difference between cell cultures and in vivo tissue is that damage in primary cultures following the 30–45 min exposures is completely dependent on activation of NMDA receptors (376). Longer insults (90 min) produce an NMDA-independent form of damage that appears largely apoptotic (405). In organotypic cultures, NMDA antagonists provide only partial protection (5), and combined glutamate receptor antagonists are required to protect against 60-min in vitro ischemia (1073). In that sense, the organotypic cultures seem better models for in vivo events and at this stage seem a better route to take.

Although damage in cultures is easily studied, it is, of course, essential to verify any inferences by studies of in vivo tissue. Neuronal gene expression is likely to alter between cells are very different, and there are no vascular cells. The latter two objections do not pertain for organotypic cultures.

P. Summary

1. In vitro systems

The goal of research on ischemia is to understand what occurs in vivo. In vitro systems are able to provide clues, but for reasons discussed above, and others, conclusions cannot be simply extended to in vivo situations. The absence of blood flow as a variable, and the absence of blood vessels themselves in cultures, facilitates interpretations. However, by the same token, the absences eliminate what may be important components of the damage process. It must be borne in mind that all tissue types
suffer ischemic damage, so to show that both neuronal cultures and in vivo brain tissue suffer delayed ischemic damage does not mean that the mechanisms are the same.

The composition of brain slices is closer to that of in vivo tissue, and there is little time for major genetic change. However, slices are in a compromised metabolic state, with low ATP values and elevated aerobic glycolysis (677), and are hypersensitive to ischemic insults. They show different morphologies from in vivo tissue, and the preparation of the slice itself induces transcription of some stress-related mRNA and immediate early genes, as well as accumulation of Fos and Jun immunoreactivities (1303). The consequences of this are not known but may alter ischemic sensitivity. Culture and slice work can be used as bases for later in vivo studies but the latter, eventually, must be done.

2. In vivo systems

Global and focal ischemias are two very different insults. Global ischemia involves a short (usually 15 min or less) very intense insult in which ATP is severely lowered. It is characterized by a slow development of cell death during reperfusion, which shows great selectivity. There is no intimation of involvement of the vasculature, although there is a real possibility that microglia/macrophages play a role in the process. Overall, the insult is straightforward and quite uniform, but in the hands of one group, residual blood flow is much higher than the usual 1–3% (1089).

Focal ischemia is a far more complex insult, with several unresolved issues. There are many variations of the model including whether or not there is reperfusion (temporary vs. permanent), where the lesion is, the strain of rat, and the degree of temperature control. These lead to a very complex literature. One goal of this section has been to describe these variations well enough so that results obtained in different systems can be related to each other.

A) CORE AND PENUMBRA. Ionic and metabolic changes in the core and penumbra are dramatically different, and these probably account for the great disparity in how well the two regions can be protected by pharmacological interventions. The fall in ATP in the penumbra is quite small (levels do not fall much below 70% of control during development of damage in permanent ischemia), and it really is not clear that this fall per se is adequate to cause damage. For example, when damage was initiated by mitochondrial blockers, ATP fell to 25% for several hours, and the lesion developed over 1–2 days (736). A real possibility is that effects in the core add to the insult in the penumbra and are essential for damage. Examples are diffusion of K+ and glutamate into the region. The intrischemic depolarizations that may be important in development of damage in the penumbra may well be driven by ions and glutamate generated in the core. Core damage is terribly severe when ischemia is maintained for >1 h.

B) TEMPORARY VERSUS PERMANENT FOCAL ISCHEMIA. Development of damage after 2- to 3-h temporary ischemia and during permanent ischemia are very similar in many detailed respects. One explanation for this is that all the important events occur in the first 2–3 h so that reperfusion does not have important effects; there is no doubt that this is true to some extent. As an important example, ATP levels in the penumbra after temporary ischemia are about the same as during permanent ischemia; they do not appear to recover very well, indicating permanently compromised metabolism. This might help maintain the similarity between the two insults. However, there are important differences; free radical and NO generation are both enhanced during the first 40–60 min of reperfusion (622, 893). Vascular damage is much greater after temporary ischemia. The most glaring difference is the apparent importance of leukocytes in damage following temporary ischemia. In some way, these cells appear necessary to allow damage to continue to develop in the reperfusion period. They are not necessary for development of damage in permanent ischemia.

C) GLIAL AND VASCULAR RESPONSES. The astroglial responses to focal and global ischemia are very different from each other. Global ischemia is characterized by quite rapid glial hypertrophy, increased protein synthesis, and hyperplasia (642), whereas the more prolonged focal ischemia is characterized by quite rapid glial swelling and, eventually, cell death during formation of the infarct (355). Glial cell death in the core, and possibly penumbra, of the infarct might well result from combination of lowered pH and ischemia; combining pH 6.2 with respiratory chain inhibition kills glia cells in culture within several hours (1090). It is important to consider that glial damage may, in some way, be enhancing neuronal damage.

Roles for vascular changes and extracellular edema in development of damage in focal ischemia are not yet compelling, although evidence that a metalloprotease that attacks the basement membrane is likely to be important in damage raises the issue of the role of the vasculature. There is currently no resolution.

3. Some outstanding issues

There are important technical issues in evaluating results from the models. The most important technical issue in producing reliable data on protective effects of drugs is careful and prolonged temperature control; control for at least 24–48 h after an insult now seems necessary. This is difficult to do and so is usually not done. Another key issue is determining whether artifactual vascular damage occurs in some focal ischemic insults and alters the sequence of events. Finally, the relationship of infarct size in to neuronal death in focal ischemia needs to be clarified as long as it is the former that is generally...
measured. To what extent does reduction in infarct size after 24 and 48 h reflect protection against neuronal death?

Of paramount importance is the question of how well these animal models replicate events during and after human stroke. This issue is outside the scope of this review. However, the difficulty to date in successfully applying principles learned from these models to human therapy trials suggests that there are added complexities in the human disease. These may include greater sensitivities to detrimental side effects of some drugs, such as MK-801 and its analogs (135, 299), or more complex processes of cell death that cannot be halted by only one pharmacological intervention.

III. MORPHOLOGIES AND BIOCHEMISTRIES OF ISCHEMIC CELL DEATH

As discussed in section I, there are at least three recognizable pathways of ischemic cell death: necrotic cell death, apoptotic cell death, and, very probably, autophagocytic cell death.

The purpose of this section is to describe the end stages of these pathways and to understand why different pathways are taken in different cases. Another elusive goal is to understand the molecular changes that constitute these end stages.

A. Morphological Characteristics of Necrotic Cell Death

Most of the morphological changes associated with ischemic cell death were defined by Spielmeyer in the early part of the 20th century (1063) and led to work in the 1960s and 1970s by Brown and Brierly (129, 130, 355) that quite rigorously defined different stages of cell damage including forms of cell death. The earlier work, as well as that of Brown and Brierly, has been nicely reviewed (121, 128, 130, 392). There are three categories of necrotic cell death.

1. Edematous or pale cell change (ECC)

   a) Description. The cytoplasm is very swollen. Some mitochondria are swollen with disrupted cristae, but others are rounded and slightly shrunken. Endoplasmic reticulum, the Golgi apparatus, and polysomes are no longer apparent as complete structures but remnants, often with attached ribosomes that exist and often accumulate close to the nucleus. Microtubules and other filamentous structures are absent, and the cytoplasm is almost clear. The plasma membrane is irregular and sometimes shows frank breaks, but this is very rare. The nucleus is normal except for irregular clumping of chromatin (520, 521, 942). These changes have some characteristics of what is termed peripheral chromatolysis (392, 897).

   b) Occurrence. This end stage is not seen very often. It is predominant after 3- to 4-h hypobaric/ischemia in young animals (490), in whom it is also produced by glutamate exposure (490, 852, 920). Agonists of NMDA induce it in striatum of adult brain (919). It occurs 90 min after 30- or 10-min global vessel-occlusion ischemia during hyperglycemic conditions (494, 522) and occurs in the final stages of delayed death in gerbil and rat global ischemia, where the existence of some “swollen cells” is described, along with other end-stage morphologies (579, 582, 897, 942) and where, in one case, electron micrographs show this morphology (897).

2. Ischemic cell change (ICC)

   a) Description. This end stage contrasts markedly with ECC and is far more common. In its more exaggerated phase, it is termed ICC with incrustations (128). There is a major darkening and shrinkage of the nucleus and cytoplasrn (128, 130, 494, 521, 896, 897), and the nucleolus often assumes a honeycomb appearance (319). The plasma membrane and the nuclear membrane are both very irregular, and the cell often assumes a triangular shape (in 2 dimensions). The cytoplasm generally contains many large (1–2 μm diameter) vacuoles, some of which are greatly swollen mitochondria with disrupted cristae (130), but most of which are swollen Golgi cisternae (896), ER (130), or unidentified. Many of the latter may well be autophagic vacuoles (128, 834). Normal ER and Golgi organization into stacks is disrupted. There are few, if any, polysomes, but there is a plethora of ribosomes. At least some microtubules are present. This end stage and its incidence are very elegantly described in a recent review (716).

   Viewed in the light microscope, these cells are intensely acidophilic (eosinophilic), which is a defining characteristic of this morphological stage; they are also intensely argyrophilic (223, 437).

   b) Occurrence. This end stage is prominent in many ischemic models. The cells coexist with the edematous cells in CA1 at the end of delayed degeneration in rat and gerbil global ischemic models (579, 582, 897), where frank eosinophilia is very apparent (889). They are the dominant form in the quite rapid (within hours) death that occurs in CA4 after global ischemia in gerbil (579) and rat (896). During or after focal ischemia, cells showing this change emerge and remain for several days in the peri-infarct cortex (814). They are abundant in the penumbra for 1–2 days and in the core of the lesion for a relatively short time before the cells disintegrate. They are widespread in adult rats after 40 min sporadic hypoxia/ischemia (121) where they take ~30 min to 1 h to appear after 40-min hypoxia and where they persist for ~24 h. Thus they constitute a relatively stable state toward which cells
tend following ischemia. Ischemic cell change may well require reperfusion to form, because it is not seen immediately after even prolonged complete ischemia (521). This has not been rigorously tested. If so, there are many possible reasons such as the requirement for free radicals or near-normal levels of ATP.

3. Homogenizing cell change/ghost cells

A) Description. The nucleus is somewhat shrunken and slightly darker than normal, often with a fragmented membrane. There is irregular chromatin clumping. The cytoplasm is slightly shrunk and appears fragmented with small vesicles and dense bodies (128, 351, 355), but still retains its delimited structure. Although there are some shrunk mitochondria, there are no other recognizable organelles. The cell membrane appears disrupted. Cells are much paler than normal cells in the electron microscope and are weakly eosinophilic, giving the cells their “ghostlike” appearance. Nuclear “integrity” is maintained longer than cytoplasmic integrity (128, 355). This is the most disrupted of the three morphologies.

B) Occurrence. This stage is generally assumed to follow from ICC, and this seems likely based on location and timing (128, 351). It arises ~24 h after anoxia/ischemia (128) and ~6–12 h into permanent focal ischemia in rat, in the core of the lesion (351), and sometime before 48 h after 2-h focal ischemia in the penumbra (652). It has not been described after global ischemic insults, although reported analyses have not been very rigorous (579, 896, 897).

4. Summary

Although these end stages are considered to represent dead cells, this has not been rigorously established except for homogenizing cell change (HCC) which, in the electron microscope is clearly associated with badly fragmented cells. Metabolism and function have not been carefully measured in cells showing ICC or ECC, and although the morphology is very distorted, the possibility of recovery cannot be ruled out. However, after focal ischemia, the disappearance of neurons showing ICC semiquantitatively parallels the decrease in neuronal density in the peri-infarct region of focal lesions (814). Furthermore, although silver-stained ICC neurons were visualized in the infarct after 24 h, several days later only silver-stained terminals remained (484). These results certainly suggest degeneration of the ICC neurons.

B. Apoptotic Cell Change

1. General considerations

Apoptosis, in its purest form, is best considered as one of the processes by which cells die in response to normal physiological stimuli (205, 565, 1227). In this sense, it is a “programmed” physiological process. The question is the extent to which this program of changes has been coopted by ischemic cell death.

This would be quite simple to answer if the morphological changes and biochemical events of apoptosis were unambiguously defined. However, there is no overwhelming consensus on this at present. In the next section, a set of criteria that allow this to be done as well as possible are described and discussed.

2. Minimal criteria for apoptosis

A large number of reviews detail changes associated with apoptosis in different cell types and conditions (32, 1004). The following are reasonable minimal criteria for identifying apoptosis in ischemia. A key is differentiating it from ischemic cell change that has somewhat similar characteristics.

A) Morphological changes. The most unequivocal morphological markers are the formation of smoothly contoured spherical- or lunar crescent-shaped masses of chromatin within the nucleus and the subsequent formation of apoptotic bodies that are cell membrane-bound structures containing cytoplasm and dark chromatin masses (32, 564, 652). This contrasts with ICC where the chromatin is irregularly clumped. Before formation of the apoptotic bodies, the cytoplasm (in electron microscopy) becomes darkened and pyknotic, as in ICC, but neither it nor the nucleus becomes nearly as dark as in ICC, so this is a reasonable way to identify apoptosis (716). The organelles are relatively normal (as distinct from the very swollen mitochondria and cisternae of ICC (32, 564, 716), but there is often some vacuolization (564, 1006), so the presence of vacuoles cannot be used to identify ICC. Mitochondria usually only become visibly swollen and malformed in the end stages of apoptotic change (541, 919), and even then, changes are often very minor (564) compared with necrotic cell death (541, 564, 919). However, they do swell so that, as with vacuolization, swollen mitochondria cannot unequivocally be used to identify necrosis. However, cell shrinkage and darkening, in the absence of mitochondrial swelling and vacuoles, can be used to identify apoptosis. This, however, is not seen after ischemia, so the issue is not simple. Importantly, in contrast to ICC, cytoplasm does not become eosinophilic. This is a very useful way to distinguish the two processes.

B) Biochemical changes. Double-stranded breakdown of DNA into nucleosomal segments is a very strong criterion (111, 859) and is manifested as DNA laddering, with fragments being multiples of ~200 bp. This is not unambiguously identification of apoptosis because it is observed in populations that almost certainly have undergone necrotic cell death (1147). Glutamate toxicity in cultures (404) and in vivo (918) leads to prominent nucleosomal fragments of DNA at the same time as the cell population shows a distinctly necrotic morphology. In situ end label-
ing of DNA using terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL), a histochemical approach, is even less selective for apoptosis because it labels both nucleosomal and nonnucleosomal fragments (918); the latter are formed during necrotic cell death. Although not at all selective for nucleosomal cleavage, TUNEL is relatively selective for double-stranded breaks as distinct from single-stranded nicks (82), so is somewhat discriminatory.

Although the biochemistry of apoptotic death is not completely understood, there are extremely useful identifying characteristics. The activation of caspases, particularly caspase-3 (719, 886), and inhibition of cell death by inhibition of these proteases (886, 1172, 1301) is the strongest criterion, although the ever-emerging complexity of the caspase system makes the situation somewhat uncertain (739). Caspases do not appear to be activated in necrotic death (e.g., Refs. 33, 387). There are several manifestations of caspase-3 activation that can be measured quite readily including cleavage of poly(ADP-ribose) polymerase (PARP) (318), cleavage of caspase itself (953), and characteristic cleavage of spectrin (811). Another important criterion is the release of cytochrome c from mitochondria (941), which activates caspase-3 by binding to the ced4 analog APAF3 and cleaving the procaspase (441). This now appears to be an almost universal step in apoptosis (591, 941); in some cases, another mitochondrial protein, apoptotic initiation factor, may play a role analogous to cytochrome c (1275). Neither of these changes has been noted in necrotic cell change to date. Another unique feature of apoptosis is the transfer of phosphatidylserine to the outer leaflet of the plasma membrane as part of the process by which the cell is targeted for phagocytosis. This can be monitored by binding of annexin V, which preferentially binds to this phospholipid in the presence of Ca$^{2+}$ (1189).

Another frequently occurring feature of apoptotic death is an increase in the ratio of Bax or other proapoptotic members of the Bcl family to the antiapoptotic members of this family, Bcl-2 or Bcl-xL, (940). Thus there are a large number of unique characteristics of apoptosis that can be measured or observed.

Blockade of cell death by inhibition of protein synthesis is sometimes used as a criterion for apoptosis (194), but it is not satisfactory. Not only is apoptosis often immune to inhibition of protein synthesis (61, 718, 1125), but more importantly there is strong evidence for postischemic synthesis of two very damaging enzymes, COX-2 and iNOS (477, 479, 837). Indeed, in a study of effects of mild focal ischemia, cycloheximide was very protective and abolished both DNA laddering and DNA smearing, indicating strong effects on necrotic death as well as apoptotic (304).

Thus, at this stage, uniformly condensed chromatin and apoptotic bodies identified in the light microscope, a shrunken cytoplasm that is not eosinophilic, activation of caspases, and/or inhibition of cell death by caspase inhibitors, extracellular binding of annexin V, and release of cytochrome c into the cytoplasm are all strong indications that the apoptosis process has been activated and is making a major contribution to ischemic cell death.

With the use of these criteria as best as can be done at this time, there is now reasonable evidence that apoptosis contributes significantly to cell death in focal ischemia and anoxia/ischemia. Evidence for its role in global ischemia is much weaker, although aspects of the process almost undoubtedly contribute to cell death there also.

3. Extent to which ischemic cell death is via apoptosis

A) Global ischemia. I) Chromosomal and morphological changes. There are now many reports of both nucleosomal DNA laddering and TUNEL labeling in extracted tissue and sections of the CA1 region of hippocampus, following short durations of global ischemia in rats (443, 698) and in gerbils (568, 848, 951, 1016). These occur during the period that neuronal death is developing. When observed in detail, TUNEL staining occurs only over cells undergoing severe cell shrinkage and not in adjacent, healthy, cells (568, 834). However, as discussed, these chromosomal changes are not unambiguous identifications of apoptosis (899).

Morphological evidence for apoptosis is weak, despite the title of one publication claiming that delayed neuronal death is apoptosis (834). In that paper, light microscope staining was intense with TUNEL when the cells were dying, but there was no light microscope evidence for chromatin condensation or apoptotic bodies. Some (it is not stated how many) nuclei showed condensed chromatin, but it was not at all smooth edged when viewed in electron microscopy, and the shrunken dark cells showing the condensed chromatin were highly vacuolated and contained autophagosomes. In fact, the process seems far more akin to autophagocytic cell death than apoptosis. Two studies of rat hippocampus, after quite mild 2-VO (251) and 4-VO (899) insults, failed to note any morphological signs of apoptosis. Only ischemic cell change was noted. This is also true in cat hippocampus after 10-min global ischemia and in Purkinje cells (716).

Apoptotic bodies probably have a short lifetime (~3 h), and it has been suggested that this might explain their absence (150). This seems unlikely because apoptotic cells are readily seen in focal ischemia, hypoxia/ischemia (see sect. II B), and also in a global ischemic-like insult to spinal cord (541).

II) Biochemical studies. There are no reported studies of cytochrome c localization changes. There is a series of studies on caspases and their inhibitors which suggest that these enzymes are involved in cell death and thus suggest a contribution from apoptotic processes.

CA1 neurons in the gerbil were almost completely...
protected against cell death by a broad-spectrum caspase inhibitor (451), with no effect on temperature. The protection persisted, albeit at a lower level, when the hippocampus was injected with IL-1β at levels that were adequate to enhance ischemic damage, indicating that protection did not result from inhibition of ICE (caspase-1) and subsequent reduction of IL-1. However, these studies did not specify the caspase that is involved. Less, but significant, protection was afforded in rat by the more specific caspase-3 inhibitor ZVAD-CHO; 4.5 μg injected into the ventricle saved ~40% of the CA1 neurons after 3 days and 20% of the neurons after 7 days (183). The specific ICE inhibitor YVAD had no effect. These protective effects are not terribly strong but do show an involvement of caspases.

Less direct, but important, evidence for caspase involvement is the upregulation of the enzyme. This occurs either by cleaving the existing procaspases (699) or by synthesizing a new enzyme that is then cleaved. Caspase-3-like mRNA is upregulated between 8 and 24 h after 15- to 30-min ischemia in rat, predominantly in CA1 pyramidal cells, and total caspase-3 protein is increased by 8 h (183, 826). Cleavage of the procaspase increased by 4 h after ischemia, indicating an early activation of the enzyme, although measurements of enzyme activity indicated a somewhat later activation. Caspase-3-like activity measured on artificial substrate was elevated ~10-fold in hippocampus between 8 and 24 h after ischemia, and PARP breakdown began sometime between 24 and 72 h after the insult (183). This timing is consistent with the appearance of active caspase-3 fragments at 24 h after 12-min global ischemia (1232). In gerbils, there was also cleavage of procaspase-3, but the timing was not ascertained (451).

Overall, these studies indicate that activation of caspase-3 occurs fairly early in development of damage, which normally takes 3–4 days. There are some timing details that are difficult to explain such as the fact that total caspase-3 was elevated slightly before the elevation in mRNA and that breakdown of a synthetic substrate by caspase was far earlier than the action on a natural substrate, PARP. These may reflect subtleties of the apoptotic process that are not yet apparent; the work is quite recent, and small discrepancies are likely to become resolved with time.

Although caspase-3 appears to be important in the rat, caspase 2 (Nedd-2) may be more important in gerbil. Nedd-2 mRNA was upregulated severalfold in CA1 and CA3 pyramidal neurons 3–6 h after 5-min ischemia in gerbil. By 12 h, it was down to baseline (572). There was no change in caspase-3 mRNA, nor was caspase-2 upregulated in rat (826). Thus there may be a real species difference here. No protein measurements have been reported.

There are further chemical changes that are suggestive of apoptosis; the proapoptotic proteins BAX (185, 420) and Bcl-Xs (264) are expressed in vulnerable CA1 cells after global ischemia before and during the time of maximal cell death.

III) Summary. Evidence is conflicting. Chemical evidence suggests a contribution of apoptotic processes to cell death after global ischemia insofar as there is DNA laddering, caspase activation that is reasonably timely, and some attenuation of damage by caspase inhibitors. BAX and Bcl-Xs expression are also consistent with an apoptotic pathway. Certainly the attenuation of cell death by caspase blockers is not nearly as strong as it is when death is clearly via apoptosis, for example, in nerve growth factor (NGF) or serum withdrawal (739, 1106), but it is substantial.

In ischemia, the caspase inhibitor was protective when added 2 h after the insult (183), and it had to be injected within 12 h of the insult (451). This is early in the cell death process, which lasts ~3–4 days. Caspase activation is a very late step in many cases of apoptosis (963, 1106, 1302), generally being activated by mitochondrial release of cytochrome c (441), but this apparently is not the case in global ischemia, or if it is, then early caspase activation is also important. The implications of this are considered in section IV C.

Although biochemical evidence strongly indicates that apoptotic processes occur, morphological evidence does not support a standard apoptotic process. Most importantly, there is no evidence of spherical nuclear chromatin clumping/apoptotic bodies at the light microscope level, and these are a hallmark of classical apoptosis. Perhaps the caspase-activated endonuclease (301) is not able to act after ischemia. At the electron microscopic level, the cytoplasms and nuclei of cells appear too dark and too disorganized (e.g., Ref. 716). Furthermore, almost all cells are eosinophilic.

The most reasonable overall assessment of the situation is that caspases are very clearly activated and that their proteolytic actions contribute to the cell death. However, other important processes occur that bias the final morphology away from classical apoptosis.

b) Focal Ischemia and Hypoxia/Ischemia. I) Morphology. There is DNA laddering in the penumbra of permanent lesions after one to several hours (669, 670, 1124), 24 h after 2-h temporary occlusion (652), and several days after very short (30 min) temporary lesions (276, 304). Unlike in global ischemia, there is also quite strong morphological evidence for apoptotic changes in both focal ischemia and hypoxia/ischemia.

Two groups showed similar effects of temporary ischemia (178, 652). Apoptotic nuclei, showing condensed chromatin/apoptotic bodies, first appeared in the infarct core ~15–30 min after beginning ischemia (653) and were very apparent there within 30 min after 1- or 2-h temporary MCA occlusion (178, 652). After this time, they were continually present at high density in the periphery of the infarct (178, 652) for at least 2 days (652). They also appeared in regions of individual cell necrosis after milder...
insults (653). Thus apoptotic neurons appear to arise quite soon after a focal insult and to predominate either early or in regions where the insult is less severe. The ratio of apoptotic cells to necrotic (ghost) cells was 9:1 in the penumbra versus 1:1 in the core 4 h after 2-h temporary ischemia (178). One study compared apoptosis in temporary and permanent ischemia (785). The time course of development and the number of apoptotic cells were identical in the penumbra in the two insults; however, there were about one-half the number of apoptotic cells in the core area of the permanent lesion, probably reflecting the continuing profound energy deprivation (785).

Cells with spherical condensed chromatin structures are present in great numbers 24 h after permanent or 1-h temporary ischemia in mouse. In the penumbra, they constitute ~5–10% of the total number of cells (601, 786), indicating a major apoptotic component to cell death. TUNEL staining was almost exclusively over neurons as opposed to glia (304); no more than 10% of the TUNEL cells were colabeled with glial fibrillary acidic protein (652). This confirms the neuronal nature of the apoptotic cells.

Apoptotic death was convincingly demonstrated after 60 min of ischemia/hypoxia in newborn piglets (745). There was coincident strong basophilia of the nuclei, TUNEL labeling, and presence of apoptotic bodies and condensed cells in the cingulate gyrus 2 days after the mild insult, which reduced ATP levels to ~50%. A similar insult, applied to young rats, yielded cells with greatly increased labeling by annexin V 48 h after the ischemia (1189), also suggesting apoptotic change.

High-resolution study of the cell changes in the various models indicate that the morphology is often not that of classical apoptosis. The cells showing apoptotic nuclear morphologies are mostly shrunken, but some of the apoptotic nuclei are in distinctly swollen cells, with no possible apoptotic morphology (178). At the level of electron microscopy, the condensed chromatin in nuclei of cells that very probably had stained positive with TUNEL did not actually have the smooth appearance of chromatin in apoptotic cells and furthermore was associated with cells that were highly vacuolated and were often undergoing quite severe homogenizing-like cell change (155). This is a secondary necrosis, following major apoptotic changes.

Overall, these morphological studies strongly indicate nuclear changes associated with apoptosis. However, the cytoplasm often shows much more degeneration than is expected in classical apoptosis, including vacuolization and disintegration. More morphological studies would be very beneficial, along the lines of the elegant studies of Portera-Cailleau and co-workers on glutamate toxicity and global ischemia (716, 919).

In most studies, apoptosis tends to be favored in regions where insult intensity is less (penumbra rather than core of lesion, following shorter rather than longer insults, Ref. 178), but the apoptotic changes certainly exist in the lesion core.

II) Biochemical studies. Mutant mice in which ICE is knocked out (993) or rendered ineffective (421) have infarcts that are 50% the volume of controls after transient focal ischemia. However, the mutants have reduced buildup of IL-1β after ischemia (421), which could account for protection. The relatively specific caspase-3 inhibitor Z-DEVD.FMK reduced infarct size by 27% in a mouse model of 2-h ischemia followed by 18-h reperfusion (422). It was only effective if added within 1 h of the end of the ischemia, not when added at 2 h (604), again indicating that caspases play a role early in the cell death process.

Caspase-3-like activity was more important to overall cell death in a less severe model (30-min ischemia). Z-DEVD.FMK reduced infarct size by ~60% when added anytime before 6 h after the insult. It was ineffective added 18 h after the insult but partially protected when added at 12 h (304). An elegant more detailed time course study showed that caspase inhibition was effective only up to to 9 h in this model, and that corresponded to the point at which caspase-3 was activated (324). As with the effects of caspases in global ischemia, this is still early relative to the final stages of cell death, which do not occur until 3 days or more after ischemia in this model.

There are many caspases (11 at present), and the full contribution of caspase activation, as distinct from its role in IL synthesis, will require improved inhibitor specificity. Also, two more universal inhibitors, V-ICEinh and BAF, which prevent neuronal apoptosis that is unaffected by the inhibitors of caspase-3 and caspase-1 (877), will be of help in showing the extent of caspase involvement in ischemic cell death. It may be greater than indicated by the ~25% inhibition produced by caspase-3 inhibition with Z-DEVD.

Several studies have been carried out on caspase changes after one or more hours of focal ischemia. As in global ischemia, the increase in de novo synthesis of caspases appears rather leisurely, but there is an earlier activation of the procaspase-3 by cleavage. Upregulation of caspase-3 mRNA in MCA territory is delayed until 16–24 h after the onset of permanent focal ischemia, whereas caspase-2 mRNA is upregulated by 4–8 h, and is down to baseline by 16 h (42). Two hours of temporary focal ischemia in mouse (807) led to an increase in caspase-3 protein levels in the penumbra after 24-h recovery; there was no change in the focus. [There is a recent report in abstract form showing a far more rapid increase in caspase-3 mRNA in layers 2 and 5 cortical neurons in spontaneously hypertensive rats, beginning within 15 min of occlusion, with no change in caspase-2 mRNA (825).] There was an early increase in the 20-kDa caspase cleavage product, peaking between 1 and 4 h after the 2-h insult but actually beginning during the ischemia. This was accompanied by a manifold increase in caspase-3-like ac-
tivity, measured with an artificial substrate, which was maximal immediately after ischemia and decayed within ~6 h. This is consistent with the requirement for very early inhibition of caspase-3 to attenuate damage in this mouse model (422, 694). The very delayed synthesis of caspase-3, and even the less-delayed synthesis of caspase-2, is perhaps too late to be of great functional significance in focal ischemic damage, which is strongly manifested between 6 and 12 h, but this needs to be tested by future studies.

Caspase activation via movement of cytochrome c from the mitochondria into the cytosol is a cardinal feature of most apoptotic systems. There is good evidence that this occurs in a large number of cells during development of focal ischemic damage (342). Substantial intracellular redistribution of cytochrome c from mitochondria to cytosol was measured both by immunocytochemistry and by Western blots within 4 h of reperfusion after 90-min ischemia, and this increased over the next 24 h. This was selective in that cytochrome oxidase did not show this change. This time course is in keeping with the measured activations of caspase.

Hypoxia/ischemia in young rats produces large numbers of apoptotic-like cells. Caspase activity increases and is half-maximal by ~12 h (191). Neuronal death and brain volume loss were markedly attenuated by the nonspecific caspase inhibitor BAF when administered within 3 h of the insult (191), at levels which did not affect core temperature for at least 2 h.

Taken together, these studies suggest that apoptosis contributes to cell death during focal ischemia and hypoxia/ischemia and also that caspases are important. Somewhat surprisingly, neither caspase antagonists nor knockouts have been tested in permanent focal ischemia, although the presence of apoptotic profiles suggests that apoptosis is important. The relative importance of the apoptosis in temporary focal damage, as evaluated by the efficacy of the caspase inhibitor, decreased as the duration of the insult increased, and this is in line with the general notion that less severe insults favor apoptosis over necrotic cell death (111). The role of caspase synthesis, as distinct from its early activation by proteolysis, is not clear. The former occurs quite late; it may play a role in ongoing cell death, perhaps that which is activated by induced enzymes such as iNOS and/or inducible COX-2.

It is, of course, possible that caspases aside from caspase-3-like forms can perpetrate apoptosis so that the effect of z-DEVD.CHO may represent a lower limit on the amount of apoptosis that occurs.

4. Basis for initiating apoptosis

A) OVERVIEW. Apoptosis can be induced by a large number of different stimuli, and many of the conditions that prevail during ischemia are capable of inducing apoptosis in one or more cell types. These conditions include free radical generation (380, 502, 938, 1207), NO production (830), reduced mitochondrial activity and membrane potential (503, 894, 1220, 1275), microtubule disaggregation (110), increased Ca$^{2+}$ (535, 831), activation of calpain (811, 1065), increased levels of ceramide (619), and increased expression of a mutant P53 (654, 978). The broad-spectrum protein kinase inhibitor staurosporine causes apoptosis in many cell types, including neurons (700), and both protein kinase C (PKC) and Ca$^{2+}$/calmodulin-dependent protein kinase II (CaMKII) are severely downregulated for many hours after global and (for CaMKII) focal ischemia; PKC has not been measured in the latter (35, 418, 780).

B) BAX, TNF, CD-95L (FAS LIGAND), AND CYCLINS. More specific inducers of apoptosis are also changed appropriately by ischemia. This includes a decreased ratio of Bcl-2 to BAX (365), upregulation of cJun (415), and changes in the “cell death domain” receptor ligands. Tumor necrosis factor-α is elevated after global ischemia (975, 1155) and has been shown to be damaging in focal ischemia (636, 746), where it is also elevated within 20 min (636). Importantly, reported in abstract form, the Fas ligand, or CD-95 ligand, was upregulated in the ischemic region for 24 h after 2- to 3-h temporary focal ischemia and, as with TNF-α, this appeared to be very damaging. Lesion size at 3 days was much reduced by injected antibody to CD-95 and also in mice in which the Fas ligand was knocked out (722).

Although TNF-α has many effects in addition to initiating apoptosis, this is not generally true for the CD-95 system. Thus these data suggest it may play a critical role in the ischemic apoptosis. Such a role is consistent with the widespread upregulation of CD-95 in postmortem brains showing many different neurodegenerative diseases (246). It would be of interest to measure ischemic damage in FADD knock-out mice, if lesion size was reduced, it would strongly support the role of the CD-95 system (43).

The cyclins are thought to be instrumental in activating apoptosis in some systems, including NGF withdrawal from sympathetic neurons. Cyclin D1 levels increase after withdrawal, and apoptosis is effectively prevented by inhibition of the cyclin-dependent kinases (876). Cyclin D1 mRNA is increased selectively in CA1 and subiculum 48–72 h after 20-min global ischemia in the rat, and cyclin D1 was expressed in individual CA1 pyramidal neurons by 72 h, before their undergoing ischemic cell change or apoptosis (1117). Neither the mechanism nor significance of this recent finding has yet been determined, but it is reasonable to think the activation of the protein plays a part in apoptotic cell death.

C) DIRECT EFFECTS ON MITOCHONDRIA. Another mechanism for cytochrome c release (and hence activation of apoptosis) is suggested by the recent observation that the combination of mitochondrial membrane depolarization and elevated perimitochondrial Ca$^{2+}$, conditions which occur during ischemia, cause release of cytochrome c from
isolated mitochondria in the absence of the mitochondrial pore transition (24).

D) TIMING OF CASPASE ACTIVATION. The timing of caspase activation with respect to cell death remains something of an enigma. In both global and focal ischemia, apparent activation of caspase-3 occurs many hours and in fact days before signs of cell death are manifested. This is true when evaluated by appearance of fragments, by appearance of proteolytic activity, and by the time at which caspase-3 inhibitor has to be added to prevent apoptotic cell death. There are several systems where caspase activation is very early, including the death-domain ligand/receptor interactions and staurosporine (618). However, this is generally an activation of a "precursor" caspase such as caspase-1 (618) or caspase-8 (1004, 1186, 1219). It is hard to envision how caspase-3, with its multiplicity of downstream critical targets, would take days to kill cells (this can be compared with NGF withdrawal where nuclear condensation and cell death follows caspase-3 activation within 1 h or less). Understanding this time course should give important insight into the control of ischemic apoptosis.

5. Importance and role of protein synthesis

In some cases apoptosis requires protein synthesis, whereas in others it does not. Whether or not it does so in ischemic death provides insight into the mechanism of its activation. Protein synthesis inhibition does ameliorate ischemic damage in many cases; however, damaging proteins such as iNOS and COX-2 are synthesized and may be the target of this protective action (as may temperature reduction; the studies are not generally well controlled for this). Thus the challenge is to demonstrate that inhibition of synthesis protects because it specifically prevents apoptosis. The most persuasive pertinent studies on in vivo ischemia are those in which mild transient focal ischemia (30-min distal MCA occlusion) was the insult and where artificial inhibition of protein synthesis, which probably lasted for 12 h, almost completely protected against the damage (276, 304). The evidence that apoptosis was occurring was not strong in one of the studies, comprising only TUNEL labeling and DNA laddering, but it was stronger in the other where there was chromatin condensation also. There is a caveat. Apoptotic cell death following ischemia is very sensitive to small decreases in temperature, being abolished by a 2°C fall (292). Unfortunately, there were no controls for possible reduction of temperature by cycloheximide at the levels that were used in the studies (1 and 10 mg/kg), beyond 1 h after ischemia. The studies thus suggest, but do not prove, that protein synthesis is required for ischemia-induced apoptosis. A similar conclusion emerged from work on cell cultures where non-NMDA-mediated cell death after ischemia showed TUNEL labeling and was blocked by cycloheximide (405); here again, independent evidence for apoptosis was not terribly strong, although the cell death was inhibited by the general caspase inhibitor Z-VAD FMK (387). There is no possibility of a temperature artifact in culture. Although more careful studies would be very useful, testing a correlation between protein synthesis inhibition and identified apoptotic-like death with good temperature control, the current evidence indicates that ischemia-induced apoptotic death requires protein synthesis.

If protein synthesis is required, it might explain the absence of apoptosis following global ischemia, where protein synthesis is severely inhibited throughout the postischemic period (see sect. ivC).

If synthesis is required, then the question is what proteins are likely to be synthesized, and what is the trigger? The answers are not known. Caspase-3 expression increases in global and focal ischemia, but the timing of the increase is quite late for it to be playing a major role in apoptosis, as discussed above. Quite extensive work on other systems, and particularly on thymocytes, strongly suggests that the newly synthesized proteins are required to enable one or more steps that cause the release of cytochrome c from mitochondria and ultimate activation of caspases (710, 909). For example, thymocyte apoptosis initiated "physiologically," by cortisone requires protein synthesis. However, thymocyte apoptosis initiated by directly opening the mitochondrial transition pore does not (710). Clearly, there may be differences in different cell types. However, it is possible that one effect of ischemia is to trigger synthesis of proteins that allow eventual release of cytochrome c by acting on the mitochondrial membranes. A challenge is to find such proteins.

6. Conclusion

The extent of apoptosis is more problematic than ECC or ICC, because it is more difficult to define. The former are straightforward morphological classifications. Presently, there is no good morphological evidence that apoptosis occurs after global ischemia, but there is such evidence, in the nucleus, after focal ischemia and hypoxia/ischemia. TUNEL staining, which has been used to imply apoptosis in global ischemia, is not a valid measure.

There is DNA laddering in all the ischemic models, although the laddering of DNA may proceed by a somewhat different mechanism than in normal apoptosis. The DNA fragments produced by either focal or global ischemia in adult rats, or by hypoxia-ischemia in young rats, have staggered ends, as distinct from blunt ends seen in classical apoptosis. The 3'-end is recessed ~8–10 bp (J. MacManus, personal communication). One possibility is that an additional endonuclease may be activated in cerebral ischemia, which acts after the classical apoptotic nuclease(s) (301). There is strong evidence for caspase activation and some attenuation of cell death by caspase inhibition in global and focal insults, which seems to be
greater for milder insults, a relatively common property of the apoptosis/necrosis dichotomy. Further work on the very rapidly developing field of caspase biology will, hopefully, clarify the roles of these enzymes.

The nuclear morphology of apoptosis is apparent in focal ischemia. What is not yet clear is how completely processes in the cytoplasm, which are almost undoubtedly the keys to cell death, mimic classical apoptosis and also what percentage of the population is showing the apoptotic events (699). At present, it is clear that the cytoplasm in neurons showing apoptotic changes is generally highly vacuolated compared with normal apoptosis, although the latter do often show vacuolation. It is also clear that there are a large number of eosinophilic or ghost neurons and that some of these show apoptotic nuclei, indicating a very mixed cell death pathway. Future studies in which characteristics of apoptotic cell death, and ischemic cell death, are defined more clearly will be of great help in determining what is occurring. Part of this should include characterizing the presence of specifically apoptotic protein changes such as spectrin and F-actin (gelsolin) cleavages, as well as phosphatidylserine redistribution in the plasmalemma. A promising line of work that is developing is the careful comparison between properties of apoptotic death in neurons, induced for example by staurosporine, and ischemic cell death (700). This issue is considered in section III.

C. Autophagocytotic Cell Death

1. Basic properties of autophagocytotic cell death

A third distinct form of cell death that may pertain during ischemia is autophagocytotic cell death. This was classified by Clarke as type II (apoptotic death is type I) cell death and has been noted in several cases during development of the nervous system (205). The possibility that this occurs has been suggested in some discussions (178), but it has not been studied at all rigorously. In fact, though, it may be quite prevalent.

Autophagosomes are fusion products of lysosomes with mono- or multilayered lipid membrane vesicles enclosing regions of cytoplasm (637), which often include organelles (371). Normal protein turnover by this mechanism occurs at ~1%/h in most cells, and it is thought to be the principal mechanism of steady-state protein breakdown (637, 1015). Its involvement as a principal player in death implies that it is strongly activated, and there are now a number of studies implicating autophagy in cell death, indicating that it is indeed capable of killing cells when activated well beyond its normal rate (205).

The characteristic morphology of autophagocytotic cell death includes a condensed cytoplasm containing many large vacuoles, most of which are autophagosomes or proliferating lysosomes, and a nucleus in which chromatin is irregularly clumped (205). This is not very different from ischemic cell change, or from cytoplasmic morphologies described for some cases of apoptosis. The nucleus is generally less pynknotic and less dense than in ICC.

2. Evidence for autophagocytosis after ischemia

The rate of autophagocytosis is greatly increased in some cells that have undergone injury (371) and is approximately doubled in liver 24 h after 60-min ischemia (724). There is only one direct identification of autophagocytosis in brain ischemia, but it is quite persuasive (834). Structures that appear to be autophagosomes proliferate ~3 days after 5-min ischemia in gerbil CA1 pyramidal cells. These structures are bordered by multiple membranes, contain organelles or parts of organelles, and in addition contain cathepsin. They thus appear to be true autophagosomes (371). This is the only study in which autophagosomes were looked for specifically, and it is possible that they are peculiar to gerbil global ischemia, because that insult is characterized by a huge proliferation of ER-like membrane, which could be the precursor to the structures. On the other hand, based on appearance of cells, it has been suggested as the principal mode of cell death in rat global ischemia and as very important in focal ischemia (178), and there is a large increase in immunoreactivity of the cathepsin B precursor protein within 24 h of 12- to 15-min global ischemia in rat (448). Further work in which lysosomal enzymes are localized and subjected to inhibition and where autophagosomes are identified would be needed to establish that the process is important in ischemic cell death. There are now relatively specific inhibitors of cathepsins, such as 3-methyladenine, that appear to work in vivo (149).

There is almost no pertinent information on what ischemic change might trigger autophagy. Basal autophagy in hepatocytes is strongly inhibited by depleting ER stores of Ca^{2+} (383), indicating that ischemia-induced changes in ER could be important. Alternatively, because phosphorylation of the ribosomal protein S6 inhibits autophagy in liver (100), it is possible that dephosphorylation of this protein occurs in the postischemic period and so activates autophagy.

D. Molecular Changes Underlying Morphological End Stages

The first three parts of this section describe the morphological and biochemical aspects of the three major pathways of cell death. These lead to the key questions, which are 1) what are the molecular changes underlying these end stages and their development, and 2) what are the relationships between the different pathways of cell death? The first of these is considered in the current section. The answer is simple, albeit disheartening. There really is no published work that describes the molecular changes that form the basis for the end stages, or that
describes metabolism in these end stages. All that can be done here is to briefly speculate.

1. Edematous cell change

The key alterations in edematous cell change are the nearly empty and structureless cytoplasm and the swelling of the cells. The nucleus shows relatively mild chromatin clumping. The clearing of the cytoplasm implies a loss of protein and of the cytoskeletal structure. The swelling is most likely to result from inhibition of the Na\(^+\)-K\(^+\)-ATPase or from plasma membrane leakiness.

2. Ischemic cell change

The cytoplasmic features of ischemic cell change are very striking. They include severe shrinkage and condensation to a quite triangular shape (in 2 dimensions), increased electron density of the cytoplasm, and darkening/pynknosis of the nucleus. There is strong eosinophilia of the cytoplasm when stained by H and E and argyrophilia when it is silver stained.

Steady-state regulation of cell volume is not well understood. Shrinkage of sympathetic neurons after NGF withdrawal is strongly correlated with loss of protein (289, 333) so that the shrinkage could be explained by massive proteolysis. There are now several very specific cell-permeant inhibitors of proteasomal activity (749, 1033), and their effects on ICC would be of interest. Indeed, a proteasome inhibitor, PS-519, does strongly decrease the size of the striatal core lesion after temporary focal ischemia (902), although this might not be related to prevention of massive protein breakdown but, rather, to prevention of activation of the transcription factor NF\(\kappa\)B (56). Individual cells were not studied in this report. Cell volume might also be decreased by activation of K\(^+\) and Cl\(^-\) channels as occurs in regulatory volume decrease (1116). The latter is triggered by increased cytosolic Ca\(^{2+}\), and it is possible that it could be activated by this or other mechanisms.

Argyrophilia might also reflect proteolysis, since it is greatly enhanced by puromycin, which produces small peptide fragments (1091). On the other hand, Gallyas et al. (350) emphasize the all-or-none quality of argyrophilia (350) and speculate that there is a cooperative macromolecular-skeletal change that exposes protein side chains, causing the argyrophilia and the increased electron density.

Eosinophilia is partly due a loss of hematoxylin or Nissl staining, whose basis is not known, perhaps loss of ribosomes or of polysomes. There may also be an increase in eosin staining. This could be due to major changes in pK\(_a\) and/or structure of proteins (1137).

Each of these speculations could be addressed experimentally to help determine the macromolecular basis for ICC.

3. Apoptotic cell change

Cell shrinkage and increased electron density are also features of apoptosis, although eosinophilia and argyrophilia do not occur. As discussed above, a net decrease in protein synthesis likely accounts for cell shrinkage in at least one model of apoptosis; it has not been studied in others. Specific cell changes in apoptosis are not very well known. However, the central role played by caspases, and by calpain (909), has revealed a wealth of potential protein changes. In cytoplasm, fodrin/spectrin is a caspase-3-like target (811), as are proteins involved in actin stabilization such as gelsolin (605). Thus a fairly massive attack on the cytoskeleton is suggested, which may well account for the apoptotic death. Caspase-3 also cleaves the endogenous regulator of protein phosphatase 2A, activating that enzyme (987). This could strongly affect interactions between cytoskeletal proteins. Of these changes, only caspase-mediated spectrin degradation has been shown during ischemia, in cultures (810). Actin breakdown has not been noted (183).

4. Summary

It is clear that much work is required if the molecular changes underlying the morphological end stages are to be determined. Almost nothing is known. Measurements of metabolism in different end stages would also be very valuable. Knowing these would show the targets of the processes that might be occurring during development of damage.

E. Relationships Between Different Forms of Cell Death

1. ECC and ICC

Edematous cell change and ICC change appear to be independent pathways of cell death. Only ECC is seen in hyperglycemic global ischemia and prolonged hypoxia/ischemia in young animals, whereas ECC has not been noted in focal ischemia. However, the two morphologies are seen together in some cases, in particular at the end stage of global ischemia and after NMDA injection in striatum. In the latter case, a detailed study indicated that they developed along parallel paths for 12 h and that by 24 h both types of damage showed major disintegration (919), suggesting no crossover. It seems most likely that when cell damage reaches a certain point it can lead to one or the other pathways; the basis for the very dramatic bifurcation is not known.

2. ICC and apoptotic cell change

Apoptotic changes and ICC are seen at the same time and in the same populations during focal ischemia, hypoxia/ischemia (292), and after glutamate injection into
striatum (919). However, evidence suggests they are quite distinct modes of death.

In piglet hypoxia/ischemia, reducing temperature by 2°C eliminated apoptotic death but caused no apparent change in the numbers of necrotic neurons (292). This is very difficult to reconcile with a sequential process. In spinal cord ischemia, different populations of cells, segregated by lamina, show almost entirely necrotic cell death, or apoptotic cell death (541). Thus different populations subjected to the same insult appear to take one or the other pathways. When two different glutamate agonists were injected into striatum of the adult rat, the NMDA analog quinolinic acid caused exclusively necrotic cell death while kainic acid caused almost exclusively apoptotic-like death (919), again indicating two distinct pathways. The distinctiveness of the two pathways is further suggested by the major morphological differences, particularly the eosinophilia/argyrophilia of the necrotic pathway as well as the very different nuclear morphology (although a transition from condensed spherical chromatin to uniformly condensed chromatin does not, at least naively, seem to be prohibitive). A recent study in cultured neurons exposed to either staurosporine (apoptosis) or high levels of glutamate (considered to be necrosis) emphasized the difference in nuclear morphology; the very darkened pynknotic nucleus in necrosis verses the less pynknotic-punctate chromatin nucleus in apoptosis (700).

If the two pathways are, indeed, independent, then the critical question becomes why different cells within the same apparent population proceed by different pathways during certain insults. One possibility is that the cells are actually from different populations, which have not yet been recognized. Other possibilities include subtle differences such as densities of NMDA receptors because these have a tendency to force cells into a necrotic rather than an apoptotic mode in some systems. This was mentioned above and is seen in some culture systems where NMDA-mediated damage is necrotic and non-NMDA-mediated damage is apoptotic (405, 870).

A quite large number of studies show that increased insult intensity appears to favor necrosis as indicated by the fraction of apoptotic-like cells following severe and mild focal insults (compare Refs. 422 and 304), by the relative abundances of apoptotic and necrotic cells in the core and penumbra of focal lesions (178, 785), and by the fraction of cultured cells taking the two pathways as NMDA or NO levels are increased (111). A nice study in kidney tubule cells shows a very strong relationship between the percentage decrement in ATP by metabolic inhibition and the cell death pathway taken. Apoptosis proceeded when ATP levels were >25% and necrosis when levels were 15% (658). Thus certain cells, because of their state at the time of the insult, may tend to one or the other pathway because the insult’s effective intensity is different. This is suggested by the apparently random sorting of cultured cortical neurons into necrotic or apoptotic-like pathways determined by whether or not there is permanent damage to the mitochondrial membrane potential when cells are exposed to NMDA (25).

The reason that different insult intensities do predispose to one or other pathways is not known. A simple idea is that when ATP levels, or other parameters such as Ca²⁺, change drastically they disrupt apoptotic pathways, thus biasing the process toward necrosis. As an example, if protein synthesis, or phosphorylation, is required for apoptosis and it is drastically inhibited, then apoptosis may not be allowed. There are many other such possibilities and, indeed, in lymphocytes (452) artifactual opening of the mitochondrial transition pore (MTP) causes apoptosis in a cell that is set up to carry out the process but causes necrosis if, for example, appropriate caspases are inhibited. Thus necrosis may be a fallback pathway if apoptosis is somehow not available!

The final stages of apoptosis after ischemia or excitotoxicity are very similar to those of necrotic cell death (699). Unlike the controlled phagocytosis in physiological apoptosis, there is a “secondary necrosis” at the final stages of apoptosis so that cell dissolution occurs, as shown in a careful study of excitotoxicity (919). This further complicates identification of apoptosis.

3. Autophagocytotic cell change and ICC

The evidence for autophagocytosis is clearly sparse because it has been studied very little. At this stage, it must be considered that it may coexist with either ICC or apoptotic cell change; that is, the vacuoles associated with both these end stages may, at least in part, be autophagocytic. The critical factor is whether this is true and, if it is, the extent to which the autophagocytosis is responsible for cell death. Alternatively, ICC and apoptotic cell change may be completely independent of autophagosomes, and autophagocytosis may occur in a separate population of cells. There really are no data that bear on this yet.

F. Summary

There is strong evidence for at least three different pathways of ischemic cell death: ECC, which occurs least frequently; ICC (leading to homogenizing cell change), which is most prevalent; and apoptotic cell change. There is preliminary evidence for a fourth programmed pathway, autophagocytotic cell death, that may be very important in global ischemia, at least in the gerbil.

There is strong morphological and biochemical evidence that populations of cells showing apoptosis and ICC coexist during focal ischemia and also during hypoxia/ischemia in neonates. In the delayed death following global ischemia, there is biochemical evidence for apoptosis but no morphological evidence, and at present, it is concluded that
autophagocytosis or ICC, and some ECC, account for main features of delayed cell death in that model.

Some insight is now being gained into how the apoptotic end stage is reached. The broad outlines of the apoptotic pathway are identified in focal ischemia and hypoxia/ischemia, and although the details including the triggers for apoptosis, the caspases involved, the molecular changes involved, whether mitochondrial release of cytochrome c is involved, are still unresolved, there is some evidence that the Fas (CD-95) ligand system, and possibly TNF-α along with protein kinases, may well be involved in initiating the process, and there is quite good evidence for the timely release of cytochrome c from mitochondria. There is almost no insight into how other end stages are reached, although the strong protective effect of proteasome inhibition indicates that large-scale proteolysis may be important in ICC.

The marked difference in nuclear morphology between focal ischemia and delayed neuronal death from global ischemia is currently a puzzle. Given that caspases play a part in death from both insults, and possibly BAX also, why do the nuclei not show the condensed spherical chromatin bodies after global ischemia? Possibly the caspase activation, or some other process, during global ischemia is not strong enough to cause this change, thus leading to some of the biochemistry of apoptosis but not the morphology.

There is also very little understanding at present of the molecular changes, or metabolic changes, underlying the gross structural changes of ICC, or ECC, and these seem particularly important to determine.

Although there do appear to be separate, predominately apoptotic or necrotic pathways, the former may be unique to ischemic cell death, different from apoptotic pathways taken in the absence of a metabolic or physical insult. Apoptotic nuclei appear associated with very highly vacuolated cells, or even cells showing changes similar to ECC and homogenizing cell change. This certainly suggests that elements of both pathways may coexist in conditions where nuclei show clear evidence of apoptotic change and where caspases are involved in the cell death process. DNA cleavage sites are also unique.

In conclusion, the ionic and biochemical changes to be discussed in ensuing parts of the review lead to the cells adopting one of several possible pathways to cell death. Some of those changes, along with the nature of the cells affected, are the determinants of the pathway that is selected, but at this stage, the basis for selecting a particular pathway is not known. Relatedly, the critical changes in molecular structures that are associated with the end stages of the different pathways are not known.

IV. CRITICAL FUNCTIONAL AND STRUCTURAL CHANGES

The basis for this section is the hypothesis that development of cell death and of the end stages results from long-term damage to key cell functions or structures. Cell functions and structures whose changes seem most likely to be the proximal cause of necrotic or apoptotic cell change (membrane damage, mitochondrial damage, inhibition of protein synthesis, and cytoskeletal damage) are examined to see if they play a critical role in ischemic cell death.

The hypothesis may be wrong. Cell death may result from continued activation of damaging biochemical processes (perpetrators) set in motion by the ischemic insult, with ultimate breakdown of the cell as a unit. Cell death may not be perpetrated by an intermediary functional defect in one or more key processes. Nevertheless, this section describes ischemic changes in major intracellular systems because they are deemed likely to be involved in cell death.

A. Altered Membrane Transport Properties

1. Membrane permeability

The question is whether there are large increases in membrane permeability to ions or metabolites that might lead to cell death. There is a paucity of data on this very important subject.

There are a great number of studies of ischemia in culture showing a correlation between the number of disintegrated or necrotic cells and membrane breakdown, measured as LDH release or uptake of vital stains including trypan blue, propidium iodide, and ethidium bromide (376, 791, 1072, 1156). However, there are almost no studies aimed at determining whether the membrane changes precede large-scale cellular changes. Propidium iodide does enter neurons in culture that look normal but are in the process of dying by necrosis, indicating that membrane leakiness may be a proximal cause of damage in cell culture (697). Unfortunately, this work in culture is the only study addressing the issue.

The mobility of a spin probe (5-NS) within the membrane bilayer of synaptosomes increased during the first hour after 10-min ischemia and then increased again transiently ~8 h later (411). This may have reflected increased disorder in the phospholipid bilayer. Unfortunately, the study has not been followed up.

Breaks were noted in the postsynaptic dendritic membrane during the first hour after 10-min complete compression ischemia in rat, and there was also a marked increase in neuronal permeability to horseradish peroxidase (256). Unfortunately, this finding was not pursued so that neither its reproducibility nor its significance are known.
Although not related to a specific function, the fact that the phosphatidylcholine precursor cytidine diphosphate choline (cyticholine or CDP-choline) is protective in ischemia indicates that membrane damage may be critical (516, 855). When administered intraperitoneally, CDP-choline very significantly ameliorates the neurological deficit days after 20- to 30-min 4-VO (516) and survival after focal ischemia (337), and it decreases infarct size either in combination with NMDA blockade (855) or when used after short insults (36). In temporary ischemia, the molecule has several effects (516); however, it does very clearly act as a precursor to phosphatidylcholine incorporation into cell membranes (516), and it dramatically decreases the net liberation of free fatty acids (FFA) during ischemia (268, 1133), presumably by activating resynthesis of phospholipid. The interpretation of the protective studies is far from unequivocal at this stage; however, they may indicate that loss of membrane integrity is contributing to the neuronal death.

In conclusion, despite a widespread assumption that gross plasma membrane changes are important in ischemic cell death, there is almost no evidence for this, particularly in vivo.

2. Na\(^+\)-K\(^+\) pump or other transporters

One to two days of ~50% inhibition of the Na\(^+\) pump with ouabain does cause an apoptotic-like death in cultures (713) so that if there is submaximal Na\(^+\) pump inhibition after ischemia it might well contribute to apoptosis. Also, profound pump inhibition might lead to cell swelling and ECC.

Reasonably short exposures to several different free radical species cause severe irreversible damage to the Na\(^+\) pump (187, 473, 1022) and also make it more susceptible to proteolytic attack (473). There is some evidence that free radical generation following various ischemic insults does inhibit the pump (382, 701, 866). Sixty minutes of 2-VO in gerbil followed by 15- to 30-min recirculation caused a 70% reduction in ATPase of frontal cortex and hippocampus, which was blocked by pretreatment with agents that should reduce free radicals (866). However, there was no pump inhibition after 15-min ischemia (866), a duration that invariably leads to delayed neuronal death. The latter suggests that pump inhibition does not play a role in delayed neuronal damage from global ischemic damage. However, it may well be important in focal damage, based on the effects of 60-min ischemia. Studies of this possibility have not been reported and would be of great interest.

Currently, there are no reports of long-term effects of ischemia on other plasma membrane transporters or channels that might cause significant ion or volume imbalance. Such studies would be very valuable.

3. Conclusion

Overall, there is little hard evidence implicating membrane changes in ischemic cell death. This is very surprising given the lability of membrane structure, the many biochemical changes associated with ischemia, and the huge potential for cell damage if membrane function is disrupted. The problem may be the lack of studies that have addressed the question.

B. Mitochondrial Damage

1. General background

Mitochondria are assuming an increasingly important role in hypotheses about both apoptotic and necrotic cell death (591, 617, 1250), but definitive demonstrations that they play roles in ischemic damage are still lacking. There are three general ways in which mitochondrial damage or change might make an important contribution to ischemic cell death.

1) Inhibition of mitochondrial oxidative phosphorylation or significant uncoupling will lower ATP levels, increase mitochondrial free radical production, and remove the Ca\(^{2+}\) buffering ability of the organelle. These effects are capable of causing grave damage. This could occur as a result of direct effects on the tricarboxylic acid cycle enzymes, on the mitochondrial electron transport/oxidative phosphorylation system or the lipid composition of the mitochondrial membrane, or as a result of long-term opening of the MTP.

2) In addition, opening of the MTP may produce damaging effects by releasing intramitochondrial molecules and ions, for example, a factor able to increase L-channel Ca\(^{2+}\) permeability as recently shown by Nowicky and Duchen (841).

3) Finally, release of cytochrome c and or at least one other protein from the intermembrane space is now recognized as a critical step in apoptosis in all systems in which it has been studied. Such release can be effected by opening of the MTP but is very frequently due to more direct effects on the integrity of the outer mitochondrial membrane, probably mediated by a member of the BCL-2/Bax protein family, such as Bar (309, 941).

Alterations in mitochondria therefore have enormous potential for causing severe cell damage. Despite this, and despite well-informed speculation (625), there is still a lack of evidence that convincingly implicates mitochondrial changes in either necrotic or apoptotic ischemic cell death. There is, though, a body of work that bears strongly on the issue.

In assessing this work, it is notable that isolating mitochondria from ischemic or damaged tissue may actually lead to mitochondrial damage (34). This is not usually considered.
2. Damage early after ischemia

A) EVIDENCE FOR DAMAGE. Mitochondria almost always undergo a transient swelling for a few hours after any form of ischemia (130, 742, 896, 897, 1002, 1236). The origin has not been studied but may be the reversible opening of the MTP due to free radical generation and Ca\(^{2+}\) accumulation (278, 410). This swelling has not been directly correlated with function, but it certainly appears that mitochondria are inhibited during this period.

Tissue metabolic rate, as measured by glucose utilization, is generally temporarily depressed by 50% or more, for at least 6 h after transient global ischemia (291, 606, 760, 931), and is depressed in the penumbra during and after focal insults (79, 1252, 1299). The depression after global ischemia does not occur in all regions but always occurs in vulnerable cells; in gerbil hippocampus, only CA1 is affected (760). Along with the decreased metabolic rate, ATP levels are reduced by 20–30% for several hours after 30-min 4-VO and 2-VO in the rat and 10-min complete ischemia in the dog (1070) and cat (1179). Furthermore, cytochrome complex aa3 is hyperoxygenated in intact tissue after global ischemia, consistent with failure of adequate substrate supply to the respiratory chain (961). These data are consistent with, but do not establish, mitochondrial dysfunction. They could equally be explained by a failure of glucose metabolism.

Unfortunately, the totality of studies on isolated mitochondria show a great deal of variability, some of which may well result from difficulties in their isolation. However, overall they do indicate inhibition in the postischemic period. State 3 respiration of isolated mitochondria is generally severely depressed (by 50% or more) immediately after global ischemia (449, 450, 668, 1012, 1054) and, in most measurements, for at least 1 h afterward (449, 944, 1080), although in one study it recovered within an hour (1054). Respiration of penumbral (and focal) tissue mitochondria is inhibited by 40% for at least 4 h after transient focal ischemia of between 1 and 2 h (624, 625). The damage almost always affects the NADH-linked portion of the respiratory chain (complex I) (743).

B) MECHANISM OF DAMAGE. Lee and co-workers (1012) suggest that the basis for the early mitochondrial inhibition is accumulation of mitochondrial Ca\(^{2+}\) during, and possibly shortly after, the ischemia. The conclusion was based on its very rapid reversal when EGTA or ruthenium red (RuRed) were added to the isolated mitochondria (1012). This is consistent with the site of action being the MTP, which is known to be rapidly activated by increased cytosolic Ca\(^{2+}\) (278), due to accumulation in the matrix (410), and which rapidly closes when isolated mitochondria are exposed to a chelator (475). This being said, it is very probable that other processes are involved; for example, the preferential blockade of complex I and hyperoxidation of cytochrome aa3 are not obvious consequences of the MPT. Calcium accumulation appears to preferentially inhibit complex I (475), which may explain the apparent involvement of this complex in inhibition.

Several electron microscope analyses show an accumulation of Ca\(^{2+}\) in mitochondria very soon after global ischemia, which persists for several hours (285, 596, 1051, 1165, 1181, 1272) (there are no equivalent studies in focal ischemia, which are at least consistent with Lee’s hypothesis).

C) ROLE FOR NO? The basis for the prolonged Ca\(^{2+}\) accumulation is not known; however, there is interesting evidence implicating NO. Accumulation in vivo was strongly blocked when an inhibitor of NOS, \(\text{N}^2\)-nitro-L-arginine, was added intraventricularly before 5-min ischemia in the gerbil (596). Intracellularly generated NO, at levels that arise during and shortly after ischemia, strongly depolarizes mitochondria, inhibiting ATP production (127). Although not tested, it is possible that this results from large-scale Ca\(^{2+}\) accumulation and so accounts for the observed effect of blocking NOS. Both the above effects could be due to NO or to peroxynitrite (see sect. V.A). In the latter case, they would also depend on free radical production.

If this is the case, and if Ca\(^{2+}\) accumulation is tied to damage, then free radical and NOS blockade should prevent the early mitochondrial damage. Although studies are not very extensive, some data are at least compatible with this. Blockade of FFA production with the platelet-activating factor (PAF) antagonist BN50739 prevented ~50% of the postischemic mitochondrial inhibition and several free radical scavengers attenuated the early decrease in ATP after global ischemia in the dog (360). Furthermore, swelling of mitochondria 1 h after 5-min ischemia in hippocampal slices was prevented by calmidazolium (316), which, among other things, prevents activation of NOS. Thus data are consistent with peroxynitrite-mediated uptake of Ca\(^{2+}\) by mitochondria and subsequent inhibition of function. A mechanism whereby NO or peroxynitrite leads to Ca\(^{2+}\) accumulation is not yet known.

2. Delayed mitochondrial damage

There are several reports indicating delayed damage in isolated mitochondria, but again results are quite variable, and timing does not always clearly show that the mitochondrial damage precedes cell death (e.g., Ref. 786). Several hours after 11-min anoxia in cats there was a reduced tissue oxygen consumption that was strongly associated with inhibited isolated mitochondria (1180). State 3 respiration measured on isolated rat mitochondria was reinhibited by ~50% 5 h after 30-min 7-VO in the rat (1012). In the very sensitive striatum, mitochondria became permanently inhibited 30–90 min after 2-VO ischemia and 6 h after 4-VO (449, 944, 1054), and they were inhibited 48 h after the ischemia in CA1 (1054), but only by 20%. There was a delayed loss of the respiratory con-
Control ratio 2–4 h after temporary MCA occlusion, in mitochondria isolated from both the core and the penumbra (625) as well as a decrease in state 3 respiration (801). There was a large decrease in mitochondrial membrane potential, measured in situ, after 24 h of focal ischemia (786). All of these results, except the last, strongly indicate mitochondrial damage in reasonably late stages of damage development. The last result could reflect changes in dead or dying cells because there is a lot of cell damage by 24 h.

There are decreases in tissue ATP levels before or around the time of cell death in global ischemia (760, 929) and in the penumbra in focal ischemia (329). Levels after global ischemia fall by 25 and 40% in rat CA1 hippocampus and striatum, respectively (929), and probably further in gerbil at 24 h (432). It is not known, though, that they result from mitochondrial damage. For example, in one study, the falls in ATP after focal ischemia could be explained by the falls in total adenylates (329). Decreased levels could also result from inhibited glucose metabolism.

Overall, these studies suggest late, functionally important, mitochondrial damage. They are not strong enough to establish this unequivocally, and further careful studies are required. Studies of the status of the MTP and also of cytochrome c leakage would be very valuable.

4. Possible bases for delayed mitochondrial dysfunction

Mitochondria are known to be susceptible to free radicals and Ca$^{2+}$ accumulation, both via the MPT and other mechanisms. However, the nature of transition from reversible to irreversible mitochondrial damage is still not well understood

A) FREE RADICAL AND PEROXYNITRITE ACTIONS. I) IN VIVO.

Free radicals seem to be at least partially responsible for the putative delayed mitochondrial damage. As described in section 4A, free radicals are elevated after both global and focal ischemia.

Pharmacological studies suggest the involvement of free radicals. The delayed loss of energy charge, and ATP, following temporary focal ischemia was protected by the spin trap N-tert-butyl-α-phenyl nitroxide (BPN) (329), and the delayed mitochondrial dysfunction measured after 7-VO in the rat was largely prevented by ascorbate (1011), as was the mitochondrial inhibition several hours after transient focal ischemia (801). The latter was also protected by the calcineurin inhibitor FK-506 (801), possibly because it prevents activation of constitutive NOS (cNOS) and subsequent accumulation of peroxynitrite, although this has not been shown.

Somewhat less directly, Mn-superoxide dismutase knockout mice generated more free radicals during permanent focal ischemia, showed a larger lesion, and showed a much larger number of cells with a loss of mitochondrial membrane potential after 24 h (786), demonstrating that excess free radical production enhances mitochondrial damage. However, this result does not explicitly show that normal free radical production causes the damage. All these studies are suggestive; however, they do not rule out the possibility that mitochondrial damage is an indirect result of free radical damage to another process (e.g., Ca$^{2+}$ homeostasis).

II) IN VITRO. Exogenously generated free radicals severely damage mitochondrial state 3 respiration, particularly in the presence of elevated Ca$^{2+}$ (119), and these effects may be the basis for the in vivo effects described above. Very short (5 min) exposures of cultured neurons to peroxynitrite cause ~50% inhibition of cytochrome-c oxidase and succinate-cytochrome c reductase that matures over 24 h (107). This is a very profound effect that has the possibility of occurring in vivo and being very important.

Nitric oxide donors (e.g., S-nitroso glutathione) applied to isolated mitochondria, and to thymocytes, open the MTP, leading to membrane depolarization and subsequent increased superoxide generation by the mitochondria (458). Generation of peroxynitrite from superoxide and NO at rates calculated to be similar to those encountered in pathological conditions, causes Ca$^{2+}$ release and depolarization of liver mitochondrial membranes, with opening of the transition pore (863).

b) EFFECTS OF Ca$^{2+}$. In at least one study, the timing of Ca$^{2+}$ accumulation is consistent with its causing delayed inhibition; it increased in mitochondria of the striatum 6 h after 4-VO, shortly before this region began to degenerate (1272). There was a large increase in total cell Ca$^{2+}$ beginning 24 h after global ischemia in the gerbil that may well reflect large scale Ca$^{2+}$ accumulation by the mitochondria. There was a concomitant fall in ATP (432). Unfortunately, there are no other good studies of delayed changes in mitochondrial Ca$^{2+}$.

There is quite a large body of evidence that Ca$^{2+}$ accumulation by mitochondria is a major mechanism of mitochondrial damage and subsequent cell toxicity in response to glutamate, raising the possibility of a similar effect after ischemia. Glutamate-induced necrosis in several culture systems (25) is associated with large-scale accumulation of Ca$^{2+}$ by mitochondria, subsequent mitochondrial depolarization (26, 994, 1206), and lowering of ATP/ADP, down to 30% of normal levels (142). The inhibitor of the mitochondrial Ca$^{2+}$/2Na$^{+}$ exchanger CGP-37157, which enhances mitochondrial Ca$^{2+}$ accumulation and decreases cytosolic Ca$^{2+}$ accumulation, enhances depolarization and damage, establishing quite nicely that the mitochondrial Ca$^{2+}$ accumulation is causing the depolarization and damage (1206). There is other strong evidence implicating mitochondrial Ca$^{2+}$ accumulation as a key factor in this necrotic event (829, 1071).

The basis for the Ca$^{2+}$ accumulation in those cases is the glutamate-induced cytosolic Ca$^{2+}$ accumulation,
which reaches several micromolar (476). If this mechanism of mitochondrial damage is to occur after ischemia, some factor must elevate mitochondrial Ca\textsuperscript{2+} uptake. This could be either increased cytosolic Ca\textsuperscript{2+} or, perhaps, an action on a mitochondrial Ca\textsuperscript{2+} transport system. Although NO or peroxynitrite generation seems capable of such action (see above), and there is delayed production of iNOS that might contribute to damage in this way, there is no evidence for such a mechanism at present.

C) MITOCHONDRIAL TRANSCRIPTION. Mitochondrial transcription of cytochrome oxidase mRNA is damaged within ~3 h of 3.5-min global ischemia in the gerbil. There was a progressive loss in enzyme activity and mRNA, with both falling to 20% of normal after 2 days, well before major manifestations of cell death. This was confined to the CA1 region (2). This is consistent with the fairly dramatic delayed lowering of ATP that was measured qualitatively in CA1 of the gerbil (432). This phenomenon has not been well-studied in other models but clearly may be important. The mechanism by which transcription is damaged is not known.

D) OTHER POSSIBILITIES. Ceramide, whose concentration rises within 6 h of starting focal ischemia (619), has been shown to reversibly inhibit isolated mitochondria (398). The elevated ceramide concentration is maintained throughout the ischemia, so it may inhibit in situ though it seems unlikely it would account for inhibition of the isolated mitochondria, since it is readily reversed on washing (398).

Another possible basis for damage is that a large release of cytochrome \(c\), the probable activator of caspases, compromises the respiratory chain.

E) SUMMARY. There are several reasonable mechanisms for delayed mitochondrial damage following ischemia. Evidence for free radicals is strong in that several free radical scavengers, or agents which should reduce their generation such as PAF inhibitors, attenuate mitochondrial damage, and also protect against ischemic damage (1025). Furthermore, free radicals and peroxynitrite severely damage mitochondria in vitro. Further studies with NOS inhibitors are essential to determine the role of NO and/or ONOO\textsuperscript{-} in vivo.

Mitochondrial Ca\textsuperscript{2+} accumulation also remains a viable possibility as a damaging agent and may certainly act along with free radical production. More evidence concerning mitochondrial Ca\textsuperscript{2+} changes that presage damage would be very useful. Inhibition of cytochrome oxidase synthesis and/or loss of cytochrome \(c\) are also very reasonable mechanisms of damage.

If Ca\textsuperscript{2+} or free radicals are the active agents, then the molecular target needs to be determined. Delayed damage is not caused by irreversible damage to any of the dehydrogenase complexes (862). Free radicals might be the agents acting on mitochondrial DNA to block transcription of cytochrome oxidase mRNA. Free radicals may oxidize cardiolipins, the principal mitochondrial lipid. This has not been measured in ischemia but does occur when the MTP is opened and mitochondria generate free radicals (452). Cardiolipin changes may well produce defects in the electron transport chain (872).

The mitochondrial pore transition represents a reasonable target for damage; it could be mediated by increased Ca\textsuperscript{2+} or by free radicals, and by both acting synergistically (410). It produces profound changes in cell function (89, 410). Although early evidence concerning the pore opening was conflicting, it now appears that it does play a role in, at least, focal ischemic damage. Cyclosporin A, which inhibits the MPT, was very protective in a global ischemic model (1145), but the drug also inhibits calcineurin, and FK-506, the calcineurin blocker, is also protective after global ischemia (272). FK0506 is also protective after temporary focal ischemia (627), but in this insult, a derivative of cyclosporin, methyl-valine cyclosporin A, which does not inhibit calcineurin, reduces the infarct size by >50% (528). This result implicates opening of the transition pore in, at least, focal ischemic damage, although it does not show whether it is early or late opening that is damaging.

5. Damaging actions of mitochondrial dysfunction

There are four apparent mechanisms by which mitochondrial dysfunction in the postischemic period could lead to cell death. These include maintaining low ATP levels, overproduction of free radicals, initiation of apoptosis or other damage by leakage of macromolecules, and decreased ability to buffer Ca\textsuperscript{2+} loads. There may be other, unknown effects of mitochondrial damage.

A) LOWERED ATP LEVELS. The issue is whether and how maintaining small (20–30%) reductions in ATP for many hours, as occurs in the posts ischemic phase, will lead to cell death. Elegant and important in vivo studies by Beale, Greenemayre, and co-workers show that brain-injected mitochondrial inhibitors cause a delayed cell death. However, the long transient fall in ATP, of ~50–60% at 3 h, more closely parallels conditions during focal ischemia, so the studies are not necessarily germane (1001). More pertinent studies have been done in culture where chronic treatment with mitochondrial inhibitors leads to cell death within 8–24 h (870, 1276, 1277). In one of these, ATP levels were maintained at 70–80% of normal. Cell death developed in 4–8 h (870) and was preventable by glutamate receptor blockade (870, 1277). Death showed selective vulnerability (1277), and there were mixed populations of apoptotic and necrotic neurons at 48 h (870). Thus low-level mitochondrial inhibition produces both apoptotic and necrotic populations in a timely fashion, but in this case in culture. Although not tested, the assumption is that MTP did not open and that there was not an enhanced production of mitochondrial free radicals so that this represents a “pure” effect of lowered ATP. Even if this is not so the results indicate that mitochondrial
inhibition, to a level which approximates the change in vivo, can lead to cell death in a timely fashion.

B) FREE RADICAL GENERATION. Although in vitro studies of mitochondria strongly indicate that their production of free radicals should be increased by ischemia (see sect. vA), the actual evidence for this is really limited to only one paper. It was shown, using the salicylate trap to measure OH production, that free radical generation during the 1 h after global ischemia was completely prevented by blocking the mitochondrial respiratory chain with rotenone (908). No effects on subsequent damage were measured, and further work is clearly required.

C) INDUCTION OF APOPTOSIS. It is now clear that mitochondria play a central role in (if not all) cases of apoptosis (1275) by releasing cytochrome c (591, 941, 1250), and possibly other proteins (1275), from the intermembrane space, and it is clear that adequate cytochrome c can induce apoptotic changes, at least in part by activating caspases (617, 941). There are several pathways by which cytochrome c could be released including the MPT (452, 617, 894, 1274). A possible mechanism for release after ischemia is by direct interaction of BAX with the outer mitochondrial membrane (941, 1161). Overexpression of Bax induces cytochrome c release from mitochondria in several systems (220, 882, 963), and recombinant Bax causes release of cytochrome c from isolated mitochondria (514). Bax is indeed upregulated in vulnerable cell populations after ischemia (185, 420, 1306), and such a pathway would explain the apparent requirement for protein synthesis in ischemic apoptosis.

D) OTHER MECHANISMS. A compromised mitochondrial membrane potential due to inhibition of oxidative phosphorylation will compromise the Ca²⁺ uptake pathway in mitochondria and hence reduce the Ca²⁺ buffering that occurs during normal high-frequency neuronal activity (1202). This would lead, on average, to chronically elevated cytosolic Ca²⁺ levels and could thus be damaging.

Another possibility is that other heretofore unidentified proteins may be released from mitochondria once the transition pore is opened, and lead to necrotic changes, e.g., proteins which enhance Ca²⁺ channel opening (841).

6. Conclusions

There are many strong heuristic arguments for the involvement of mitochondrial change or damage in ischemic cell death. These include measured effects of conditions prevailing during ischemia, such as peroxynitrite generation, on isolated mitochondria and also effects of mitochondrial damage on variables such as free radical generation, protein leakage, and indeed on cell viability in several model systems. However, at this stage, there is very little positive evidence that these changes play a role in ischemic cell death. In fact, these are confined to protective effects of methyl-valine cyclosporin A in focal ischemia (global ischemia has not yet been tested) and the movement of cytochrome c from mitochondria to cytosol also seen after that insult.

There are many reports of damage to isolated mitochondria after global and focal ischemia, but these are somewhat unsettling because no consistent basis for the dysfunction has been pinpointed; damage to respiratory chain complexes, blockade of synthesis of, or leakage of, respiratory chain components, opening of MTP, and accumulation of Ca²⁺ have all been noted or suggested, but no coherent account has arisen to date. Furthermore, there is not a strong consensus among different laboratories on times at which mitochondria are inhibited. Much of the uncertainty probably arises from difficulties in preparation from damaged tissue, tissue heterogeneity, and difficulties inherent in making measurements on isolated mitochondria, such as appropriate choice of buffer. Thus, at this stage, it is difficult to be definitive about the timing and severity of damage to isolated mitochondria.

Damage in situ has not been well established at all except in one case where measurements were made 24 and 48 h after focal ischemia, when cell damage was widespread. Perhaps the best evidence is the widespread pallor observed within the developing infarct when tissue is exposed to the mitochondrial function dye TTC. This certainly indicates compromised mitochondria, although decreased metabolic rate or glucose metabolism would lead to the same result. Certainly mitochondria appear to be greatly swollen and disorganized in cells showing ICC, which is consistent with damage, but this does not necessarily translate to functional damage.

If mitochondrial function is indeed compromised after ischemia, then evidence from cell cultures indicates that even the low-level inhibition that appears to exist at that time, evaluated by ATP concentrations, could cause apoptotic and necrotic cell death. Unfortunately, there are no pertinent studies in vivo, in which effects of prolonged mild inhibition of mitochondrial function have been tested. The studies of glutamate toxicity indicate that profound mitochondrial Ca²⁺ accumulation is a major factor leading to cell death, although the mechanisms are not well understood (829). There is, though, no particular reason to think this occurs before ischemic cell death.

Thus, although mitochondrial lesions seem to be strong candidates for sites of cell death, or severe damage leading to death (1044), there is no compelling evidence at the present time except the very recent data on the effects of methyl-valine cyclosporin A, presently in abstract form. Whether or not lesions in this organelle are important in cell death, this section has pointed out that there is much to be done in determining the nature of mitochondrial damage associated with ischemia.
C. Global Inhibition of Protein Synthesis

Prolonged inhibition of protein synthesis, if uncompensated by a decrease in breakdown of protein, would clearly lead to massive changes in cell properties and cell death. Protein synthesis is a complex process that is critically dependent on energy charge (936), intracellular K⁺/Na⁺ (672), and the integrity and phosphorylation level of a large number of proteins and RNA species (1230). It is strongly and permanently inhibited by ischemia in vulnerable cells, and the question is whether or not this is responsible for any forms of ischemic cell death. The question has to be considered in light of the marked activation of synthesis of certain damaging proteins in those same areas in which global synthesis is inhibited, making it a complex but very intriguing issue.

1. Problems of measurement

Glial proliferation occurs after most global ischemic episodes, and the increased protein synthesis (which is seen as soon as 3 h after ischemia in the hippocampal slice, Ref. 936) can more than compensate for any decrease in neuronal synthesis (332). Thus autoradiographic measurements are necessary to properly assess changes in neuronal protein synthesis.

2. Inhibition of synthesis by ischemia

There is a profound brain-wide depression of protein synthesis in the period during and immediately after global ischemia that persists in most brain regions of many different species for 1–24 h depending on the model (218, 257, 348, 790, 1113, 1209). Over 12–48 h there is complete, or near complete, recovery in the regions where cells will recover from the insult. In contrast, regions in which the cells will die never regain normal levels of synthesis or in some cases regain and lose them again (102, 257, 348, 790, 1113). In gerbils, CA1 pyramidal layer synthesis fell to unmeasurably low values 12 h after 5-min ischemia and recovered no further; polysomes remained dissociated (581, 790, 1113). In another study of gerbils, synthesis in the CA1 region was ~30% of basal values after 24 h (348). In the monkey, synthesis in hippocampus showed a transient recovery 6 h after complete compression ischemia, but became strongly inhibited between 12 and 24 h afterward. Synthesis was depressed in core and penumbra during at least 12 h of focal ischemia (757, 758); there are no reports of protein synthesis after temporary focal ischemia. Protein synthesis in CA1 pyramidal cells is reduced to ~40% 3 h after ischemia in rat hippocampal slices (936).

Thus, overall, neuronal protein synthesis remains profoundly depressed in vulnerable areas after global ischemia and during permanent focal ischemia. It would be useful to know what occurs after temporary focal ischemia.

3. Basis for prolonged inhibition of protein synthesis

Protein synthesis is extremely sensitive to cell energy charge and ion contents (672, 936). However, the persistent postischemic inhibition of synthesis occurs despite return of energy charge to normal values in slices (936) and of ATP to normal levels in vivo (12). Ribosomes from ischemic tissue seem competent (243) so are unlikely to be the site of damage.

A) INITIATION FACTORS. At this stage, the only changes that have been positively implicated in the decreased synthesis are changes in the two initiation factors, eIF-2 and eIF-4, consistent with the major loss in polyribosomes after ischemia (104).

eIF-4G is a substrate for calpain, and there is a 30–50% loss in its immunoreactivity after 10- and 20-min decapitation ischemia in rabbit (821). This is very likely due to calpain-mediated proteolysis because the 70% loss of immunoreactivity at the end of 20-min cardiac arrest is completely prevented by MDL-28170, the selective calpain antagonist (820). There is a prolonged inhibition of protein synthesis in vascular endothelial cells as a result of free radical attack on eIF-4 family-like proteins (513), showing that eIF-4 is also inactivated for a long time by free radicals. Thus it is a very likely target.

There is also evidence that eIF-2 is involved in the inhibition. Ternary initiation complex formation in tissue homogenates is decreased throughout the cerebrum 30 min after ischemia and is decreased in vulnerable regions only (striatum and CA1 of hippocampus) 6 h after 15-min 2-VO ischemia (146, 466). There is conflict as to the basis for this inhibition. A priori, phosphorylation of ERF-2α or dephosphorylation of guanine nucleotide exchange factor (GEF) are the most likely inhibitory points (466, 1230). One set of studies concluded that there was no increase in EIF-2α phosphorylation but demonstrated a decrease in GEF activity that appeared to be due to dephosphorylation of a site that was normally phosphorylated by a tyrosine kinase. The decreased activity lasted at least 6 h in striatum and CA1 of hippocampus (466, 470). There was a correlation between decreased phosphorylation of MAP kinase and cell damage/protein synthesis inhibition (468), and it was speculated that inhibition of synthesis resulted from the lack of neurotrophin upregulation in vulnerable populations and resulting dephosphorylation of GEF. Certainly neurotrophin withdrawal dramatically and rapidly lowers protein synthesis in dependent cultures (239).

With the use of somewhat more sensitive methodology than used previously (466), inhibition of synthesis at 30-min postischemia was actually associated with a 20–30% increase in phosphorylation of eIF-2 (146). Furthermore, there is a threefold increase in the eIF-2 kinase activity of neocortical brain homogenates 30 min after 30-min ischemia in the rat, suggesting activation of protein kinase R (PKR) and providing a basis for the phosphorylation (147). More recent work, in which the anti-
body for this phosphorylated form of the initiation factor was used, amplified this considerably (244). There was a 20-fold increase in the phosphorylated form of eIF-2a that was confined to vulnerable CA1 cells, and some CA3 cells, 10 min to 1 h after 10-min cardiac arrest in rats. At 4 h, the antibody to this form was still present in the vulnerable cells, and although a great deal had shifted to the nucleus (244), a significant amount remained in the cytoplasm (1078). Importantly, when insulin, which dephosphorylated eIF-2, was injected several hours after ischemia, protein synthesis was restored to its normal level, strongly suggesting that the eIF-2 phosphorylation was the basis for the inhibition of synthesis (1078).

There is at least one caveat, however. The absence of the phosphorylated form in dentate granule cells after 10-min reperfusion (244) is inconsistent with the very profound inhibition of synthesis that occurs even in nonvulnerable cell populations for the first 6–24 h (348). Furthermore, it is essential to explain the very prolonged inhibition in CA1 (days). It is not clear that phosphorylation of eIF-2 would be maintained that long. For example, glutamate activates eIF-2 phosphorylation and inhibits protein synthesis. However, the phosphorylation is transient; the eIF-2 dephosphorylates within 1–2 h of removing glutamate (712). Prolonged maintenance of GEF dephosphorylation, which may result from chronically lowered brain-derived neurotrophic factor (BDNF) (599), as occurs in vulnerable populations. Clearly, proteolysis of eIF-4 is likely to be an enduring effect. However, it is not established that this factor is adequately rate limiting in brain to account for the inhibition of synthesis. This could be tested fairly readily by measuring the effects of the cell-permeant calpain inhibitors on long-term inhibition of protein synthesis.

b) RNA levels. Unlike protein synthesis, global RNA synthesis seems to be remarkably stable, showing no decrease in one study up to at least 4 h after 5-min ischemia in the gerbil (103). However, there is a decrease in total mRNA in CA1 3 h after 5-min global ischemia in the same species (723). Thus there may be activation of mRNA breakdown, which could account for loss of mRNA and inhibition of synthesis. This needs to be tested further.

4. Ischemic changes that might lead to inhibition of synthesis

A) Increased cytosolic Ca\(^{2+}\). There is evidence from different sources that increased cytosolic Ca\(^{2+}\) is involved in inhibition of synthesis.

N-methyl-d-aspartate antagonism, which blocks much of the increase in cell Ca\(^{2+}\) in focal ischemia, protected against inhibition of synthesis in that model (758). Inhibition of synthesis in the CA1 pyramidal cell layer of hippocampal slices does not occur if the rise in cytosolic Ca\(^{2+}\) is prevented by incubating slices in 0-Ca\(^{2+}\) and 200 \(\mu\)M ketamine during ischemia (936), a combination which almost completely suppresses the increase in cytosolic Ca\(^{2+}\) (1288). Combined NMDA and AMPA/kainate receptor blockade also attenuated the inhibition (164, 1171), and this combination largely prevents the increase in cytosolic Ca\(^{2+}\) during ischemia (1288). It has been suggested that Ca\(^{2+}\) is not involved in inhibition because in one study protein synthesis in brain slices was inhibited by ischemia even in the absence of extracellular Ca\(^{2+}\) (265). However, there is a large rise in cytosolic Ca\(^{2+}\) in that condition that was not explicitly recognized by the authors (769, 1288).

Although increased cytosolic Ca\(^{2+}\) certainly should activate calpain-mediated breakdown of eIF-4, it might also act on eIF-2. A-23187 leads to a prolonged 70% increase in eIF-2a phosphorylation in cultured neurons (11) as does glutamate (712). The Ca\(^{2+}\) might activate PKR, which phosphorylates eIF-2. The protein is regulated in a complex way (507) and might be activated by Ca\(^{2+}\)-mediated effects.

B) Free radical generation. The free radical-mediated inhibition of protein synthesis in vascular endothelial cells described above (513) indicates that the abundant free radical production in ischemia might cause the damage.

c) Depletion of ER Ca\(^{2+}\). It has been suggested (244) and previously theorized (880) that depletion of ER Ca\(^{2+}\) might inhibit synthesis after ischemia. This depletion is known to inhibit protein synthesis, by activating PKR and hence phosphorylating and inhibiting eIF-2a (925). It is not known whether this ER depletion occurs during ischemia, but there are reasons to think it might. Arachidonic acid, at levels that are present after ischemia (30 \(\mu\)M), causes depletion of Ca\(^{2+}\) from the ER and inhibition of protein synthesis in cells (244, 969). Also, Ca\(^{2+}\) uptake into isolated ER microsomes is inhibited after ischemia, although the mechanism is not known. If this occurs in vivo, it would certainly lower ER Ca\(^{2+}\) (878). Presently, though, there is no definitive evidence for a change in ER Ca\(^{2+}\) at all so the mechanism is purely speculative (879).

5. Evidence linking inhibition of protein synthesis and cell death

This is most crucial. There are strong correlations between inhibition of synthesis and eventual cell death (460). Although 2-min ischemia does not cause cell death, 3-min ischemia leads to persistent inhibition of protein synthesis in CA1 and to cell death in the gerbil (31). Ischemic tolerance in gerbil (see sect. vizF) is associated with restoration of protein synthesis in CA1 (348, 544); hypothermia protects against histological damage and also prevents delayed inhibition of protein synthesis in the 4-VO model in rat (1209) as do barbiturates (114). Unfortunately, such correlations are by no means unique to global protein synthesis and so cannot be thought to implicate the process in cell death.
There is a paucity of good evidence linking synthesis inhibition with damage. For example, there are no reports on effects of agents that prevent cell death, such as calpain inhibitors or free radical scavengers on protein synthesis after ischemia. It is important to know if they prevent the inhibition of protein synthesis before preventing cell death. Also, it would be very useful to have good pharmacological studies using inhibitors of protein synthesis to see whether partial inhibition would eventually cause cell death in normoxia. In this context, the existing data argue against a role for protein synthesis inhibition in the cell death. In particular, 2-min ischemia in the gerbil does not cause any delayed morphological damage in spite of strongly inhibiting protein synthesis in CA1 for between 5 and 24 h (348). The best test of the role of protein synthesis would be an injection of insulin soon after the global ischemia. As described above, this dephosphorylates eIF-2 and restores protein synthesis, without causing significant hypoglycemia. If it is very protective it would suggest protein synthesis inhibition is an important factor.

Although the studies of inhibition of global synthesis in normoxic or only mildly insulted brain tend to argue against a crucial role for overall inhibition of synthesis in cell death, studies of the role of protein synthesis in ischemia, described in section VIII, suggest otherwise. The inability to synthesize protective proteins after ischemia, shown by vulnerable cells, may well be the basis for their ultimate demise. Furthermore, several key proteins are inactivated by ischemic events (e.g., protein kinases), and the inability to resynthesize these proteins could be very detrimental to the cell. These effects of inhibited protein synthesis would only be manifested following very damaging insults and so would not be observed in the mild insult of 2-min ischemia.

6. Protein degradation

There is very little known about rates of protein degradation after ischemia. The average turnover times of brain proteins are 3–5 days (390, 1020). If normal protein degradation is blocked after ischemia, it would reduce the impact of synthesis inhibition. Such a close coupling has been seen in cultured sympathetic neurons where a 50% inhibition of synthesis is matched by an ~50% inhibition of degradation rates, although this matching requires the presence of growth factors (333).

There is a marked decrease in ubiquitin immunoreactivity in the CA1 region after global ischemia (703), suggesting that a major degradation pathway is blocked, and this might well lessen the impact of protein synthesis inhibition. However, degradation rates really need to be measured.

During focal ischemia, blocking proteasome activity using PS-519 was extremely protective to the core of the lesion (902). One explanation for this is that protein degradation makes a major contribution to core damage, in focal ischemia. Indeed, the protein content within the core of a focal lesion is severely reduced. However, another possible explanation is that proteasome activity is damaging because it allows activation of NFκB (56). Protein degradation after both global and focal ischemia needs to be measured to resolve this issue.

7. Protective effects of inhibitors of protein synthesis during an ischemic insult

There are studies that seem to directly contradict the possibility that protein synthesis inhibition causes damage, in that damage is prevented by inhibitors of synthesis.

Two studies showed that fairly well-maintained inhibition of synthesis for 2 days was remarkably protective against global ischemic damage in rat (386) and gerbil (1027). Unfortunately, however, temperature regulation was very transient and, as acknowledged by the authors, the protection may well have resulted from hypothermia.

Inhibition of protein synthesis for ~12 h after the insult also attenuates damage after mild (30-min ischemia) (276, 304) or relatively mild (90-min ischemia) (275) transient focal ischemia. This is consistent with the large component of apoptotic cell death in these paradigms. In one of these studies (275), temperature was actually monitored for 2 days after the insult and was unaffected by the cycloheximide treatment, so temperature is almost undoubtedly not an artifact. While important for these mild insults, these results do not show that profound inhibition of synthesis for one or more days, as occurs after global ischemia, is not damaging. Synthesis was inhibited for only several hours after the insult.

8. Conclusions

Protein synthesis is profoundly and permanently inhibited in vulnerable cells after ischemia. The mechanism is not known, but there is reasonable evidence suggesting that changes in initiation factor complexes (eIF2/GEF or eIF4) are responsible and one study indicating that mRNA breakdown may be responsible. The latter would probably reflect activated RNase activity. Evidence from brain slice studies suggests that increased Ca\(^{2+}\) is involved in causing the inhibition of synthesis. Another suggestion is that it results from depletion of Ca\(^{2+}\) from the ER and another that it results from free radical action. The last two have not been demonstrated in brain.

The localization and time course of bulk protein synthesis inhibition is completely consistent with its involvement in cell death, as is the correlation between inhibition of synthesis and cell death in several artifactual paradigms. However, it is difficult to positively implicate inhibition of synthesis in the ischemic death, as has been suggested (460, 757), particularly for focal ischemia.

The bulk turnover times of proteins are so long that even profound inhibition of protein synthesis should not
alter composition enough to cause cell death in the 12–24 h after focal ischemia. This is supported by the absence of any ill effects after 2-min ischemia in gerbil despite a profoundly depressed protein synthesis for between 5 and 24 h after the ischemia. Damage would be still less likely if turnover of protein was diminished by the ischemic insult.

These considerations are not germane to short global ischemic episodes, where death often requires 3–4 days. If synthesis is depressed for that long it might well affect function severely. Effects of prolonged pharmacological inhibition of protein synthesis would be very informative, along with measurements of protein turnover after ischemia.

Independently of the effects of long-term loss of protein, the global inhibition of synthesis after ischemia may be extremely important in death of vulnerable cell populations by preventing synthesis of protective proteins at critical times and preventing resynthesis of proteins that are inactivated by the immediate sequelae of ischemia.

D. Damage to the Cytoskeleton

Much of cell structure, protein localization, and almost all transport into and from dendrites and axons is mediated by cytoskeletal molecules. Their gross disruption would be expected to cause major losses in ordering and in transport within the cell and hence cell death by necrosis or by triggering apoptotic changes.

1. Microtubule changes during ischemia

It is clear that microtubule dissolution occurs in ECC (495, 521, 522) and may play a major role in allowing this form of damage. This has not been tested with microtubule stabilizing drugs such as taxol, and little is known about the importance of microtubule dissolution (1122, 1123, 1236, 1237).

In the gerbil, microtubules in the distal dendrites of the very vulnerable CA1/subiculum region disassemble immediately after 5-min ischemia; this disassembly reaches the proximal dendrites after 10 min (1237). Loss of MAP2 and tubulin antibody staining accompanies the microtubule breakdown (1237, 1242). The cells within CA1 that are somewhat less vulnerable require longer durations of ischemia to show this early dissolution. However, between 6 and 24 h after 5-min ischemia there is a major microtubule disassembly in proximal (and distal) apical dendrites of all CA1 pyramidal cells (348, 1236). Semiquantitative estimates show a 50% loss in microtubules at this time (348). In general, these changes are irreversible in cells that are destined to die. They also occur in nonvulnerable cells where they recover after 12–24 h (348). There is complete loss of MAP2 antibody staining in apical dendrites of the CA1 pyramidal cells at this time also (726).

Kinesin and cytoplasmic dynein immunoreactivities are greatly decreased in CA1 at 3 and 8 h after 5-min ischemia (29), before the major microtubule disruption, suggesting an early blockade of dendritic transport of most proteins (29). This could be very important, and the result will, hopefully, be pursued.

Microtubule changes following global ischemia in the rat are much less dramatic than in gerbil, even up to 24 h after 2-VO (251, 357, 581, 1127). However, microtubules do become disorganized between 24 and 48 h, well before cell death. It is not at all clear why the gerbil and rat behave so differently; there may be more stabilizing elements such as calpastatin in rat, but this is not known. There are no measurements to see whether motor proteins might be compromised, as they are in gerbil.

There are no reports concerning early microtubule changes in focal ischemia.

2. Mechanisms of microtubule changes

Known mechanisms of microtubule dissociation include MAP2 phosphorylation or proteolysis, dissociation of the putative microtubule-stabilizing protein STOP decreased GTP/GDP, or proteolysis of tubulin (708, 770). All of these are activated by Ca$^{2+}$ with the probable exception of the decrease in GTP/GDP (273). MAP2 phosphorylation and dissociation from microtubules is also activated by PKC and other kinases that may be activated very early during ischemia (132, 459, 491). Thus there are many potential mechanisms for microtubule dissolution.

Recent studies from our laboratory (316, 1291) on rat hippocampal slices, where there was significant breakdown of microtubules in CA1 dendrites during 6-min ischemia, indicate that the breakdown is almost certainly mediated by Ca$^{2+}$. It was greatly attenuated when slices were incubated in 0 Ca$^{2+}$ buffer and ketamine, a combination that prevents 80% of the normal rise in cytosolic Ca$^{2+}$ (1288), or lidocaine and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), which similarly blocked the cytosolic Ca$^{2+}$ rise. It was also blocked by inclusion of 25 μM calmidazolium, the calmodulin antagonist (316) (which did not attenuate the cytosolic Ca$^{2+}$ increase), and by the calpain inhibitor MDL-27,180 (1291).

The mechanism of breakdown is not known, but it is not mediated by MAP2 proteolysis or by proteolysis of microtubule only stabilizing protein, STOP or tubulin (Zhang and Lipton, unpublished data). The involvement of calmodulin might be because it causes dissociation of STOP from the microtubules, which it does in vitro (912), but this has not been shown for ischemia. These considerations suggest that prolonged, or delayed, elevation of Ca$^{2+}$ might be responsible for the profound microtubule dissociation in gerbil and the milder disorganization in rat, but there is no current evidence.
3. Evidence linking microtubule breakdown to ischemic cell death

The microtubule-dissociating drug colchicine causes cell death in cultured cerebellar granule cells (110), as well as other cell types (659, 1140, 1193). Death appears to be apoptotic. Furthermore, photoactivated disruption of microtubules leads to loss of intracellular organelle motility and quite rapid cell death in cultured kidney epithelial cells (639). Thus there is reason to think that the ischemic breakdown of microtubules would contribute to the cell death. Unfortunately, there are no data that support (or contradict) the possibility.

There are data showing correlations between microtubule dissolution and ischemic cell death. Ischemic tolerance in gerbils is associated with the reestablishment of the microtubules in the CA1 pyramidal cells (348), and MAP2 breakdown is affected by temperature in the same way as cell death. Reducing temperature to 33°C during ischemia markedly decreased loss of MAP2 immunoreactivity, whereas increasing temperature to 39.7°C markedly enhanced it (293).

The most exciting study to see would be the effect of the microtubule-stabilizing drug taxol on development of ischemic cell death. Unfortunately, there are no such reports. Taxol does attenuate ischemic microtubule breakdown in the slice (Zhang and Lipton, unpublished data), so it may well provide useful information if used in vivo.

4. Other cytoskeletal proteins

A) SPECTRIN AND ANKYSIN. Spectrin provides major linkages between the cell membrane and membrane-associated proteins, which in turn are involved in maintaining integrity and localization of integral membrane proteins (80, 81, 471). It is very susceptible to calpain (471) and to caspase-3-like proteases (810), which cleave it at different sites (810).

After 5-min ischemia in the gerbil there is a rapid (within 30 min) breakdown of spectrin by calpain (641, 952, 973) in many brain regions but oddly, perhaps, not in CA1 pyramidal cells of hippocampus (952). However, there is a delayed breakdown that is measurable in dendrites of CA1 pyramidal cells after 1 day, peaking at 2 days, and persisting until cell degeneration (952, 973, 1258). This suggests a delayed activation of calpain in CA1 that is probably very important and is discussed in section B. There is breakdown at the core of a focal ischemic lesion after 3 h (455), but no substantial studies have been reported for focal ischemia.

These results are certainly consistent with a role for spectrin breakdown in the delayed cell death. However, arguing against this somewhat is that only a very small percentage of total spectrin in a region is broken down. Although several studies clearly show the appearance of spectrin breakdown product, there are no notable decreases in band intensities of native spectrin on Western blots (13, 455, 641, 952, 973), even in the core of the lesion after 3-h focal ischemia (455). Thus the percentage reduction is small. It may, however, be important.

Caspase-activated spectrin breakdown, which may well be an important component of apoptosis, has not been investigated in vivo, but it is prominent during in vitro ischemia in rat cortical cell cultures (810).

Ankyrin plays a major role in linking membrane proteins, such as Na$^+$-K$^+$-ATPase and Na$^+$ channels to the cytoskeleton. Two major isoforms, ankyrin B and ankyrin R, are localized to membrane fractions including the crude synaptosomal fraction (426). Thirty minutes of global ischemia in rat, followed by 60-min reperfusion, caused a 20% loss of immunoreactivity of ankyrin B and breakdown of ankyrin R, possibly mediated by calpain. The breakdown was largely in the posts ischemic period, suggesting that reperfusion sensitizes the system. These changes could certainly lead to malfunction of the Na$^+$ pump or other membrane proteins and so contribute to cell death. This may account for the observed inhibition of the Na$^+$ pump after 60-min ischemia (866). Effects of shorter, but still lethal, durations of global ischemia need to be demonstrated, as do effects of focal ischemia.

b) OTHER CYTOSKELETAL PROTEINS. There is a 30% loss of immunoreactivity of a 200-kDa neurofilament subunit 1 day after either 5- or 8-min 2-VO in the rat (519); the role of the protein is not known.

5. Conclusions

The data and the literature support the possibility that microtubule dissolution contributes significantly to apoptotic or necrotic cell death. However, there are major uncertainties. There are no studies of microtubule changes in focal ischemia, and changes in the rat during global ischemia are much later, and less profound, than in gerbil. Mechanisms of cell death in the gerbil and rat may differ, or changes in rat may be more subtle but still important. Certainly, though, in the gerbil, changes are very rapid and profound and highly correlated with eventual cell death. It is difficult to think that these do not strongly influence cell death given the profound effects of microtubule dissolution on cell viability. Unfortunately, though, there are no data showing a causal relationship between microtubule breakdown and ischemic cell death, and these are essential. If microtubule changes are important, then a key question becomes how their dissolution is maintained over time. Early effects of Ca$^{2+}$ and phosphorylation might well be expected to be reversed. Indeed, it is possible that an insult causes irreversible damage and death when it is intense enough to cause a loss of the ability of microtubules to reassemble. This could be via proteolysis of key components.

The decrease in motor proteins seen in the gerbil is potentially very important, since it would stop distribu-
tion of substances by microtubule-based flow processes. This could be very damaging. In light of the large difference in microtubule dissolution between rats and gerbils, it would be very interesting if rats also showed the loss of motor proteins. It would add weight to the conclusion that there is a cytoskeletal component to cell death.

Other cytoskeletal proteins, such as spectrin and ankyrin, also undergo breakdown, and many more as yet unidentified probably do also, since they are prime targets for calpain, which seems to play a major role in apoptotic and necrotic cell death. Many important cytoskeletal changes are also effected by caspasases.

There are many reasons to think cytoskeletal changes would play a major role in cell death. Cytoskeleton plays essential roles in distribution of protein and lipid and has a major role in maintaining cell structure, which is clearly dramatically altered in ischemic cell death. To date, though, there is no strong positive evidence that the cytoskeletal changes are important in the ischemic damage.

E. Summary

Four functional changes have been examined which, a priori, seemed most likely to account for necrotic cell death. Two of these changes, in mitochondrial function and microtubular/cytoskeletal structure, could also be important in apoptotic death. Unfortunately, there is no compelling evidence that any of these functional changes cause the cell death, although it is difficult to think that they are not, among them, largely responsible.

Both plasmalemma and mitochondrial damage could clearly cause cell death but, so far, there are no convincing demonstrations that either occurs to the extent that would do this. Evidence that appropriate mitochondrial changes occur is better than for plasmalemma changes, including leakage of cytochrome c and protective effects of methyl-valine cyclosporin A in focal ischemia but is not yet compelling. Global protein synthesis is clearly drastically inhibited, but in this case, the difficulty is in showing that such inhibition will actually cause cell death; judicious use of insulin may be very helpful in this regard. Microtubule dissolution in gerbil is dramatic, and independent studies suggest this should lead to cell death. However, no pharmacological interventions have been tried to see if the changes are important in this case. Also of concern is the great disparity between the extent of microtubule changes in the gerbil and rat. Changes in the latter are much less, at the ultrastructural level. They may still be important, but this would need to be shown.

As noted at the beginning of section IV, cell death might not result from a functional defect in one or more of the key processes examined here; rather, it may result from continued activation of perpetrators (see sect. V) set in motion by the ischemic insult, with ultimate breakdown of the cell as a unit. In this regard, there is some evidence for the importance of purely degradative processes. There is equivocal evidence that large-scale proteolysis mediated by proteasomes may be important in focal ischemia and that autophagocytosis may be important in global ischemia.

V. PERPETRATORS OF FUNCTIONAL OR STRUCTURAL DAMAGE

This section examines biochemical processes that are likely to play a part in cell death by causing long-term changes in macromolecules. These will lead to the functional changes discussed in section IV or other effects related to cell death. They are considered perpetrators of damage.

A. Free Radical and Peroxynitrite Actions

I. Free radical, NO, and peroxynitrite changes during and after ischemia

The possible roles of free radicals, NO, and peroxynitrite in ischemic cell damage have been extensively reviewed (170, 985, 1043, 1046) since the early suggestions of Siesjo (1041). As discussed in section V A2, there are many ways in which free radicals (including NO) and peroxynitrite may be generated during ischemia. There is massive evidence from many systems that these species can cause cell damage. The question addressed in this section is the extent to which they are actually involved in ischemic cell death and the ways in which they are involved.

A) GLOBAL ISCHEMIA. I) Free radicals. Several studies show increased free radical formation during vessel occlusion. The electron-spin resonance signal increases over 10-fold during 15 min of 4-VO, measured on extracellular fluid collected in a cortical cup (906). There is a similar increase during this period in the striatum (1305) and in hippocampus (908) as measured with microprobes containing a spin trap or salicylate, respectively. There is also an increase in PBN spin adducts after 20-min 2-VO (976). There are some contradictory results. In one study there was no evidence of increased free radicals at the end of 10-min ischemia in gerbil (851), and no change in reduced glutathione was measured after 30 min of 4-VO in the rat (217). However, overall the evidence showing increases is persuasive.

The increase in free radicals becomes larger and unambiguous during the early reperfusion period (263, 851, 906, 908, 976, 1260, 1305). The persistence of the increase depends on the duration of the ischemia. After 10-min ischemia in rat, lucigen chemiluminescence levels fell from two times baseline to baseline after 40 min. However, after 15-min ischemia, the levels of 2,3-DHBA, a measure of free radical or peroxynitrite attack on micro-
probe salicylate, was still rising 60 min after the end of ischemia (908), and after 20-min ischemia, chemiluminescence levels remained at 150% baseline for at least 2 h. Thus durations of global ischemia that lead to profound delayed neuronal death cause free radical increases that are probably maintained for at least 2 h. Most interestingly, 24 h after 10-min global ischemia, there is a large elevation in superoxide that is restricted to the vulnerable CA1 pyramidal layer in the hippocampus (173). This was measured using hydroethidine fluorescence. The increase is approximately coincident with an upregulation of the enzyme COX-2 in CA1 pyramidal neurons. This occurs between 8 and 24 h after the ischemia (806) and is a very likely source of free radicals.

II) NO and peroxynitrite. There was an increase in NO, to ~11 μM, at the end of 15-min 2-VO global ischemia (1126), as measured with an NO-sensing electrode, and there was also an increase after 7-min 2-VO in hippocampus (850). No on-line measurements have been made after the end of global ischemia, so it is not known if the radical is continually generated. Peroxynitrite, which is generated from NO and superoxide, is specifically monitored by measuring formation of nitrosotyrosine. The latter is elevated 4 h after 30-min global ischemia in rats (330), but this is the only reported study. Further quite strong evidence for peroxynitrite formation, from the same study, is that blocking NOS with N\(^{G}\)-nitro-L-arginine (L-NNA) increased the net production of superoxide (330), suggesting the latter is normally metabolized to peroxynitrite. Thus insofar as measured, there is evidence for NO and peroxynitrite accumulation in global ischemia. At present, there is no detailed knowledge about the time course of peroxynitrite except that it occurs at some time during the first 4 h.

There may well be delayed production. Inducible NOS is greatly increased in the astroglia that border the CA1 pyramidal layer after 10-min 4-VO global ischemia (302, 303). The increase is first seen 1 day after the insult and becomes prominent 2 days later. The increase is confined to the CA1 region and is adjacent to pyramidal cells, which die at about this time. No measurements of nitrosotyrosine have been reported.

b) Focal Ischemia. Early indirect measurements of thiobutryric acid reactants (TBAR) accumulation (777), of loss of scavengers such as α-tocopherol, and of reduced forms of ascorbate and ubiquinones (577) strongly indicated that free radicals were produced within 30 min of initiating focal ischemia. These early studies were very important in establishing that there was production of free radicals. Moreover, the study in which depletion of scavengers was measured indicated that there was continual production of free radicals for at least 6–12 h of ischemia (577).

More sophisticated on-line measurements, using a cytochrome c electrode (312), or measuring lucigenen enhanced chemiluminescence in a cranial window (893), show marked increases in free radical formation in the peri-infarct penumbra (where flow levels are ~50%) during and after focal ischemia. During the ischemia, there were about twofold increases in indicator signal that occurred within 15 min of onset in one study (312) and within 100-min of onset in another (773). These persisted throughout 2–3 h of ischemia, indicating continual generation. Reperfusion after 1 or 2 h gave a large burst that lasted ~40 min and persisted throughout the remaining 2 h of the study at a steady level about twofold over baseline (312, 773).

Free radical production appears largely restricted to the penumbral region. When localized salicylate microprobes were used, continued generation was measured in the penumbra during 3 h of ischemia and for 6 h after ischemia. There was no production in the core through 3-h ischemia and until after 3-h reperfusion (1060). There is major damage by this time. This is an important study, suggesting that damage in the core of the lesion is unlikely to result from free radicals, although, as seen below, NO and peroxynitrite do appear to rise in the core. The absence of free radical generation in the core is somewhat surprising given the apparent generation of free radicals during global ischemia, where O\(_2\) levels are lower than in the ischemic core, and confirming studies will be important.

Actual rates of superoxide generation within the ischemic region, measured by the cytochrome c electrode, were ~25 μM/min (312). This is undoubtedly much lower than actual values, because some free radicals would have been scavenged before measurement.

I) NO and peroxynitrite. Nitric oxide appears to rise during focal ischemia. There are three reports of a transient (20 min) increase (622, 707, 1293) to ~2 μM (707). There is a continual generation in the postischemic period (622), although it is somewhat smaller than the early increase during ischemia (~0.6 μM) (707, 1293). It lasts for at least 60 min.

The increase in NO was enhanced by superoxide dismutase (SOD) infusion, which competes for superoxide radicals (74), indicating that the NO is normally reacting with O\(_2\) to produce peroxynitrite (622). Measurements of 3-nitrosotyrosine confirmed a substantial generation of peroxynitrite (347). There was about equal formation in core and penumbra at the end of 2 h of focal ischemia and approximate doubling of this in the penumbral region during 3 h of reperfusion. This was all blocked when NOS was inhibited by N\(^{G}\)-monomethyl-L-arginine (347). Levels of 3-nitrosotyrosine rose to ~1% of all tyrosine residues, far higher than in basal conditions when there is no measurable nitrosotyrosine, and 5 times the levels seen in Huntington’s disease. This is a major increase.

The rise of nitrosotyrosine in the core appears to conflict with the lack of free radical generation in the core (1060). More studies are required to resolve this, but it is quite possible that the enhanced NO production com-
bined with a normal steady-state superoxide production will allow formation of peroxynitrite (74).

C) INDUCED EXPRESSION OF NOS AND OF CYCLOOXYGENASE AFTER FOCAL ISCHEMIA. Two enzymes that are intimately related to free radical production are upregulated during the 6- to 24-h period after focal ischemia, as they are after global ischemia. Both iNOS (479) and COX-2 (837) are upregulated in the penumbral region during this period. The former is in endothelial cells and invading neutrophils (479, 836), whereas the latter is in neurons (836). Cyclooxygenase-2 (prostaglandin H synthase) generates superoxide radicals (837). The upregulation of these enzymes might lead to NO and superoxide synthesis during the later phases of reperfusion. Although they are in different cell types, they might well react to form peroxynitrite because both NO and superoxide can diffuse for several hundred microns (74). Further evidence for communication between the two processes is that about one-half of the enhanced COX-2 activity, although not the enzyme upregulation, results from the action of the iNOS (836). This was determined both by pharmacological and knockout studies.

D) SUMMARY. The studies definitively show that free radical production is elevated during both global and focal ischemia, for at least 2 h after focal ischemia and very probably for at least 6–12 h. Levels are elevated 24 h after global ischemia in vulnerable cells. Nitric oxide production is elevated to the low micromolar range during global and focal ischemia; it is also elevated after focal ischemia; no measurements have been made after global ischemia. Peroxynitrite is produced during or after both focal and global ischemia. Superoxide levels of 25 μM and NO levels of 1–15 μM have been measured. They should (74), and apparently do, yield a high level of peroxynitrite formation.

Critical questions that remain include the time course of free radical generation throughout the postischemic period and further localization and quantitation.

2. Mechanisms of net free radical production during ischemia

Normally the rates of free radical production and elimination are equal, leading to a steady state that is presumably tolerated by the cell. Ischemia creates several conditions that could account for the increased net production of free radicals (336, 413, 488).

A) XANTHINE/HYPOXANTHINE OXIDATION. The breakdown of adenine nucleotides during ischemia leads to accumulation of hypoxanthine within 10 min (the earliest published measurement, Ref. 407), which is then metabolized by xanthine oxidase (XO) or xanthine dehydrogenate (XDH). Xanthine oxidase produces free radicals, but XDH does not. The basal level of XO in brain tissue is similar to that in other organs such as liver and intestine (92).

The reaction for generating superoxide is as follows

\[ \text{Hypoxanthine} + O_2 \rightarrow O_2^- + H_2O_2 + \text{urate} \]

Accumulation of urate also results from the action of xanthine dehydrogenase (XD), and allopurinol, which inhibits the XO, also scavenges free radicals. Thus unequivocally demonstrating this mode of free radical production in the tissue is difficult.

At the moment, the evidence favors its importance in global ischemia (578) but not in focal ischemia (92). This is reasonable because changes in ATP and, probably, Ca\(^{2+}\), are larger during the global ischemic insult. The activity of XO, produced by proteolytic cleavage of XDH, was increased about fivefold 30 min after 15-min global ischemia in rat, and there was concomitant urate production (578). This is reasonable evidence that free radicals are being produced by XO. Although there is a large production of urate (tens of μM) during focal ischemia (92, 577, 1152), there is no particular evidence that it reflects XO activity rather than xanthine dehydrogenase activity. There is no protease-mediated conversion of XD into XO during focal ischemia, measured at 24 h (92), and, more importantly, allopurinol does not prevent edema at doses that almost completely block the urate production (92). Other free radical scavengers do protect against edema in the same condition. The results thus suggest that free radical formation does not result from this reaction in permanent focal ischemia. There are no data for temporary focal ischemia.

B) ACCUMULATION OF EICOSANOIDS. Oxidative metabolism of accumulated arachidonic acid (549, 1111) via the cyclooxygenase pathway (602, 620, 623) or via the lipooxygenase pathway (488, 623) leads to superoxide or OH\(^-\) production. Phospholipid breakdown into arachidonic acid is then further activated by free radicals (45, 175).

There is a manyfold increase of FFA, including arachidonic acid, during global and focal ischemia (804, 1111, 1292), and arachidonic acid metabolites are still 7–30 times basal 30 min after global ischemia (1111). No measurements were made right after focal ischemia, but by 4 h, there was no elevation (1292). The metabolism of arachidonic acid must thus be considered a likely source of the free radicals during and soon after both global and focal ischemia. However, there are no pharmacological studies directly establishing this in brain. There are such studies in retina, where inhibition of arachidonic acid metabolism reduced lipid peroxide formation and retinal damage (179).

A major mechanism for eicosanoid accumulation is the ischemia-induced synthesis of COX-2 that occurs 6–24 h after both global (806) and focal (837) ischemia. Inhibitors of this enzyme, such as SC-58125 or NS-398, dramatically reduce the delayed accumulation of prostaglandin E\(_2\) in both insults (806, 836). This COX-2-induced eico-
sanoid production occurs at 6–48 h and so constitutes a major source of delayed free radical production.

c) Altered mitochondrial function. Generation of free radicals by mitochondria has been well reviewed (740, 1142). The organelles normally generate free radicals at a rapid rate (117, 1142), but one which is handled by normally functioning cells. When the electron carriers become highly reduced, as much as 2% of the electron flow leads to direct single-electron reduction of oxygen and formation of superoxide (117, 336, 561). This very probably occurs during focal ischemia, and low-flow global ischemia when the members of the respiratory chain are in a relatively reduced form. Accumulation of Ca\(^{2+}\) (1143) and opening of the MTP (617, 710, 1275) are among other factors that may well occur at some point after ischemia, and which very probably cause mitochondrial free radical production by mechanisms that are not yet known (see sect. IV).

Despite the great potential for mitochondria as a source of free radicals, there is only one study that actually demonstrates their involvement (908). Direct measurement of free radical concentration, using the salicylate trap, revealed a steady increase during 15-min 2-VO and for the following 1-h reperfusion. This was completely prevented when mitochondrial respiration was blocked by including rotenone in the dialysate. It was restored if the rotenone block was by-passed by succinate, further implicating mitochondrial electron transport as the source. The free radical production was also prevented by haloperidol, which blocks respiration at complex I (908). As the authors point out, these results do not exclude other possible sources of free radicals, particularly prostaglandin metabolism, because salicylate is a potent inhibitor of cyclooxygenase and so could have been blocking that pathway while measuring free radical production.

Free radical production from electron transport also occurred in cultured neurons exposed to NMDA (282). The authors suggested this was due to accumulation of Ca\(^{2+}\). Consistent with this idea, the combination of elevated Ca\(^{2+}\) (2.5 \(\mu\)M) and ADP potently activated OH\(^-\) production in isolated mitochondria (287), with the OH\(^-\) acting as a positive feedback to enhance OH\(^-\) production. Although the mechanism was not determined, the conditions approximate conditions after ischemia and after glutamate application.

Knockouts of mitochondrial SOD (Mn-dependent SOD) enhance the extent of the lesion after focal ischemia (786), whereas overexpression of the same enzyme decreases infarct size (562). If this enzyme selectively inactivates mitochondrially generated O\(_2^-\); then mitochondria are definitely a source. However, this selectivity has not been established (74).

d) Neutrophil accumulation and activation. As discussed in section II, these cells adhere to the vessel walls and invade the parenchyma to a limited extent during and after focal ischemia (488, 1046). Neutrophils oxidize NADPH to generate superoxide and thus are important potential free radical donors during and after focal ischemia. In addition, neutrophils synthesize iNOS 1–2 days after focal ischemia (480). Evidence as to their role in free radical production is conflicting as discussed in section II. Neutrophils may play a priming role in activation of free radical production. There is no evidence concerning the roles of neutrophils in global ischemia free radical production.

e) Mechanisms of NO and peroxynitrite generation. Nitric oxide is generated by neural or endothelial NOS in an oxygen-dependent reaction that is activated by Ca\(^{2+}\)/calmodulin in most neurons and endothelial cells (477, 996). The Ca\(^{2+}\)/calmodulin directly activates the enzyme and may also remove a phosphorylation-induced block by activating calcineurin (235). Activation of NOS by the Ca\(^{2+}\)/calmodulin system has not been explicitly demonstrated by measuring, directly or indirectly, effects of appropriate agents on NO production during ischemia. The conclusion that the Ca\(^{2+}\)/calmodulin system is important is based on the importance of Ca\(^{2+}\) in NOS-mediated glutamate toxicity, as well as the ability of calmodulin inhibitors and calcineurin inhibitors to prevent such toxicity (235). The latter studies are not persuasive, however, because calcineurin inhibitors prevent NMDA toxicity in cerebellar cultures where NOS is not mediating the toxicity (26). Thus proving the involvement of calmodulin remains an issue.

Both neuronal (1294) and inducible (478) NOS are upregulated after focal ischemia, and iNOS is also upregulated in glia 1–2 days after global ischemia (303, 477). There is no explicit demonstration that these cause NO accumulation, but it must be considered very likely.

Peroxynitrite is generated by the reaction between superoxide and NO:

\[
O_2^- + NO \rightarrow ONOO^-
\]

It can be protonated to produce the very reactive peroxynitrous acid that dissociates into OH\(^-\) and various nitrogen/oxygen species (74). The unprotonated form, which is about 75% of total at pH 7.4, is also very reactive, with a half-life in biological systems of \(~1–2\) s and long diffusion distances of \(~100\ \mu\)m. There is good evidence that both the acid and the anion participate fairly equally in most reactions (1013). One or other of the forms readily crosses cell membranes so that peroxynitrite, like superoxide, can act in cells other than those in which it is generated (74, 1162).

Direct evidence for peroxynitrite formation was described above in all forms of ischemia, as accumulation of nitrotyrosine. As well-argued by Beckman (74), the peroxynitrite formation occurs because the rate constant for this reaction is very high \((6.7 \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1})\) so that NO
can effectively compete with the very active SOD for superoxide when it rises to 1–2 µM, as it does in ischemia. Peroxynitrite formation is not expected in the absence of NO generation unless superoxide generation is high enough to effectively saturate the competing SOD reaction. Mass action would then allow significant formation of peroxynitrite. This may, in principle, occur endogenously and does appear to occur when superoxide is artifactually generated in cultured PC6 cells (562).

**F) MONOAmine ACCUMULATION.** There is quite strong evidence that H₂O₂ is produced from accumulated catecholamines (51) via monoamine oxidase (MAO) in the 5 min after 15-min global ischemia in rat (1053). Measurements were indirect, but rapid oxidation of glutathione was blocked by MAO inhibitors, suggesting MAO activation of H₂O₂ production. This certainly makes sense given the large release of monoamines during ischemia. Blockade of MAO was not protective (1053), indicating that this early H₂O₂ production was not damaging.

**G) INTERACTIONS BETWEEN DIFFERENT SPECIES: FORMATION OF OH FREE RADICAL.** Superoxide and NO are usually the first free radicals formed, but superoxide is not very reactive. The formation of ONOO⁻ is likely to mediate much of its toxicity. In addition, though, the reaction products of superoxide, OH⁻ or H₂O₂, are very reactive and more likely to mediate toxic effects (413). Formation of OH⁻ is strongly favored by the decreased pH that prevails during and after ischemia (75, 488) and also by free iron (413), as shown below in the Fenton reaction

\[ \text{O}_2^- + 2\text{H}_2\text{O} \rightarrow + \text{H}_2\text{O}_2 \]

\[ \text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{OH}^- \]

Brain iron is ~63 µM/g dry tissue, a concentration of ~12 mM. Most of this is in heme enzymes or bound to ferritin. Iron appears to be delocalized from the proteins in the postischemic phase and possibly also during ischemia (1205), probably as a result of lowered pH (488) or superoxide-induced reduction of Fe³⁺ (692). One hour of anoxia in cortical homogenates tripled the amount of delocalized iron (118). The increase in free iron should greatly enhance OH⁻ production, and iron conjugation has been used somewhat successfully to reduce ischemic damage (46, 1205), probably by blocking this reaction. Decreased pH is an important initiator of iron delocalization and hence OH⁻ production.

In contrast to this scenario, it has been argued that the Fenton reaction is of little importance. The reactivity of OH⁻ may paradoxically render it less harmful than other free radicals because it will oxidize many small organic molecules and so be effectively "inactivated" with regard to damaging important macromolecules (74). Furthermore, the superoxide-driven Fenton reaction is very slow, even in the presence of iron, and it has been argued that superoxide would be largely metabolized by SOD and also by NO to peroxynitrite (74). Although sound, these considerations have not been verified experimentally and may not be applicable; lowered pH does actually enhance damage (see sect. viD) arguing for the importance of the Fenton reaction. However, protonated ONOO⁻ may well be more damaging than ONOO⁻ itself, providing an alternate way by which lowered pH could enhance damage (1013). At this stage, the importance of the Fenton reaction needs to be established. The efficacy of iron chelation suggests it is important.

**H) CONCLUSION.** Free radicals including NO, and also peroxynitrite, certainly rise as a result of ischemia. Despite the multiplicity of possible pathways, the only explicit demonstration of a mechanism for free radical formation is via the mitochondrial redox chain during and for 45 min after global ischemia. Monoamine oxidase acting on released monoamines very probably releases H₂O₂, which could then be metabolized to OH⁻ in the Fenton reaction. This may be an important source of early free radical production, but it does not appear to contribute to damage. Specific demonstrations of NO-induced free radical generation, arachidonic acid metabolite-induced free radical generation, calmodulin-induced NO production, and neutrophil-induced superoxide formation from NADPH are lacking at this time. The fact that inhibitors of iNOS and of COX-2, as well as iNOS knockouts, attenuate damage suggests strongly that synthesis of these two enzymes generates NO and superoxide 12 h to several days after ischemia, but no measurements have been made.

There is good evidence that ONOO⁻ is formed from increased NO and superoxide. Indirect evidence suggests that it can also be formed after activation of NOS alone, using basal levels of superoxide, or from superoxide generation alone.

Peroxynitrite, superoxide, and OH⁻ represent three very active species. It is, in fact, quite difficult to determine which of these contributes to the measured elevations in free radicals described in section viA1 because most of the standard measuring techniques for free radicals also respond to peroxynitrite. Lucigenin fluorescence is quite specific to OH⁻, but PBN will also respond to peroxynitrite or its acid (1013) as will the hydroxylation of salicylate to DBA (809).

3. Evidence that free radicals are involved in ischemic cell death

Free radicals are generated during and after both global and focal ischemia. If they are damaging, then conditions which reduce their accumulation should ameliorate damage.

Studies in which free radical or NO/ONOO⁻ accumulation is attenuated, or enhanced, strongly suggest these species make critical contributions to ischemic cell death.
Unfortunately, most studies are not accompanied by measurements of changes in free radical accumulation during or after the ischemia. That makes it very difficult to derive any quantitative relationship between the level of free radical increase and damage.

**A) GLOBAL ISCHEMIA.** Several free radical scavenger systems protect against global ischemia in the gerbil. α-Tocopherol rescued ~75% of the cells that normally died 7 days after 5-min ischemia (424); the animals were on heating pads, but their temperatures were not monitored. A similar degree of protection was afforded by the spin trap PBN when injected for 2 days starting right after the 5-min ischemia (1270). The PBN had no measurable effect on body temperature, although at higher concentrations it did strongly lower temperature, so there is a small suspicion that temperature may be a factor even at the lower concentrations. When infused into ventricles just before the ischemia, human recombinant Cu/Zn SOD attenuated damage in CA1 (1157) as did the XO inhibitor and general free radical scavenger oxypurinol (40 mg/kg), when injected intravenously. Temperature was not regulated in this study (903), but subsequent studies in rat pups (8 days) showed that the very similar drug, allopurinol, at higher doses did not lower body temperature (865). Intraventricular SOD (1135, 1136) protected against damage from three successive 2-min ischemic episodes, and as predicted, if free radicals are involved, the combined administration of SOD and catalase was more effective than either enzyme alone (1135, 1136).

Free radicals also play a major role in damage in the rat. The membrane-permeant free radical scavenger U-101033E attenuated damage in CA1 by 50% after 20-min 2-VO; temperature was unaffected for at least 70 min after U-101033E attenuated damage in CA1 by 50% after 20-min ischemia (1270). The PBN had no measurable effect on body temperature, although at higher concentrations it did strongly lower temperature, so there is a small suspicion that temperature may be a factor even at the lower concentrations. When infused into ventricles just before the ischemia, human recombinant Cu/Zn SOD attenuated damage in CA1 (1157) as did the XO inhibitor and general free radical scavenger oxypurinol (40 mg/kg), when injected intravenously. Temperature was not regulated in this study (903), but subsequent studies in rat pups (8 days) showed that the very similar drug, allopurinol, at higher doses did not lower body temperature (865). Intraventricular SOD (1135, 1136) protected against damage from three successive 2-min ischemic episodes, and as predicted, if free radicals are involved, the combined administration of SOD and catalase was more effective than either enzyme alone (1135, 1136).

Free radicals also play a major role in damage in the rat. The membrane-permeant free radical scavenger U-101033E attenuated damage in CA1 by 50% after 20-min 2-VO; temperature was unaffected for at least 70 min after the ischemia (1059). This protection contrasts with earlier results with another free radical scavenger that was found not to penetrate the blood-brain barrier (72). The OH-scavenger MCI-186 reduced CA1 pyramidal cell damage by 70% 3 days after 10-min 4-VO in a well temperature-controlled study (1238). This reduction in damage was associated with about a 50% reduction in OH radicals in that region, as measured by the salicylate-DHBA method. Oral administration of a flavinoid antioxidant, (-)-catechin, for 2 wk before and 1 wk after completely protected CA1 in gerbil hippocampus against 5-min ischemia (496). There was an associated 25% increase in the free radical scavenging activity of cortical homogenates. Temperature was not controlled in the study. Fivefold overexpression of the cytosolic Cu/Zn SOD reduced CA1 damage in rat by ~50% 3 days after 10-min 2-VO (173), further implicating free radicals in the damage. The implication is supported by the protective effect of the COX-2 inhibitor SC-58125. This reduces damage 14 days after ischemia by ~35%, even when given 1 h after ischemia (836).

**B) FOCAL ISCHEMIA.**

1. **Temporary ischemia.** Even the blood-brain-barrier-impermeant scavenger U-74006F strongly protected against damage in temporary focal ischemia, probably because of the more extensive involvement of the vasculature in this insult. It almost completely blocked the twofold increase in TBAR that usually occurred 24 h after 3-h MCA occlusion and reduced infarct area by ~25% (1154). Temperature was well-regulated. Superoxide dismutase (30), SOD conjugated with polyethylene glycol (679), and SOD entrapped in liposomes (492) consistently reduced infarct volumes by 25–40% in well temperature-controlled focal ischemia studies. These studies have been pursued and confirmed in a very sophisticated series, principally by Chan and colleagues (573, 1246), using mouse mutants. Overexpression of the mitochondrial Mn SOD by about threefold reduced the infarct volume in two different models of temporary focal ischemia in mouse. The penumbra was significantly preserved 24 h after 1-h cortical ischemia (573) and 3 h after a 3-h proximal occlusion (26%) (1246). In both cases, there was also preservation of scavenging systems (reduced glutathione and/or ascorbate), strongly indicating that the mutants were more effectively dissipating free radicals. Overexpression of the mitochondrial Mn SOD, at a level twice its normal value, reduced infarct volume by ~20% 24 h after 1-h focal ischemia (502). The similar effect of the two overexpression mutants suggests that mitochondrial and cytosolic SOD might both act on the same pool of superoxide.

Uric acid, a very effective scavenger of peroxynitrite and OH-, reduced the infarct measured 24 h after 2-h MCA occlusion by 75% when injected either before or immediately after the ischemia (1266). This dramatic effect, which extended to the striatal core of the infarct, suggests free radicals play no role. On the other hand, PBN should scavenge free radicals produced as intermediates in reactions of peroxynitrite with targets such as amino acid side chains or scavenge peroxynitrite or peroxynitrous acid themselves (1013).

2. **Permanent ischemia.** There are apparently conflicting data on effects during permanent lesions; however, they can be resolved. The mice in which overexpression of the cytosolic Cu/Zn SOD prevented the temporary focal ischemic damage described above were not at all protected when subjected to 24-h permanent focal ischemia (172), suggesting free radicals play no role. On the other hand, the spin trap PBN reduced infarct size by >50% at the end of 48-h transient or permanent MCA occlusion, even when administered 3–12 h after the onset of the ischemia (160, 1298). This discrepancy may be readily resolved. Permanent ischemia is protected by inhibiting NOS (see sect. vA), implying damage is mediated by peroxynitrite. This might have been formed from excessive NO production and residual superoxide even when superoxide production was attenuated in the mutants. On the other hand, PBN should scavenge free radicals produced as intermediates in reactions of peroxynitrite with targets such as amino acid side chains or scavenge peroxynitrite or peroxynitrous acid themselves (1013).
The time window for damaging free radical action of between 3 and 12 h after the onset of ischemia has been further refined in a nice study on a mixed permanent/temporary model of thrombotic occlusion (1099). A short-lasting OH• scavenger, EPC-k-1, whose brain turnover half time is ~1 h was very protective when administered at 3 h after the onset of ischemia, but not at 0 or 6 h. This study very nicely pinpoints the first damaging action of OH• to between 3 and 6 h after the start of ischemia.

Further evidence that free radicals are important in permanent focal ischemia comes from the effects of altered dietary vitamin E in a well temperature-controlled study. Forty-eight hours after proximal MCA occlusion, the lesion size was twice as large in rats that had been fed a vitamin E-deprived diet as compared with those on a vitamin E-supplemented diet. Lesion size correlated well with blood levels of vitamin E in the two populations (542). This is a dramatic effect, indicating the great importance of free radical scavenging in preventing permanent focal ischemic damage.

C) ENDOGENOUS SOD CHANGES IN VULNERABLE REGIONS. Given the apparent importance of free radical changes in damage, it is extremely interesting that levels of both forms of SOD decrease in the vulnerable CA1 and CA4 regions 1 day after 10-min global ischemia in gerbil (734). Levels are unchanged or elevated in CA3 and dentate gyrus, two regions which are not vulnerable. There is a similarly selective downregulation of the predominantly mitochondrial Mn SOD after 5- to 10-min 4-VO in rat (680). It is clear from other systems that downregulation of these enzymes by using antioxidants to Cu/Zn SOD hastens apoptotic cell death (393, 967) and also increases the size of infarcts resulting from focal ischemia (601, 786). Thus the enzyme decrease following ischemia might well contribute to the vulnerability of CA1 pyramidal cells. This is rendered more likely by the quite dramatic selectivity of the superoxide increase that occurs 24 h after a global insult in rat. Although COX-2, a likely perpetrator of the delayed rise, increases throughout the hippocampus (806), superoxide only rises in the CA1 pyramidal layer (173).

D) SUMMARY. Various factors that should reduce free radical buildup provide reasonable but not complete protection against global ischemia in rats and gerbils. Protection against temporary focal ischemic damage ranges from reasonable (25% reduction in infarct size) to very strong when uric acid or the spin trap PBN were used. PBN was also very protective against permanent focal ischemia. Damage from permanent ischemia was also very sensitive to dietary vitamin E, again indicating a dependence on free radicals. Unfortunately, none of these studies included measurements of free radical levels, so the degree of free radical suppression is not known. Studies in which actual free radical levels are measured and in which the full time course of protection is measured are needed to help determine the mechanism of free radical damage.

4. Role of NO and peroxynitrite in ischemic cell death

A) NO. Interpretive difficulties in early studies were caused by conflicting effects of endothelial NOS activation and neuronal NOS (nNOS) activation (230). It is now clear that nNOS activity is damaging during and after ischemia in all major models and that endothelial cell NOS activation is beneficial. Although more sophisticated measurements may later sort out subtleties, the current evidence for this conclusion is quite compelling (861).

I) Focal ischemia. In focal ischemia, relatively specific inhibitors of nNOS, when used at levels that do not decrease blood flow or prevent the vessel response to ACh, are strongly protective against permanent (1263) and transient (308, 1296) ischemia in well-controlled studies. ARL-17477 reduced the infarct volume measured 7 days after 2-h MCA occlusion by 55%, at a dose that had no effect on blood flow and 7-nitroindazole (7-NI), and another more long-lasting inhibitor, TRIM, decreased infarct by ~45% 24 h after 2-h MCA occlusion. 7-Nitroindazole decreased infarct volume by 25% after 24-h permanent ischemia (1263). The reduction may have been smaller than with ARL because the occlusion was permanent rather than transient or, more probably, because the lifetime of the 7-NI is only ~2 h. In one study (1296), the drug was fully effective when injected at the start of reperfusion. In addition to effects of these pharmacological agents, nNOS knockout mice showed 40% reductions in infarct size measured after 24- and 72-h permanent MCA occlusion (474) and measured 24 h after temporary ischemia (423).

Inducible NOS knockout mice showed about a 20% decrease in infarct size measured after 96 h, but not 24 h, of permanent focal ischemia (482). These studies are consistent with the delayed (24 – 48 h) increase in iNOS in invading neutrophils. They indicate that the late progress of damage may be due to production of NO from this source. These studies are more persuasive than effects of the iNOS blocker aminoguanidine (799) because the latter also inhibits other potentially damaging enzymes.

Thus there is strong evidence that NOS is very important in focal ischemic damage.

II) Global ischemia. Neuronal NOS knockout mice showed greatly reduced damage 72 h after 10-min global ischemia (868). A normal ischemic effect of 85% cell loss was reduced to a 32% cell loss in the CA1 pyramidal cell layer. This is a substantial effect and argues for an important role of NO generation. Earlier pharmacological studies did not support a role for NO in global ischemic damage because they showed no protection against CA1 damage in either rat or cat (138, 585). However, when the specific inhibitor of nNOS 7-NI was used in well temperature-controlled studies, it reduced damage 7 days after 20-min 2-VO in the rat by ~35% (808). The level of inhibition of NO production is not known, but the result indicates that NO generation during the first 2 h of reper-
fusion contributes to damage. Greater protection may have been achieved if the 7-NI was injected before the ischemia as NO is generated during global ischemia.

III) Cell cultures. Very dramatic effects are manifested in cell cultures where delayed cell death after 60-min in vitro ischemia is almost completely blocked in cultures from mutant mice lacking nNOS (237) and also by NOS inhibition (704).

IV) Localization of nNOS. In the resting state, almost all nNOS in the forebrain is localized to interneurons (261, 956, 1159, 1198). It is possible that a few pyramidal cell neurons contain nNOS, but several studies, referenced above, show very clearly that most nNOS neurons in forebrain structures are interneurons. The interneuron processes with nNOS are very ramified among the parenchyma, with good evidence that nNOS-containing processes are very close to non-nNOS-containing cells (956) and so could readily affect them (236). Thus it seems likely that interneurons, which are generally much less vulnerable to ischemia, must receive the (presumed Ca2+) signal to enhance NO production, release it in the vicinity of other neurons and, in this way, help cause toxicity.

Added to this, there is a major and very rapid increase in the number of nNOS-positive neurons during focal ischemia that peaks within 4 h (1294). This no doubt reflects de novo synthesis of the enzyme in these neurons (as mRNA also goes up) and may well contribute to the damage. The cells that nNOS is in are not clear, although in one micrograph there was quite extensive involvement of what appear to be principal neurons (1294). The role of this process has not been pursued since the original publication.

b) Peroxynitrite. Peroxynitrite is currently considered to be the major mediator of NO toxicity (107, 231, 678, 1093). Evidence for this is only indirect because there are no reliably specific scavengers. Urate, which has been used in some cases (1095), and does react with peroxynitrite, is not necessarily specific (74) and is not very active; it fails to prevent oxidation of protein thiol groups (1162). On the other hand, its actual effects in smooth muscle are most consistent with a selective blockade of processes with nNOS (1095), and also in lipid peroxidation, as revealed by conjugated dienes. These are both prevented by pretreatment with the NOS inhibitor L-NNA (843). This strongly suggests that peroxynitrite, formed from NO and existing superoxide, is the active agent in this case.

The fact that either free radical scavengers or NOS inhibition block global and focal damage is consistent with peroxynitrite being the active agent. Another explanation for these results is that NO and a superoxide-derived product are independently acting on a molecule or function and that both actions are necessary for ischemic damage. This seems less likely than the involvement of the one peroxynitrite species.

It is worth considering why peroxynitrite might be the damaging agent in so many cases. Although NO alone is not necessarily toxic, superoxide or its products, H2O2, or the OH- radical, certainly can be (630). One possibility is that ONOO− attacks specific molecules that are critical. There is no particular precedent for this. For example, single-strand DNA breaks, which seem very important as activators of PARP (see below), are induced very rapidly by ONOO− (1093), O2− or OH− (1112), or H2O2 (999). It is more likely that the reason is quantitative rather than qualitative. If superoxide (and probably therefore OH- or H2O2) generation is fast enough, toxicity is likely to be independent of NO; if it is not, then NO and peroxynitrite formation will become important. This could be tested.

c) Conclusion. Overall, the studies are quite persuasive in ascribing a major role to free radical generation in ischemic cell death. A large number of agents that prevent free radical buildup, or NO buildup, strongly protect against global ischemia and against penumbral damage in permanent or temporary focal ischemia. In several of the studies, temperature has been well regulated. There are, though, critical details that need to be understood.

The degree of protection is variable, depending on the agents used to prevent accumulation. The basis for this is unknown. It could be that treatments have different efficacies on free radical or NO buildup; measurements are required to resolve this. It could be that different species (e.g., OH-; ONOO−) have different efficacies in
different systems and that they are differentially affected by inhibiting NO or superoxide accumulation. Details of buildup and targets are really necessary to get at this next level of understanding.

5. Molecular targets of free radical action

Free radicals affect the structures of both lipids and proteins. They interact strongly with unsaturated bonds in lipids, leading to a chain reaction formation of peroxides, hydroperoxides, and aldehydes (488, 561). They activate phospholipase (PL) A$_2$, possibly because the peroxidation makes the lipids better substrates (1163). Probably as a result of the formation of aldehydes, they markedly reduce membrane fluidity (186). Free radicals oxidize protein side chains, forming carbonyl groups (336, 1067) or disulfides, and can also act as reductants, causing SH formation from S-S bonds (336).

Quite short exposures (10–90 min) strongly inhibit several very important enzymes, including plasmalemma Na$^+$ (300, 446, 684) and Ca$^{2+}$ (1017) pumps, creatine kinase (1267, 1271), and several mitochondrial dehydrogenases (1290). In the case of the Na$^+$.K$^+$.ATPase, oxidation by free radicals makes the enzyme susceptible to calpain-mediated proteolysis (1307). Peroxynitrite is equally toxic, permanently inactivating the cytochrome b and aa$_3$ complexes of neuronal cultures 24 h after a short exposure to 500 µM concentrations; smaller concentrations are also effective (107). Free radicals and peroxynitrite also produce single-strand breaks in DNA (999, 1093).

Any or many of these effects may account for damaging effects of free radicals, but in no case yet has a specific molecular change been tied to free radicals, and also been shown to be instrumental in damage. The most clear-cut change is probably DNA cleavage, as discussed in section vA6.

6. DNA cleavage and activation of PARP

Activation of the enzyme PARP (or PARS) appears to play a critical role in damage following transient focal ischemia. The basic findings, from at least four different groups, are that blockade of PARP using either of two different classes of drug (683, 1098), one of which is highly specific (683, 1280), and also using knockout mice (298, 305) greatly decreases infarct size measured by different staining techniques 24 h after beginning 90-min to 2-h transient ischemia. Decreases in infarct size range from ~45 to 85%, the latter of which, in the mouse, and the rat (1280) is the largest effect of any drug or knockout treatment yet noted. In at least one case, the drug GPI-6150 is effective when given 1 h after 2-h ischemia (1280). Only one group has studied permanent ischemia, and although data are not presented, there is said to be little elevation of polyadenine-ribosylated protein and little protection against infarct by inhibition of PARP (305).

This is a dramatic difference between the two forms of ischemia. There are no reports of measurements in global ischemia.

Cortical cell cultures from Parp$^{-/-}$ mice were completely protected against cell death after 60-min in vitro ischemia (298). Cell death following 5-min exposure of cultured cortical neurons to 0.5 mM NMDA is essentially completely blocked by the quite specific PARP inhibitor DPQ (298).

Two major issues are how the PARP is activated and what its activity may be doing to cause damage.

a) Activation of PARP. Poly(ADP-ribose) polymerase is a nuclear enzyme that is strongly activated by single-stranded DNA breaks; there are no other known activators (88, 1093). It is extremely likely that these breaks are caused by peroxynitrite (1093), although free radicals such as superoxide or hydroxyl are also capable of doing this and almost surely do so following ischemia in heart muscle (1112). Hydrogen peroxide has the same effect (999).

Such single-strand breaks, detected by DNA polymerase I-mediated biotin-dATP nick translation (PANT) have been observed in both core and penumbra neurons early during reperfusion after 1-h focal ischemia (182). Production of the single-strand breaks seems to require reperfusion. There were no breaks at the end of 60-min focal ischemia, yet there were a sizable number after just 1 min of reperfusion (182). This requirement may derive from the large spikelike increase in free radical formation that occurs at the onset of reperfusion after 1-h MCA occlusion (893). The requirement for reperfusion is consistent with the lack of involvement of PARP in permanent ischemia damage. The very rapid production of single-strand DNA breaks that was observed is also consistent with the early appearance of poly(ADP-ribose), within 5 min of terminating 2-h focal ischemia (305). It would be very important to know if blockade of free radical or NO buildup diminished the number of single-strand breaks and also the extent of PARP activation, which can be measured (305).

The importance of peroxynitrite in the strand breaks is suggested by work in several model systems including thymocytes (983), smooth muscle cells, and macrophages (1094), where 25–50 µM peroxynitrite, probably acting in its protonated form, causes strand breaks and PARP activation, followed by cell death, with timely kinetics (1304). Nitric oxide is very ineffective in this process (1093).

b) Mechanism of PARP toxicity. This has not been well defined. The PARP-catalyzed reaction involves the cleavage of NAD, and one commonly considered basis for toxicity is depletion of NAD, with resultant inhibition of mitochondrial function. This would cause depletion of ATP and possibly the opening of the MTP. Single-stranded DNA breaks lead to this sequence (absent any measurements of MPT) in several systems, and ATP and NAD are
almost completely depleted if there is enough strand breakage (88, 1094). Hydrogen peroxide very likely causes cell death by this mechanism in several cases (216, 999).

The evidence for such an energy metabolism-mediated cell death in temporary focal ischemia is not great. In general, effects of PARP inhibitors on ATP changes have not been measured. In one study, there was a 65% fall in NAD in the ischemic territory, and both PARP knockouts and a PARP inhibitor 3-aminobenzamide attenuated this fall by ~40% (305). This result is somewhat difficult to interpret because measurements were made at 24 h, by which time there were a lot of dead cells. Thus the cell saving by PARP inhibition could be responsible for the higher NAD levels. Measurements before significant cell death would be more useful. The other difficulty is that there is no evidence for dramatic falls in ATP in temporary focal ischemia. It is possible that the small reductions in ATP may be damaging over a long period, as discussed in section ivB. Effects of PARP inhibition on these ATP levels need to be measured. Alternatively, ATP may fall dramatically for a short period during reperfusion, and this may trigger damage.

Thus, at this stage, there is no well-established mechanism for the damaging effects of PARP after ischemia. Possibilities other than inhibited energy metabolism include additional effects of NAD(H) depletion and resultant metabolite changes, or effects on histones and other nuclear proteins as a result of the transient ADP ribosylation (it lasts ~3 h; Ref. 305). Alternatively, this ADP-ribosylation appears to be a critical step in apoptosis in many model systems (1049) and could certainly play such a role in ischemia.

7. Gene-mediated effects of free radicals: effects on NFκB, cytokines, and CD-95 ligand

A) NFκB. There is accumulating evidence in many tissues, including brain, that free radical damage following ischemia is at least partially mediated by the cytosolically located transcription factor NFκB. NFκB is activated by the inactivation of an inhibitory protein, IκB, which occurs in two major steps. Phosphorylation is mediated by effects of free radicals (1000) and other pathways (56, 747, 1005) on IκB kinases (254). Subsequent to this, the phosphorylated IκB is broken down by the 20S proteasome and rendered inactive (56). Two active subunits of NFκB are then translocated to the nucleus. NFκB activates synthesis of many proteins including iNOS and COX-2 (1005); the latter has been particularly nicely demonstrated during hypoxia, where it was shown that the p65 unit is responsible (995). It also upregulates the CD-95 ligand (1173), which is upregulated after global ischemia. It may upregulate proapoptotic BCl-xshort after global ischemia, but this is speculative (264). Although the NFκB pathway can be antiapoptotic in some conditions (1005, 1186), there is evidence that the overall pathway is damaging after ischemia. The evidence for this, and its mediation by free radicals, is much stronger in noncerebral tissues such as liver (1309), vascular endothelium (464), and heart (176) than it is in brain, but there is emerging evidence that it may be instrumental in the latter also.

NFκB is activated and translocated to the nuclei of hippocampal neurons after 30-min 4-VO global ischemia in rats (206); this occurs in all neuronal populations 24 h after ischemia, but only in the soon-to-die CA1 population 72 h after the ischemia. The delayed translocation is blocked by the antioxidant LY-231617 (207), indicating that it is mediated by free radicals. However, somewhat surprisingly, the early translocation is not blocked by the antioxidant, suggesting another pathway is responsible for that process, probably a kinase cascade (640, 1005). This differs from other tissues such as liver (1309), cerebral blood vessels (464), and heart (176), where early postischemic activation of NFκB is prevented by free radical scavengers. The antioxidant was very protective against global ischemic damage (207), consistent with, but certainly not proving, the involvement of NFκB in damage.

NFκB is activated in the core and penumbra 1 day after 90-min temporary focal ischemia of the cortex (984). The specific proteasome inhibitor PS-519 strongly attenuated damage measured 24 h after 2-h ischemia (902). As discussed previously, this might reflect damage via proteolysis. However, because the proteasome pathway is required for NFκB activation, the result may reflect the importance of NFκB in focal damage. If so, it shows that the ischemic core is most susceptible to damage via this system.

Aspirin and salicylate, which directly block activation of NFκB, protect strongly against glutamate toxicity in cultures and seem to do so by blocking NFκB. Effects are not mediated by reducing Ca2+ entry or by inhibiting prostaglandin metabolism and are strongly correlated with inhibition of the activation of NFκB by 50 μM glutamate (395).

NFκB is likely to be instrumental in upregulation of iNOS and COX-2, which are damaging in both focal and global ischemia, and also of cell adhesion molecules that are damaging in focal ischemia. However, it is not at all established that this is its mode of action. The near-complete blockade of glutamate toxicity, which is not thought to be mediated by either of the enzymes or adhesion molecules, certainly suggests alternate modes of action.

B) CD-95 ligand. The 45-kDa CD-95 ligand is upregulated after focal ischemia and is a reasonable candidate for initiation of apoptosis, as discussed previously. This ligand is upregulated in 12 h by H2O2 in cultured microglial cells, and the mRNA is upregulated after hypoxia/reoxygenation (no measurements were made of protein) (1173). In the former case, the upregulation coincides with activation of NFκB; this and the fact that the CD-95
ligand gene has a putative NFκB binding region suggests that the transcription factor may mediate activation of the CD-95 ligand (1173). If this mechanism also pertains after cerebral ischemia, it would be an important way by which free radicals mediate apoptotic cell death. Further work along these lines is necessary.

C) ROLE OF CYTOKINES. Cytokines that are upregulated early in ischemia and reperfusion, particularly TNF-α and IL-1, are strong activators of NFκB (1039). Thus they, along with free radicals generated from other sources, are potential activators of the transcription factor. The mechanism by which cytokines activate NFκB is not fully known, but it does appear likely that free radicals generated by the cytokines mediate the effect because several free radical scavengers strongly attenuate the effect (381, 1000). However, other pathways activated by the cytokines, such as ceramide synthesis and MEKK kinase cascades (1005, 1039, 1186), may also be important. It is not currently known what is responsible for the ischemic upregulation of cytokines TNF-α or IL-1 (747).

At this stage, the actual activators of NFκB during and after ischemia are not known.

8. Summary: possible mechanisms of free radical-mediated cell death after ischemia

The studies described above make it quite clear that free radical production makes a major contribution to ischemic cell death. Six likely mechanisms of action are described here.

1) As described above, activation of PARP which probably, but not surely, results from free radical/peroxynitrite-mediated cleavage of DNA may well mediate a lethal effect of free radicals after temporary focal ischemia. The very large saving produced by uric acid (1266), which is a good peroxynitrite scavenger, is consistent with this because PARP inhibitors also greatly reduce the size of the lesion (1280).

2) Synthesis of CD-95 ligand via activation of NFκB might be a mechanism by which free radicals cause apoptotic cell damage, as may be the increase in TNF-α. However, the pathways by which the CD-95 ligand and TNF-α are produced after focal ischemia have not yet been determined, so this is very speculative. Free radicals appear to initiate apoptosis in several systems, so there may also be other pathways by which they do so in ischemia.

3) Other known functional targets of free radicals that could well lead to cell death include increased plasmalemma permeabilities (201, 849), prolonged inhibition of protein synthesis in (vascular endothelial) cells via effects on eIF-4-like proteins (513), and major changes in mitochondrial function, including opening of the MTP, as discussed extensively in section IV B. There is, though, no evidence positively identifying the importance of these phenomena in ischemic cell death.

4) Effects of free radicals on Ca2+ homeostasis could be important. After 60-min focal ischemia, cytoplasmic Ca2+ remains elevated for at least 30 min. This elevation is prevented if ventricles are perfused with SOD at high levels (30), suggesting that free radicals affect some aspect(s) of Ca2+ homeostasis, possibly by elevating Ca2+ fluxes through existing or new pathways, or possibly by inhibiting Ca2+-ATPase. This could reflect the same underlying process as the apparent dependency of postglo- bal ischemic mitochondrial Ca2+ accumulation on NOS activation (1208).

5) Inhibiting NOS during focal ischemia prevents the normal fall in pH in the penumbra, suggesting that NO synthesis in some way causes the pH drop (943). The mechanism of this somewhat surprising effect is not clear, but if it does do this, it could certainly be a way that NO enhances damage (see sect. vD). One mechanism for this may be the effect described immediately below, where NO contributes to the release of glutamate in focal ischemia. Glutamate generally acidifies neurons (159).

6) Nitric oxide (possibly via peroxynitrite produc- tion) appears to play an important role in the accumulation of glutamate in the core of a focal lesion and the subsequent spread of glutamate to the penumbra and the resulting intraschismic depolarizations. Release of glutamate in the core, and subsequent intraschismic depolarizations, were greatly attenuated in nNOS knockout mice (1031). The mechanism may be the peroxynitrite-mediated inhibition of glutamate transporters (1132).

Of course, because of their wide-spread effects on different proteins, free radicals might alter protein or proteins whose importance has yet to be shown.

9. Summary: apoptosis or necrosis

Exogenous free radicals are able to cause necrosis (111), or apoptosis (393, 502, 788, 789, 967, 1134), in cultured neurons or neuronal-like cells; at a much more extreme level, free radicals (largely OH·) generated by the xanthine/XO/Fe2+ system in the intact brain caused dramatic cellular degeneration within 2 h, with neurons appearing as in ischemic-cell change and the blood-brain barrier becoming highly permeable to Evans blue. Infarct developed within 24 h (174). Thus whether free radicals selectively promote apoptosis or necrosis after ischemia may well be a function of the level of free radical production, as seen in cultures (111).

When excess free radicals were produced as a result of −/− SOD null mutants in mice, the region that became more vulnerable showed a greater number of apoptotic neurons than other regions (601), suggesting free radicals caused apoptosis. However, this could have arisen from the fact that the insult intensity in the newly vulnerable region (the former peri-infarct zone) was low. It would be very useful to explicitly count the effect of free radical, or NO, on attenuation of apoptotic and necrotic neurons in...
regions that were protected by free radical scavengers or by SOD overexpression to determine whether free radicals preferentially induce one or the other forms of cell death. They certainly have the potential to induce both forms.

10. Summary: timing of damaging free radical actions

The data suggest that free radicals persist for well beyond 24 h after the start of the insult in temporary focal ischemia; they have been measured explicitly for at least 6 h in the penumbra and the core, and iNOS and COX-2 are induced between 6 and 24 h of reperfusion, suggesting there will be prolonged production of free radicals and peroxynitrite. The latter accumulates for at least 3 h in the penumbra; later measurements are not reported. Free radicals have not been measured more than 3 h after the start of permanent focal ischemia but may well persist. Thus free radicals persist in the tissue for many hours and possibly days. What is important is when these free radicals are actually damaging. One can envision their being important early, perhaps as a trigger, as occurs in sympathetic ganglia following NGF withdrawal (239), perhaps to important early, perhaps as a trigger, as occurs in sympathetics are actually damaging. One can envision their being possibly days. What is important is when these free radicals play or may well play roles in ischemic cell death, which the time they remain in the brain is measured, would help sort out the times at which free radicals are acting and thus help to determine what they are doing.

The most likely bases for prolonged maintenance are COX-2 and iNOS inductions and possible ongoing production by mitochondria. These possibilities can be tested.

B. Ca²⁺-Dependent Proteases

1. Inhibitor specificities

There is strong, albeit not very plentiful, evidence that the Ca²⁺-activated neutral cysteine protease calpain plays an extremely important role in focal ischemic damage and very possibly in global damage (66, 379, 750, 971). This evidence is based on cell-permeant inhibitors of calpain that have been developed in the last few years (66, 715) and relies on their specificity. Knockout animals have not yet been made. Inhibitor specificity is somewhat problematic because there are at least three currently known alternate proteolytic systems that might play a role in ischemia. These are caspases and cathepsins, both of which play or may well play roles in ischemic cell death, and also the 20S proteasome.

The current generation of cell-permeant intracellular protease inhibitors are aldehyde-linked di- or tripeptides in which the peptides mimic the substrate specificity of the protease (715). Amino acid sequences that most potently inhibit calpain contain two hydrophobic residues. Val-Phe (MDL-28170) and Leu-aminobutyrate (AK-275). These are the two inhibitors that have been shown to possess the greatest potency in focal ischemia (67, 715). A much less cell-permeant molecule, leupeptin, was very protective in global ischemia (641).

Caspases have very different substrate specificities from calpain, and caspase inhibitors have two or three hydrophobic amino acid chains but they must be accompanied by an aspartate. It is thus considered extremely unlikely that the calpain inhibitors will inhibit caspases. This being said, there is only one reported study in which it was shown explicitly that E64 and antipain, inhibitors that are similar but not identical to MDL-28170 and AK-275, have no effect on caspase-1 (ICE) (1214). One indirect but quite strong piece of evidence that calpain inhibitors do not affect caspases is that MDL-28170 does not prevent apoptosis in thymocytes when the apoptosis is triggered by insults that do not require protein synthesis (1064). This is very likely a caspase-mediated event.

Some three or four hydrophobic amino acid sequences that effectively block calpain (inhibitory constant ~10⁻⁷) also block proteasomal activity, although at 100-fold lower potency (749); potency may be greater in
situ (1033). Thus, at the doses used in vivo (≈10 mg/kg or 30 µM to get strong protection), it is possible that the calpain inhibitors are also blocking proteasomal activity. Neither of the dipeptides used against ischemia has been tested against proteasomal activity yet. Leupeptin may also inhibit proteasomes; it blocks the trypsinlike activity of proteasomes (but not the chymotrypsin-like activity) (408). This is an important issue because a specific proteasomal inhibitor has recently been found to provide strong protection in focal ischemia (902). This issue would be best resolved by examining effects of calpain inhibitors with widely different specificities for proteasomal action (749, 1033) or by using the peptide calpain inhibitors at very low doses.

The protease that is most likely to be confounding the results is cathepsin B which, although it is a serine protease, has a very similar substrate specificity to calpain (715) and is thus inhibited by MDL-28170, and by leupeptin with potencies that are similar to those for calpain (66, 657, 744, 1191). There is at least one cell-permeant inhibitor that is much more effective against cathepsin than against calpain, ZYA-CHN2 (224), but this has not yet been used in ischemia. This would be a good way to test whether the inhibitors are acting on cathepsins.

Another way to attain reliable calpain specificity has been to develop a cell-permeant inhibitor that blocks at the Ca²⁺ binding site of calpain. This has been done (1190), and although it is effective in cell culture (1064), it is unfortunately not effective in brain slices because of penetration problems (Lipton and Zhang, unpublished data) and has not been studied in vivo. It is likely to be ineffective there too.

2. Regulation of calpain

Although quite high concentrations of Ca²⁺ are necessary to initially activate the isolated enzymes µ- and M-calpain (10 µM and 1 mM, respectively), these concentrations are lowered by binding to polyphosphoinositides, particularly phosphatidylinositol 4,5-bisphosphate (PIP₂) at the cell membrane and by products of lecithin breakdown (37). The mean affinity constant for Ca²⁺ activation of µ-calpain is reduced from ≈10 µM to ≈100 nM, and the sensitivity of M-calpain is also increased (971, 972, 1087, 1240). The Ca²⁺ sensitivity is also greatly increased by a membrane protein that has been found in several tissues, including the erythrocyte and brain (979). Once activated by Ca²⁺, the isolated enzyme often undergoes autoproteolysis, and the smaller protein is active at normal cell levels of Ca²⁺ (622). Another mode of activation may include changes in calpastatin, but little is known about this. Thus calpains may well be activated by quite small increases in Ca²⁺.

Even without mechanisms of increasing its sensitivity, at least µ-calpain can be expected to be activated during ischemia as both in situ (1048) and in slices (397) cytosolic Ca²⁺ appears to rise into the tens of micromolar range.

An important unresolved issue is how the enzyme might be inactivated after ischemia.

3. Localization of calpain and calpastatin

The localization of calpain may well be relevant to selective vulnerability of neuronal populations. µ-Calpain is widely distributed throughout the neuron, including spines, dendrites, cell somata, and axons (892). In rabbit hippocampus, it is primarily localized to pyramidal neurons, whereas M-calpain, the less sensitive isozyme, is primarily localized to inhibitory interneurons (344). This is interesting because of the great resistance that interneurons show to ischemic damage. The localization of calpastatin is interesting in this regard also. Although it is present in most cells, it is at a far lower concentration in (vulnerable) CA1 pyramidal cells than (resistant) CA3 pyramidal cells (344).

4. Increased calpain activity during and after ischemia

A) Global ischemia. Many measurements of calpain activity are actually measurements of spectrin breakdown into a fragment that is characteristic of calpain action (although all proteases have not been tested). Early studies showed spectrin proteolysis 30 min after 10-min global ischemia in CA1 of gerbil that was inhibited by leupeptin (641). However, for reasons that remain somewhat of a mystery, more recent immunohistochemical studies have revealed that the protease activation is biphasic. Five minutes after ischemia there is spectrin breakdown in CA3 and in cortex, but not in CA1 of hippocampus (952). After 10-min ischemia, there is a small amount of proteolysis in CA1 (973), but not until 24 h after ischemia is there a large spectrin breakdown in CA1 (952, 973, 1258). The very delayed nature of calpain activation is difficult to understand because Ca²⁺ certainly seems to rise in CA1, and previous studies had demonstrated an early calpain increase. If this holds up, and it has so far been demonstrated by two different groups, it suggests that delayed calpain activation may be more important to global ischemic damage than early activation (which occurs in non-vulnerable neurons). This could be tested with appropriately timed inhibitor studies.

Finally, there is a very large increase (to 40%) in spectrin breakdown around the time of cell degeneration (1258). If this in fact precedes cell death, it indicates a very strong calpain activation at a critical time, but the detailed timing is not yet known.

A direct measure of calpain autolysis indicated activation in whole rabbit brain during the first 10 min of global ischemia (823).

B) Focal ischemia. There was a large increase in spec-
trin proteolysis during 3 h of focal ischemia in regions that later developed infarct. This had begun by the time of the first measurement, at 1 h. There was a delayed increase in the peri-infarct regions, where individual cell death generally occurs. That increase was noted after 21-h reperfusion and was as large as the increase in the infarcted regions (455). Because cell damage was developing in the lesion throughout the study, it is not clear whether the calpain activation preceded the damage, although the significant proteolysis by 1–3 h in core and penumbra certainly preceded major cell death.

C) BRAIN SLICES. In rat brain slices, where calpain inhibitor-sensitive MAP2 proteolysis and microtubule dissociations were used to monitor calpain activity, there was a clear calpain activation during the first 5 min of in vitro ischemia, with continued activity in the reperfusion period (1291).

5. Mechanism of calpain activation during and after ischemia

The mechanism of calpain activation is not clearly established. Although increased intracellular Ca\(^{2+}\) is of course the most likely mechanism, it is notable that Ca\(^{2+}\) levels needed to activate the \(\mu\)-form are very high unless it is bound to phosphoinositides or to specific proteins.

In liver cells, calpain was activated twofold during anoxia, without an increase in intracellular Ca\(^{2+}\). Activation appeared to be dependent on PLA\(_2\) breakdown (37, 38). This is consistent with the ability of phosphatidylcholine breakdown products to activate calpain (37).

In global ischemia, most of the early and delayed calpain upregulation is blocked by MK-801, suggesting that Ca\(^{2+}\) entering via NMDA receptors is important (952). This is consistent with either direct activation or activation via PLA\(_2\) since the latter is Ca\(^{2+}\) dependent.

6. Involvement of calpain in ischemic damage

As far as tested, calpain inhibitors appear to reduce cell death very effectively. In the global gerbil model, leupeptin infusion into the ventricles prevented the early breakdown in spectrin and almost completely prevented cell death in CA1 7 days later (641). Temperature effects were not measured. This is the only reported study in global ischemia.

In focal ischemia, superfusing the cell-permeant calpain inhibitor AK-275 into cortical tissue at various times before during and after a temporary distal MCA/CCA occlusion reduced the size of the cortical infarct by 50–70% (67). The drug was very effective when administered 3 h after starting the ischemia but not 4 h after ischemia. This is a massive reduction in infarct volume and suggests that calpain plays a crucial role in the process, possibly even in damage to the core of the infarct. A similar level of protection was found against 3-h temporary focal ischemia where another dipeptide/aldehyde inhibitor, MDL-28170, reduced infarct size by \(\sim 70\%\) when administered intravenously within 30 min of starting ischemia. The drug was still very effective when administered up to 6 h after starting the ischemia (3 h into the reperfusion) (715). In both studies, damage was only measured 24 h after occlusion so that very long-term protective effects were not tested. Temperature was quite well controlled in one study (67), where no effect of inhibitor on temperature was noted for at least 1 h after MCA occlusion, but was not discussed in the other study (715). The fact that the two inhibitors are very similar certainly suggests that temperature did not vary in that study either.

Cell death following in vitro ischemia in cerebrocortical cultures was attenuated by the cell-permeant calpain inhibitor that has almost no effect on cathepsins (1190), strongly implicating calpain in cell death in cultures. In conclusion, the effects of calpain inhibition on focal lesion size are more profound than all other ways of intervening. Unless these drugs are protecting by inhibiting cathepsin B activity, or proteasome activity, the results show a major contribution of calpain to cell damage in transient focal ischemia. There are, though, only very few studies, particularly in global ischemia.

7. Possible sites of calpain action

The apparent importance of calpain in focal ischemic cell death, and probably global also, makes knowing its targets very important.

A) SPECIFIC TARGETS. The enzyme attacks many cytoskeletal proteins, including MAP2 (900, 1291), spectrin, tubulin (971), and very probably ankyrin. It leads to loosening of the postsynaptic density (269) and participates in the breakdown of microtubules in an unknown way, as evaluated from effects of MDL-28170 and calpain inhibitor 1 (1291). Hence, dissolution of critical cytoskeletal elements may well be how it participates in cell death.

Opening of the MTP is another possible site of action. In hepatocytes, mitochondrial calpain was activated by increased intramitochondrial Ca\(^{2+}\) and by oxidative stress generated by tert-butyl hydroperoxide, leading to opening of the MTP (7).

Another possible site of action is eIF-4, which is broken down by calpain to one-third its normal level during 20-min global ischemia in the rabbit (820, 822). However, a role for global protein synthesis inhibition in focal ischemic death is quite unlikely, so this is probably not its mode of action. It may well be important in global ischemia.

Calpain may play a role in activating autophagocytosis. The activated form of \(\mu\)-calpain was identified immunocytochemically on lysosomal membranes after global ischemia in gerbils (1240), before the formation of the autophagic vacuoles (834).
tion and the effects of calpain inhibitors. For example, T-cell apoptosis induced by anti-CD3 monoclonal antibody was blocked by calpain inhibitors, and there was a calpain blocker-sensitive breakdown of spectrin to the 150-kDa fragment during the process (720). This calpain-mediated cleavage of spectrin occurs after several different apoptotic stimuli in different types of cultured cells including hippocampal neurons (511, 720). There are many instances in which cell-permeant calpain inhibitors including calpeptin in neuroblastoma (78), MDL-28170 in cultured hippocampal cells (511), calpeptin and MDL-28170 in thymocytes and metamyelocytes (1065), and leupeptin in T cells (989), prevent apoptosis. One of the studies (1065) very nicely demonstrated the autoproteolysis of calpain I, showing calpain activity is turned on, using an antibody that was specific to the native form.

In thymocytes, calpain inhibition by the Ca\(^{2+}\) binding site-specific inhibitor PD-150656 produced a clear inhibition of all aspects of apoptosis when apoptosis required protein synthesis (for example, when induced by dexamethasone), but not when protein synthesis was unnecessary for apoptosis (1064), suggesting calpain is involved in a relatively early event in apoptosis.

Taken together, these results provide a picture of a strong involvement of calpain in apoptosis.

The role of calpain is not known and may be quite complex. It required protein synthesis to be activated in staurosporine-treated neuronal glial culture (909) where its inhibition not only prevented cell death but also DNA breakdown. A possible site of action is actin breakdown, a feature of apoptosis in most cell types. In chick neurons deprived of growth factor (1169), calpain inhibitors I and II blocked both apoptosis and actin proteolysis. The effect on actin is almost undoubtedly indirect. Prevalent forms of actin are not substrates for calpain (269, 971), whereas several actin-binding proteins are substrates (971).

c) A CAVEAT. Although this section has focused on damaging effects of calpain, the enzyme also appears to play an important role in remodeling dendritic structure after injury. In cultured neurons, NMDA induces reversible dendritic varicosities that result, at least in part, from disassembled microtubules. The reestablishment of normal dendritic structure was blocked by calpain inhibitors, implying that calpain helps in recovery from morphological damage (313). It is not clear if these effects relate to ischemia, but the results point out that calpain may be beneficial in reestablishing neuronal function during late phases of recovery. This is important when considering therapeutic aspects of the drug.

8. Conclusions

The existing evidence strongly suggests involvement of calpain in ischemic cell death; protections by AK-275 and MDL-28170 against temporary focal ischemia are the most profound reported with any protectant except for one set of studies on the PARP knockout mice, and the protection by leupeptin against global ischemia in gerbil was also very profound. There are some caveats, however. Although temperature control was good during the occlusion in the case of AK-275, there are no reports of temperature control during the study of global ischemia. This is of concern. A second concern is the specificity of the agents for calpain. Although it is very unlikely that they act on caspases, given the marked differences in targets and hence inhibitor construction, the inhibitors may well act on cathepsins, or on proteasomes. It will be necessary to judiciously use newly developed selective inhibitors against these different proteolytic systems to firmly establish the importance of calpains in ischemia.

The inhibitor studies with AK-275 and MDL-28170 indicate that the lethal calpain action in temporary focal ischemia is somewhere between 3 and 6 h after the start of ischemia, a time consistent with its involvement in early events of apoptosis and in development of necrotic death. This is quite similar to the time at which free radicals are critical in permanent focal damage. If the two events are comparable, then a critical time for damage development is the 3- to 6-h period after the onset of focal ischemia. Although there are no timed inhibitor studies in global ischemia, there is certainly an apparent dramatic upregulation of calpain activity in vulnerable CA1 at \(\sim 24\ h\). This is the same time as the appearance of significant free radical changes in this region and suggests that this period may well be a critical one for both global ischemic damage. As with free radicals, it would be very useful to have studies in which the periods during which drugs effectively prevent damage became known. This necessitates knowing the period for which an injected drug is efficacious.

At this stage of our understanding the most likely damaging action of calpain is on apoptotic biochemistry. Effects on the cytoskeleton may mediate necrosis but the role of the cytoskeleton in necrotic cell death is not well established; the same is true for effects on protein synthesis. The other known functional effects of calpain do not seem likely to be lethal. It would be interesting to see whether calpain inhibition selectively prevented apoptotic cell death.

C. Phospholipid Metabolism

Breakdown of phospholipids could, in principle, cause major changes in membrane function and also changes in signaling capabilities resulting from loss of phosphoinositides. Furthermore, the breakdown leads to major increases in FFA production and hence, also, free radical formation. All these are potentially damaging. Cytidine diphosphate choline, which can act as a phospholipid precursor, appears to attenuate ischemic damage (1014), highlighting the potential importance of phospho-
lipid breakdown in damage. However, the dearth of good inhibitors makes study of this topic very difficult.

1. Changes in FFA and phospholipids in ischemia

A) FFA in Global Ischemia. There is a very rapid increase in FFA during global ischemia, first noted about 25 years ago by Bazan (69) and since reported by a large number of workers in all models and species tested. Levels of total FFA rise approximately fourfold during the first 5 min of ischemia (69, 945, 1083) and reach a plateau after ~15 min at ~8–10 times basal values (69, 945, 1262). There are marked differences in the percentage increases of different FFA. With an average of five different studies between the 1970s and 1990s, arachidonic acid (20:4) increased from ~30 to 300 μM, 22:6 rose from ~10 to 30 μM, and oleic acid (18:1) went from ~40 to 100 μM. Of the two principle saturated fatty acids, palmitic (16:0) rose from ~60 to 180 μM and stearic (18:0) went from ~50 to 350 μM (3, 945, 1034, 1261, 1262). Thus there are very sizable concentrations of FFA at the end of ischemia.

B) Maintenance of FFA After Global Ischemic Insults. The oxidative metabolism of arachidonic acid, which is where free radicals are generated, will be greatest during reoxygenation. In the rat, fatty acid levels in whole brain or cortex largely resolve by 30-min postischemia, and in most studies, they completely recover by 60–90 min (574, 945, 1260). However, in gerbils, levels are much elevated as long as 90 min after 10-min ischemia (869); FFA and arachidonic acid in the CA1 region remained elevated for at least 1 day after 5 min of ischemia (4). Unfortunately, this delayed elevation has only been studied once. It suggests a prolonged generation of FFA in a vulnerable region, and this could be important in damage. Consistent with this, PLA2 activity was elevated 10 min after 10-min ischemia in gerbils (960), and it might stay elevated; this has not been measured.

C) FFA in Focal Ischemia. Total FFA were increased 4- to 10-fold between the first 15 min and the end of 60 min of MCA occlusion (1292). As with global ischemia, the largest percentage increases were in 20:4, 22:6, and 18:0 (1292). If ischemia was reversed after 1 h, FFA returned toward baseline, and there was then a large secondary increase at 16 and 24 h of reperfusion. In this case, 18:0 rose to a very high level, but arachidonic acid (20:4) was not elevated (1292). Thus the contribution of this increase to free radical generation would probably not be great.

D) Changes in Phospholipids. Both PIP2 and phosphatidylinositol 4-monophosphate (PIP) fell by ~60% during 2–3 min of ischemia (487). However, despite the large increases in FFA, there were generally no measurable decreases in other phospholipids (3, 945). An exception was a report showing a 20% decrease in phosphatidylcholine after 10-min global ischemia in the gerbil (1133). The absence of other measured falls is probably because the percentage decreases in the more plentiful phospholipids are too small to reliably measure.

E) Inositol Trisphosphate Changes. These are very small and last no more than 30 s in vivo or in vitro despite the large falls in phosphoinositides (662, 663, 674, 1082). There are large and prolonged increases of inositol monophosphate and inositol bisphosphate in vivo (663, 1082) and of IP3 in slices (674). The reason that the increases in inositol trisphosphate are small and unsustained is very probably because the low ATP levels make it impossible to regenerate the substrate, PIP2 (487, 663).

F) Summary. Changes in arachidonic acid both during and after global and focal insults are substantial and should contribute to superoxide production. They may be prolonged in vulnerable tissue. Decreases in phosphoinositides could lead to deficits in signaling pathways.

2. Mechanisms of net phospholipid breakdown

A) Phospholipases that are Involved. Measurements of reactants and products showed that the FFA increases during the first 2 min largely result from phospholipase C-mediated breakdown of the phosphoinositides, PIP2 and PIP, and resultant lipolysis of the diacylglycerols (3, 487, 554, 1082, 1083). This was confirmed by the action of the (quite nonspecific) phospholipase C inhibitor, phenylmethylsulfonyl fluoride (PMSF), which largely blocked the FFA increase during that time (1153). Phospholipase A2 activation might contribute somewhat to production of 22:4 and palmitic acid (16:0) during this early time (3, 554). Release of FFA later than 2 min is basically unaffected by PMSF and so must largely be due to ongoing activity of PLA2 or other lipases (3, 487, 1083).

B) Phospholipase Activation. 1) Activation of PLC. Brain PAF may be responsible for increased PLC activity. The PAF activity increases 10-fold at the end of 10-min global ischemia in gerbils, and regional effects on exogenous PAF binding suggest that the largest increase is in the hippocampus (266). The mechanism of the increase in PAF is not known. It may be synthesized by "remodeling," which requires activation of PLA2 followed by the action of 1-alkyl GPC acyltransferase (54). Both these enzymes can be activated by Ca2+, kinase-mediated phosphorylations, and G protein activation (54, 55, 250), events which are increased by ischemia. De novo synthesis of PAF is also quite possible, since this should be strongly activated by the decrease in ATP levels; the key enzyme is inhibited by 0.5–1 mM MgATP (55).

In normoxic neuronal cultures (1268), PAF activates PLC via PAF receptors and G protein activation (166, 661). This pathway remains to be established in ischemia. Certainly activation of PLC seems far too early to result from neurotransmitter release and binding to a metabotropic receptor.

2) Activation of PLA2. Possible mechanisms of PLA2 activation have been nicely reviewed (250, 738). Pres-
ently, it is not known which form of the enzyme is activated by ischemia. The Ca\(^{2+}\)-dependent form should be elevated by the rapid rise in cytosolic Ca\(^{2+}\) levels. The Ca\(^{2+}\)-independent form of the enzyme is strongly activated in cardiac ischemia (436), and its inhibition is protective (988). The same could be occurring in brain, although the Ca\(^{2+}\)-independent form in cardiac tissue is unique; it primarily targets plasmalogens that are the principal lipids of the cardiac sarcolemma.

C) DECREASED RESYNTHESIS OF PHOSPHOLIPID. During global ischemia, the large rise in FFA is coincident with the large fall in ATP (553), and it is possible that the FFA increase occurs because resynthesis of phospholipid is blocked by the lowered ATP (1084). This receives support from effects of intracerebrally injected CDP-choline, a precursor for phosphatidylcholine synthesis. This very strongly attenuated the early appearance of FFA in the gerbil and rat (268, 1133). Thus, at the least, phospholipid resynthesis plays a major role in the net breakdown during and shortly after ischemia.

The very delayed increase in FFA discussed above, ~1 day after temporary focal ischemia, is correlated with a reduction in a major phospholipid-synthesizing enzyme, lysophosphatidyl:acyl-CoA acyltransferase (1281). The mechanism of the activity loss is not known, but it could lead to a change in membrane properties.

3. Mitochondrial phospholipid changes

Mitochondrial lipid changes are profound and are different from those in the cell as a whole. During 30 min of 4-VO, mitochondrial contents of all FFA, saturated and unsaturated, increased severalfold with no apparent preference for specific FFA (1079, 1080). The increased level of FFA is maintained for at least 60 min of reperfusion. Unlike cells as a whole, there is a measurable decrease in phospholipid contents of the mitochondria (1079).

The basis for the phospholipid breakdown is not known, but the high mitochondrial contents of phosphatidylcholine and phosphatidylethanolamine, and their measured breakdown (1079), suggests that PLA\(_2\) is activated, very probably by the early accumulation of Ca\(^{2+}\). Phospholipase A\(_2\) activity in the mitochondrial fraction of gerbil forebrain 10 min after 10-min ischemia is two times normal (960).

4. Role of phospholipid changes in ischemic cell damage

Whether these dramatic changes in cellular phospholipid metabolism actually play a role in cell death has been very difficult to resolve.

A) EFFECTS OF ARACHIDONIC ACID. This would be expected to be a major source of free radicals. However, effects of blocking arachidonic acid metabolism, with its subsequent free radical production, have not been reported, except for retina where they are protective against anoxic damage. Inhibition of COX-2, which is upregulated in neurons several hours after ischemia, is also protective in temporary focal and global ischemia, indicating that delayed metabolism of arachidonic acid is damaging. In the absence of more pharmacological studies, one can only speculate as to possible effects of early arachidonic acid increase.

On the basis of in vitro studies, the early rise in arachidonic acid (200 \(\mu\)M) is more than adequate to account for the early inhibition of mitochondrial respiration. Furthermore, at 500 \(\mu\)M, which is about twice the level during and shortly after ischemia, arachidonic acid exposure causes profound swelling of cortical slices within 40 min, very probably as a result of free radical formation (171, 175). It is possible that the rapid ECC seen in some models is a result of such a process. However, it is more surprising that such swelling is generally not seen in ischemia despite the large increase in arachidonic acid.

Between 1 and 50 \(\mu\)M, arachidonic acid inhibits glutamate uptake on the Na\(^{+}\)-coupled transporters. This should increase ambient glutamate and may thus be damaging.

B) EFFECTS OF PAF. Platelet-activating factor may well play an important role in ischemic damage (666). In the gerbil, there is a ninefold increase in PAF during and right after ischemia, which may persist (this has not been measured). In the rat, there is a small increase in PAF in a microdialysate during ischemia, but an extremely large increase ~2 h after the end of 20-min 4-VO (901).

Platelet-activating factor antagonists strongly reduce the increase in FFA that occurs after global ischemia in whole tissue (869) and in mitochondria (1080), implying that PAF causes these. Platelet-activating factor does rapidly increase cytosolic Ca\(^{2+}\) about twofold in cultured neurons and other cell types, by a currently unknown mechanism (98, 661, 1269), but it is not clear that this accounts for its very large FFA mobilization. In principle, though, it could certainly enhance damage.

The PAF antagonist BN-52021 (25 mg/kg) modestly reduced neuronal damage in rat hippocampus by ~20% 7 days after 10-min 2-VO (921) in a study in which temperature was well controlled. The antagonist strongly reduced infarct volume during permanent MCA occlusion, with no effect on blood flow (97). It also strongly protected neuronal cultures against NMDA receptor-mediated glutamate toxicity (921).

These experiments are all consistent with the conclusion that PAF production may act to enhance fatty acid production during ischemia, and possibly Ca\(^{2+}\) accumulation, and in these two ways enhance the probability of cell death. Platelet-activating factor may also activate transcription factors. It activates expression of COX-2 in cultured rabbit cornea where the effect is mediated by an increase in cytosolic Ca\(^{2+}\) (68), whereas in alveolar macrophages, the effect also requires lipopolysaccharides.
It is not clear that this action is important in ischemia.

c) Effects of Changes in Phospholipid Content. I) Phosphoinositides. Although levels of triphosphoinositides and diphosphoinositides in the cortex recover completely after 5-min ischemia in gerbils, those in CA1 region of hippocampus stay very low. They remain at 30 and 20% of normal throughout the period during which there is delayed neuronal degeneration (4). The reason that levels remain low is not clear, possibly a prolonged activation of PLC, but the effect of the large decrease may be to prevent signaling mechanisms that rely on polyphosphoinositides (238). Some of these, for example, activation of phosphatidylinositol 3'-hydroxykinases, are critical in preventing apoptosis in the face of neurotrophin withdrawal (232). Thus this phenomenon is potentially very important in enhancing apoptotic death. More work is needed here.

II) Phospholipids and membrane structure. There is quite good evidence that depletion of membrane phospholipids, and presumed loss of membrane integrity, is a major factor in ischemic damage to liver (314, 1069), but this is not the case for brain where the loss in phospholipid content in nonmitochondrial membrane is very small. However, when CDP-choline was given systemically for several days after 20- or 30-min 4-VO, it greatly ameliorated neurological deficits measured 10 days later. Neurological status was assessed on a 0–5 scale (5 worst), and the CDP-choline improved the score from 2.5 to <1 (516). Also, CDP-choline administration, when combined with a low dose of MK-801 which was not normally protective, reduced infarct volume 7 days after 90-min focal ischemia by ~50% (855). These protective effects may be mediated by enhancing synthesis of phospholipid in the brain, since the combination of choline and cytidine does this in striatal slices (992). If so, it would point to the importance of phospholipid depletion in cell death and, hence, the likely importance of membrane damage, although the effect may be in an organelle other than the plasmalemma (e.g., mitochondria). Again, more studies are required to follow up these important possibilities.

III) Phospholipids and calpain. In liver cells, phospholipid breakdown very probably leads to activation of calpain (37). This would be a very potent mechanism by which the change in phospholipid metabolism could lead to damage. There is no evidence yet as to whether this is important in brain tissue.

5. Conclusions

Ischemia causes profound changes in phospholipid metabolism with large increases in FFA in mitochondria and in the rest of the cell. The bases for the increased mobilization have not been completely determined but very probably involve activation of PLC and PLA_2 and also decreased resynthesis of phospholipid due to low ATP. Whether or not these changes in FFA and phospholipids help cause functional damage and cell death has not been established to date. This is partly because there are no satisfactory inhibitors, although informative studies could still be carried out with the current crop, such as PLA_2 inhibitors and cyclooxygenase inhibitors. The protective effects of compounds that enhance phospholipid synthesis suggest the importance of the phospholipid breakdown, but the studies are not complete. The protective effects of PAF inhibitors, combined with their strong inhibition of total and mitochondrial FFA production, also suggest the importance of phospholipid changes, perhaps in the mitochondria. However, PAF may be damaging at the level of the genome rather than by mobilizing FFA.

Reasonable mechanisms of damage by net phospholipid breakdown include altered membrane function, free radical generation, activation of calpain, or in the case of PAF, genetic mechanisms.

Overall, there is good reason to think that the very dramatic changes in phospholipid metabolism are important in ischemic cell death. However, studies have not yet established this.

D. Long-Term and Short-Term Changes in Protein Kinase and Phosphatases

Permanent, or long-term, inactivation of protein kinases or phosphatases could lead to initiation of apoptosis or could lead to the permanent alteration of proteins involved in cell membrane or mitochondrial function, the cytoskeleton or protein synthesis. Such effects could thus make a major contribution to ischemic cell damage.

1. PKC

Five minutes of global ischemia in rat does not affect overall PKC activity, nor does it cause substantial damage. However, 10-min ischemia causes damage and causes major reductions in soluble and particulate enzyme activity in hippocampus (35), striatum (161, 1211), and cortex (227). Decreases of ~30% occur within minutes or a few hours and persist for several days. At present, the basis for this change in PKC activity is not known. There is no measurable decrease in total PKC, and there is a major shift of immunoreactivity from the cytosol to the membrane (161, 163).

Enzyme activities in focal ischemia have not been reported; there is a small (10%) reduction in total PKC in the ischemic core, measured as binding sites for phorbol esters, 3 h after 90-min focal ischemia (797) but no reports of activity changes.

There are no direct studies linking the very profound PKC changes in global ischemia to ischemic cell death. However, there is an important study linking such PKC changes to glutamate-induced cell death in cultured neurons (283). Glutamate is not toxic in young cultures (<8
cell divisions) but is toxic in older cultures. The differential toxicity was not related to differences in glutamate effects on cytosolic Ca\(^{2+}\). However, it was strongly correlated with an absence of PKC inhibition in the younger cultures. Protein kinase C was inhibited by 50% in older cultures in a Ca\(^{2+}\)-dependent manner. However, it was not blocked in young ones. Furthermore, when excitatory amino acids were combined with artifactual inhibition of PKC in these younger cultures, there was major cell death. Thus it was concluded that the combination of a Ca\(^{2+}\) increase and PKC inactivation was necessary, and sufficient, for cell death (283). This ascribes a key role in damage to the PKC inhibition, and it is anticipated that similar studies will be carried out using ischemia as the insult.

2. CaMKII

There is also a profound, rapid, and prolonged decrease in CaMKII following ischemia. Activity actually increases about fourfold after 2-min ischemia (1273) but is reduced by ~50% in both particulate and cytosolic fractions, at the end of 5-min global ischemia in rat, and stays reduced for at least 24 h. Ten minutes of ischemia reduces activity by ~70% (35, 447, 1204). Twenty minutes of 4-VO led to equal falls in activity in CA1, CA3, and dentate gyrus 12 h after ischemia. There was partial recovery in the two nonvulnerable regions but no recovery over the next days in CA1 (780).

Ca\(^{2+}\)/calmodulin-dependent protein kinase II was also strongly inactivated in both the core and the penumbra during focal ischemia. This occurred within 5 min and was maintained throughout ischemia. After reperfusion, activity was restored when ischemia was short (5 min) but not if ischemia was longer (30 and 60 min) (418). Thus, in both global and focal ischemia, there is a very good correlation between permanent inhibition of CaMKII and cell death.

As for PKC, the mechanism of the activity decrease is not yet known. After global ischemia, there is a major decrease in immunoreactivity (200, 469, 780) and a marked shift of remaining immunoreactivity from the cytosol to the particulate fraction of the tissue (1019), as with PKC. The shift is largely to the postsynaptic density (469, 600). This persists in CA1 (469). Although there may be a small decrease in the total amount of CaMKII, that is not the major reason for the loss of activity, or immunoreactivity (200, 469). Thus the loss of activity and movements must reflect structural changes in the kinase (200, 1019). The changes are accompanied by a large decrease in ATP affinity and in the ability of an ATP analog to bind to the enzyme, indicating a structural change in the ATP binding region of the protein (200). This conclusion is supported by phosphopeptide mapping, which reveals regions in CaMKII from ischemic tissue that become inaccessible to autophosphorylation (1019). If this change in structure is the basis for the ischemic change in CaMKII activity and immunoreactivity, it is notable that it is extremely long lasting.

As with PKC, CaMKII suffers long-term inhibition during glutamate toxicity in cultured cells (199, 779), and also following the application of A-23187 (779), suggesting the inhibition is Ca\(^{2+}\)-mediated. Unlike PKC, there are, as of yet, no studies that establish the importance of the decrease in CaMKII in a cell death paradigm.

In this vein, it would be most interesting to know the effects on cell viability of combining PKC and CaMKII inhibition, as actually occurs after ischemia. The resultant (presumed) reduced level of phosphorylation, possibly localized to specific cell regions, could surely have profound effects.

3. Other protein kinases

In experiments where PKC and CaMKII were both strongly inactivated by ischemia there was no change in PKA (35).

However, there does appear to be an important downregulation of tyrosine kinase activity. After a 10-min global insult in rat, basal tyrosine phosphorylation was upregulated in nonvulnerable regions but was the same or was downregulated in vulnerable regions (468), a pattern that persisted for at least 24 h. The same microanatomy pertained for casein kinase II. The activity decreased by 25–50% in CA1 of hippocampus and striatum within 1 h of global ischemia while activity increased in nonvulnerable regions, CA3 and neocortex, within 15 min and remained elevated (467).

Thus two kinase systems whose level of phosphorylation reflect trophic effects on cells are significantly lower in vulnerable regions of the brain after global ischemia. This is certainly consistent with a role in damage.

4. Calcineurin

As measured immunohistochemically, the calmodulin-dependent phosphatase begins to decay in hippocampal CA1 somata and dendrites ~1.5 days after 20 min of 4-VO. This is before the major phase of cell death, which occurs ~12 h later (780). The ramifications of this are not obvious at present.

5. Short-term activation of protein kinases and cell death

The long-term effects of protein kinase decreases, which may well be damaging, need to be contrasted with acute effects, determined by introducing inhibitors of the kinases just before an insult. Kinase activation is damaging in this early phase.

Inhibition of tyrosine kinases (571) strongly protected against 5-min global ischemia in gerbil; inhibition of PKC with staurosporine strongly protected against...
5-min ischemia in the gerbil and 20-min 4-VO in the rat (425), and inhibition of PKC and CaMKII during the insult protected against ischemia in cultured neurons (409, 705).

These studies imply that there is early activation of the protein kinases, as has been explicitly shown for tyrosine kinase phosphorylation of MAP2 (157) via MAP kinase kinase (MEK), CaMKII as discussed above (1273), as well as others (1257), and that these are important in initiating damage. The targets of these kinases are not yet established. Some of these include activation of PLAr, which will activate PAF synthesis (907), enhancement of \( \text{Ca}^{2+} \) entry through NMDA receptors (691), and, in the case of tyrosine kinase, ischemic release of glutamate; the latter is strongly inhibited by genistein during global ischemia (907). The mechanism of this effect may well be MAP kinase phosphorylation of synapsin. Inhibition of MEK with PD-98059 blocks ischemia-induced neural injury in culture and coincidentally blocks glutamate release as well as phosphorylation of synapsin (924).

Gene activation may also be an important mediator of the early kinase activations; this has not yet been determined.

6. Conclusions

Almost all of the protein kinases show activity decreases after global ischemia, whose timing and localization are consistent with their importance in cell death.

The mechanisms of the kinase activity decreases are not currently known. Although direct evidence for involvement in cell death is lacking, there is good evidence that a similar decrease in PKC is damaging in excitotoxicity. Certainly, the ability of staurosporine to induce apoptosis is consistent with the possibility that inhibition of multiple kinases may lead to apoptosis, but it is not firmly established that this staurosporine effect is mediated by its inhibition of kinases. Knowing the effects of prolonged multiple kinase inhibitions or multiple kinase knockouts on survival of cells in normoxic conditions would help determine whether the ischemic kinase changes mediate cell death.

If the kinase downregulation is important, then it becomes very important to understand why this occurs, including what causes it, and what the changes in the kinases are. Regarding the former, it would be very interesting to know the involvement of free radicals, calpain, or phospholipid breakdown in the loss of activity.

E. Summary

Several potential perpetrators of the molecular changes causing cell death have been considered in this section. On the basis of measured changes and effects of pharmacological agents, free radicals and calpain must be considered very likely mediators of cell death in temporary focal and global ischemia models, although there are important caveats, which were pointed out in the summaries at the end of each section. The most important one for free radicals is that most “scavenger studies” do not measure or correlate protective effects with changes in free radical production. For calpain, the most important caveat is the paucity of studies and the important question of inhibitor specificities. In both cases, whether temperature is controlled well in all the studies and for long enough is also of some concern. Free radicals also appear to be important in permanent focal ischemia, but there are no studies of effects of calpain inhibition on that insult. Phospholipid and protein kinase changes are large and a priori seem likely to contribute to cell death. However, there are no really solid pharmacological or knockout studies implicating them as yet.

The fact that inhibition of either calpain, or free radical accumulation, are (very probably) strongly protective in all models implies that both calpain activation and free radical generation are necessary for damage. This receives (very indirect but intriguing) support from the apparent coincident activation of both these variables ~1 day after global ischemia. The basis for this could be either that two or more independent macromolecular changes are required to produce the critical functional changes (say in mitochondria) or that free radicals and calpain are synergistic at the molecular level. The fact that free radical action tends to inhibit calpain (402) argues against the latter, but free radical sensitization of the Na\(^+\)-K\(^+\)-ATPase to calpain sets a precedent for such synergism (1307). The combined effects of calpain inhibition and free radical scavengers might be very protective.

VI. ACTIVATORS OF PROCESSES CAUSING FUNCTIONAL CHANGES

This section considers ionic and molecular changes that may be activating the damaging processes discussed in section v.

A. \( \text{Ca}^{2+} \)

Increases in \( \text{Ca}^{2+} \) concentration in appropriate compartments can effect all the changes that were discussed in section v. The ion has generally been considered to be a major effector of necrotic cell death during ischemia (614), and this has received a major boost from the fact that \( \text{Ca}^{2+} \) very clearly plays a critical role in the normal cell death caused by ischemia in cell culture systems (376). The goal of this section is to critically evaluate the evidence that \( \text{Ca}^{2+} \) also plays a crucial role in vivo. Although the evidence is consistent with such a role, there is little solid demonstration of it.
1. Changes in cell Ca\(^{2+}\) during ischemia

A) INCREASES IN TOTAL CELL Ca\(^{2+}\). There are large increases in total intracellular Ca\(^{2+}\) during ischemia in all systems.

During global ischemia, extracellular Ca\(^{2+}\) abruptly falls to ~0.1 mM at the time of the anoxic depolarization, 60–90 s after the start of ischemia (85, 1040, 1231). The Ca\(^{2+}\) is moving into the intracellular space and, with the assumption the extracellular space is ~20% of total tissue volume, the calculated rise in cell Ca\(^{2+}\) is ~250 μM, about a 25% increase in total cell Ca\(^{2+}\) (532).

During focal ischemia, extracellular Ca\(^{2+}\) decreases to ~0.1 mM for at least 2 h in the core after ~10 min (430, 612). This occurs at a flow rates of ~15 ml · 100 g~1 · min~1. In the penumbra, where flow is much higher, extracellular Ca\(^{2+}\) decreases are limited to the times of the intraischemic depolarizations (612) and are thus very much less than in the core. When studied over the course of permanent 24-h focal ischemia, total Ca\(^{2+}\) in the core of the lesion gradually increased and was far greater than in the penumbra (570, 937). In one case, total Ca\(^{2+}\) in the core rose at ~1 mM/h from the beginning of the insult until ~24 h (937). It is not clear whether accumulation preceded or followed cell death because the latter was also increasing over this time. However, the earliest increases, at 2 h, are before any significant level of cell death. This slow increase reached very profound levels (~15 times control levels at 24 h).

There are decreases in extracellular Ca\(^{2+}\) and increases in intracellular Ca\(^{2+}\) during in vitro ischemia in cortical and hippocampal slices that occur within the first few minutes (6, 611, 686). There are quite delayed increases of Ca\(^{2+}\) in neuronal cell cultures (376). The rise in cultures may be delayed because the ATP fall is small, only 30% after 60 min (see Table 1), or because the cultures are from embryonic rat tissue (533). The changes in slices follow similar kinetics and have similar properties to those in global insults (see below) and so are better models for this phenomenon.

B) INCREASES IN CYTOSOLIC Ca\(^{2+}\). A critical question in terms of damage is the level to which cytosolic Ca\(^{2+}\) rises during and after ischemia. Unfortunately, there are only two reported studies of cytosolic Ca\(^{2+}\) in vivo, and in one of these the technology is not reliable.

In an important study, Silver and Erecinska (1048) used Ca\(^{2+}\) microelectrodes and recorded very large increases in cytosolic Ca\(^{2+}\) in CA1 and CA3 hippocampal neurons during 4-VO or compression ischemia in the rat (1048). There was a small, 10–30 nM, increase within the first minute of ischemia (1047), and within the next 2–3 min, cytosolic Ca\(^{2+}\) rose from ~90 nM to 30 μM. This increase seems very large, but the fact that it was delayed by MK-801 tends to exclude artifact (1047, 1048). A delayed increase in cytosolic Ca\(^{2+}\) of CA1 pyramidal cells was also measured with dual-wavelength excitation of fura 2 (803). The increase was not quantified. The large increase noted by Silver and Erecinska (1048) also occurs in slices where, using a low-affinity Ca\(^{2+}\) dye in a dual wavelength mode, a rapid increase from 60 nM to 24 μM was shown (397).

Cytosolic Ca\(^{2+}\) was also measured in cortex during 60 min of MCA occlusion in the rat, using surface fluorimetry of indo 1 (1151). Unfortunately, although the authors described how they corrected for NADH and reflectance changes, no actual data were given, and others who tried to reproduce these results after making appropriate corrections were unable to show any 400/506-nm fluorescence changes when Ca\(^{2+}\) moved into the cell (660). It was noted that accurate measurements were very difficult because indo fluorescence was smaller than the basal fluorescence of the tissue and also because the method for correcting the NADH fluorescence changes was not accurate enough. In the absence of data showing the NADH and indo fluorences, it must be considered that the earlier studies may have been contaminated by artifact and thus, unfortunately, they must be regarded as questionable at this stage. This is unfortunate since they are the only studies of cytosolic Ca\(^{2+}\) during focal ischemia.

The only other measurement of cytosolic Ca\(^{2+}\) is indirect but useful; the loss of tissue staining by a calmodulin antibody that binds only to calmodulin that is not complexed with Ca\(^{2+}\) was measured (242). Loss of staining corresponds to increased cytosolic Ca\(^{2+}\), and there was a near-maximal decrease of staining within 2 h of starting focal ischemia in the core of the lesion and within 4 h in the penumbra, qualitatively indicating increases in cytosolic Ca\(^{2+}\) that occurred more rapidly in focus than penumbra.

There is an increase in cytosolic Ca\(^{2+}\) during in vitro ischemia in gerbil (765, 769) and rat (95, 1288) hippocampal slices, measured by fluorimetric dyes. In the one case, where actual concentrations were measured, using fura 2 and correcting for NADH fluorescence, cytosolic Ca\(^{2+}\) rose from 180 to 600 nM after 5-min anoxia and to 1.5, then 1.8 μM after 10 and 15 min (95). As pointed out, when cytosolic Ca\(^{2+}\) was measured with a low-affinity dye, it rose to 24 μM. The higher values are more accurate (476).

The only reliable measure of cytosolic Ca\(^{2+}\) in vivo during ischemia shows a rapid increase to 30 μM during global ischemia. Such increases are large and would clearly activate all Ca\(^{2+}\)-dependent processes discussed, including M-calpain. However, there is a paucity of studies, and more are quite urgently needed, particularly in focal ischemia where change in calmodulin binding is the only semidirect measure. Indirect evidence,
such as spectrin breakdown, MAP2 breakdown, and NOS activation, certainly strongly suggests an increase in cytosolic Ca\textsuperscript{2+} during focal ischemia. However, there are other possible explanations for each of these events, so the conclusion is not firm.

Thus, although total cell Ca\textsuperscript{2+} surely increases during ischemia, more data are required to confirm the increase in cytosolic Ca\textsuperscript{2+}, particularly in focal ischemia, and to estimate its magnitude.

2. Mechanisms of Ca\textsuperscript{2+} increases during ischemia

Cytosolic Ca\textsuperscript{2+} could rise as a result of a net entry of Ca\textsuperscript{2+} across the plasmalemma or, transiently, due to liberation of Ca\textsuperscript{2+} from intracellular stores.

In vivo the early increase in total cell Ca\textsuperscript{2+} during global ischemia is via NMDA receptors. \textit{d}-2-Amino-5-phosphonovalerate (\textit{d}-APV) reduced the fall in Ca\textsuperscript{2+} in the extracellular space during global ischemia by \~60–70\% and slowed the fall so it took \~8 min (85). In accord with this, the early increase in cytosolic Ca\textsuperscript{2+} during the first 3 min of global ischemia was largely blocked by either MK-801 or ketamine. However, by the end of 8-min ischemia, Ca\textsuperscript{2+} levels were the same whether or not the NMDA receptors were blocked (1047, 1048). Thus there is an early influx of Ca\textsuperscript{2+} via NMDA receptors that ceases and that is replaced by influx through other pathways, or release from intracellular stores. L-channel blockers did not affect the increase in cytosolic Ca\textsuperscript{2+}, showing that the latter does not result from Ca\textsuperscript{2+} entry via those channels.

In vitro systems are far more accessible and provide more information. In cortical cell cultures at least 80\% of the bulk Ca\textsuperscript{2+} entry during in vitro ischemia is prevented by NMDA blockers (376) (as is the increased cytosolic Ca\textsuperscript{2+}, Refs. 884, 1199), and the Ca\textsuperscript{2+} influx occurs at the same time as the increase in extracellular glutamate (376). Thus Ca\textsuperscript{2+} entry and increased cytosolic Ca\textsuperscript{2+} are very largely mediated by NMDA receptors.

In the hippocampal slice, influx of Ca\textsuperscript{2+} during the first 2.5 min of in vitro ischemia is via NMDA receptors, but these appear to inactivate after this time (686). Influx during the next 2.5 min is via L channels (25\%) and Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange (35\%) and via an unidentified pathway (686). The very early rise in cytosolic Ca\textsuperscript{2+}, measured using the high-affinity dye calcium-green I, was blocked by MK-801, as expected from the total Ca\textsuperscript{2+} measurements. Also, as in vivo, the rise in cytosolic Ca\textsuperscript{2+} after the first few minutes was unaffected by MK-801, and the final level of cytosolic Ca\textsuperscript{2+} after 10-min ischemia was independent of NMDA receptors (1289).

The later sustained cytosolic Ca\textsuperscript{2+} increase in slices was greatly attenuated when mitochondrial Ca\textsuperscript{2+} efflux was blocked by a novel inhibitor of the mitochondrial Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger, CGP-37157 (10 \textmu M) (1289). This Ca\textsuperscript{2+} efflux depends on Na\textsuperscript{+} influx during ischemia because the cytosolic Ca\textsuperscript{2+} rise was similarly blocked by the Na\textsuperscript{+} channel blocker lidocaine (10 \textmu M) and the non-NMDA glutamate blocker CNQX (10 \textmu M). Thus some of the Ca\textsuperscript{2+} that enters the cell during ischemia is taken up by mitochondria, and this uptake is normally attenuated by the efflux of Ca\textsuperscript{2+} on 2Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger, which is activated by the entering Na\textsuperscript{+} (1289). Thus the entering Na\textsuperscript{+} effectively acts to increase cytosolic Ca\textsuperscript{2+} during ischemia. Whether or not this occurs in vivo is not known. It ascribes an important role to entering Na\textsuperscript{+}, and such a role is evidenced in vivo by the very strong protection afforded by Na\textsuperscript{+} channel blockers (see sect. viC).

Two other features emerge from the measurements of cytosolic Ca\textsuperscript{2+}. There is fairly strong evidence that Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR) from ER contributes to the rise in cytosolic Ca\textsuperscript{2+}. Dantrolene, the specific blocker of CICR, has the same effect as NMDA blockers in ischemic hippocampal slices (1288) and in cultures treated with cyanide (279). In vivo the evidence for CICR is indirect in that dantrolene significantly protects gerbil hippocampus against damage (1196). The in vitro studies suggest that effects of NMDA-mediated Ca\textsuperscript{2+} entry on cytosolic Ca\textsuperscript{2+} are greatly amplified by CICR. Such amplification occurs after Ca\textsuperscript{2+} entry caused by high-frequency stimulation (14). The same amplification probably occurs in vivo and accounts for the protective effect of dantrolene.

The other feature of the results is the rapid inactivation of the NMDA receptors during global ischemia and during in vitro ischemia in the slice. This is probably due to dephosphorylation, since it was eliminated by okadaic acid in the slice (686). The dephosphorylation is probably a consequence of the precipitous ATP fall (696) and may well explain the relative unimportance of NMDA-mediated Ca\textsuperscript{2+} entry in global ischemic damage (see below). Damage would presumably be greater, and more NMDA dependent, if the receptors did not inactivate. Adenosine 5’-triphosphate does not fall nearly as much in the penumbra of focal lesions, and this probably explains the far greater importance of NMDA receptors in that insult, although inactivation has not been tested in any way.

A) Intracellular Localization of Entering Ca\textsuperscript{2+}. Even if cytosolic Ca\textsuperscript{2+} rises to 30 \textmu M, most of the Ca\textsuperscript{2+} that enters the cells during ischemia will not be free but will be on binding proteins or in organelles. The total initial increase during global ischemia and in the ischemic core is \~250 \textmu M, and levels appear to rise continually in the core. The amount on cytoplasmic binding proteins can be very roughly estimated because their dissociation constants and concentrations are not at all precisely known. The approximate concentration of Ca\textsuperscript{2+} binding sites in the cytoplasm is \~200 \textmu M and the average dissociation constant of these proteins is 1–10 \textmu M (50, 1217, 1224). Thus, if cytosolic Ca\textsuperscript{2+} rises to 20–30 \textmu M, then the proteins will be largely saturated so they will contain \~200 \textmu M Ca\textsuperscript{2+}. A significant portion of the entering Ca\textsuperscript{2+} will locate on nuclear binding proteins (917) as the free
nuclear Ca2+ is likely to equilibrate (854), or nearly equilibrate (16), with the free Ca2+ in the cytosol.

There may also be Ca2+ accumulation in the ER and mitochondria; recent studies show uptake into mitochondria even during ischemia in the hippocampal slice (1110). Although the importance of NOS activation, and very probable effects on other cytosolic proteins such as calpain and phospholipases, suggests that the rise in cytosolic Ca2+ is important, changes in other structures may also be important, for example, in activating genes and in affecting mitochondrial integrity.

**B) SUMMARY.** There is very little known about mechanisms of Ca2+ entry or accumulation in focal ischemia, although there is almost undoubtedly significant entry in the penumbra during the intraischemic depolarizations. This has not been explicitly shown.

The early entry of Ca2+ and buildup of cytosolic Ca2+ in global ischemia is probably due to Ca2+ entry via NMDA receptors and its effect in eliciting CICR (14). N-methyl-d-aspartate receptors appear to inactivate within a few minutes, probably due to the profound ATP decrease and resulting receptor dephosphorylation (686). The steady-state increase in cytosolic Ca2+ during ischemia, in slices, is largely due to Na+ entry and resultant activation of 2Na+/Ca2+ transporter-mediated efflux from the mitochondria. This may persist in vivo also, although it has not been studied. An important unknown is whether an augmented influx pathway contributes to the maintained cytosolic Ca2+ after the NMDA receptors are inactivated. There is entry via L channels and plasmalemma 3Na+/Ca2+ exchange in the slice, but these do not appear to contribute to the elevated cytosolic Ca2+ (1047, 1048, 1289). The maintained elevation could result simply from the Na+-induced Ca2+ release from the mitochondria coupled with somewhat impaired removal of Ca2+ across the plasmalemma due to lowered Ca2+-ATPase.

### 3. Postischemic changes in Ca2+

There is an eclectic body of evidence that cytosolic Ca2+ levels are elevated in the postischemic period. This could be very important in development of damage.

**A) GLOBAL ISCHEMIA.** There is a large increase in total Ca2+ in the vulnerable medial-CA1 region of the gerbil hippocampus immediately after 5-min global ischemia. This returns to normal by 30 min, although this area is subsequently badly damaged (802). A small, 40%, elevation in free Ca2+ has been noted 2–8 h after 8-min global ischemia in the rat, but these are very difficult measurements (1048).

Six to 24 h after 2-VO in rats there is a threefold enhancement of the Ca2+ entry from the extracellular space during burst (15 Hz) firing (22), which is mediated by NMDA receptors. Such firing is fairly normal for hippocampal cells, so a significant increase in cytosolic Ca2+ may well occur. The result suggests prolonged hyperactivation of NMDA receptors. This is supported by our studies of MAP2 proteolysis (Zhang and Lipton, unpublished data) that show continual Ca2+-dependent breakdown in the postischemic period that is mediated by NMDA receptors. It would be of interest to see if very delayed application of MK-801 would attenuate cell death. The drug generally does not do so if administered in the immediate postischemic period (327, 819, 1023).

There is excess mitochondrial Ca2+ accumulation 6–24 h after global ischemia in gerbil (285) and rat (1272) hippocampus and also during the first 6 h in gerbil (846). The most straightforward explanation is that this reflects increased cytosolic Ca2+ leading to greater uptake via the Ca2+ uniporter, whose mean affinity constant is in the micromolar range (400). This is suggested by the qualitative observation of elevated numbers of Ca2+ deposits in the cytoplasm between 2 and 6 h after ischemia (846). No measurements were made after 6 h in that study. The NMDA, AMPA, or voltage-dependent Ca2+ channel dependencies of the elevated postischemic Ca2+ have not been checked.

**B) FOCAL ISCHEMIA.** There is a persistent elevation of cytosolic Ca2+, measured by antibody binding to calmodulin, out to 24 h after 1-to 2-h focal ischemia in the rat (242), implying a prolonged elevation of cytosolic Ca2+. This is supported by measurements of total Ca2+ levels using 45Ca2+. Calcium levels remain two- to threefold elevated in the core throughout a 24-h reperfusion period, despite some extrusion upon reoxygenation, and they are elevated, but not as much, in the penumbra. In the latter case, the increase does not occur until 6 h of reperfusion (612).

The studies described in this section are almost anecdotal because they have not been followed up in a satisfactory way. Nonetheless, they highlight the real possibility that elevated cytosolic Ca2+ in the postischemic period may contribute to damage. Coupled with the observation of delayed calpain activation in CA1 of gerbil, described in section B, they certainly become important.

### 4. Role of increased cytosolic Ca2+ in ischemic cell death

**A) CELL CULTURES.** N-methyl-d-aspartate antagonists almost completely block Ca2+ entry and almost fully protect against anoxic/ischemic and CN-induced damage in neuronal cultures, arguing that Ca2+ entry, and thus probably cytosolic Ca2+, is critical for damage (237, 376, 405, 603, 884). Although this may well be, it is notable that in vitro ischemia induces cell death in zero Ca2+ buffer (377), and this is also NMDA dependent (376). It is dependent on extracellular Na+, showing that NMDA receptors can mediate cell death via Na+ entry. The speed of that process, and the fact that it is accompanied by more cell swelling than normal, suggests that it proceeds by a different mechanism from the cell death in normal buffer...
(376), and zero-Ca\(^{2+}\) incubation has been shown to sensitize other tissues to free radical damage (315, 881). Thus it is generally concluded that the normal ischemic damage is Ca\(^{2+}\)-mediated and that the processes in zero Ca\(^{2+}\) do not occur when extracellular Ca\(^{2+}\) is normal. This is probably correct.

Neuronal cell death in organotypic cultures is partly dependent on NMDA receptor activation (5, 923, 1073), implying a Ca\(^{2+}\)-dependence as above. Successfully loading cells with the Ca\(^{2+}\) chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-ethanesulfonic acid attenuated damage. However, this was very probably due to blocking glutamate release presynaptically rather than preventing the cytosolic Ca\(^{2+}\) increase in the pyramidal cells themselves (5). Thus it does not particularly support a direct role for Ca\(^{2+}\) in cell damage.

Another way to determine whether Ca\(^{2+}\) is important in damage is to determine whether Ca\(^{2+}\)-dependent processes are involved. Consistent with the importance of Ca\(^{2+}\), calpain and calmodulin antagonists both substantially, but not completely, protect against ischemic damage 24 h after 30-min ischemia in organotypic cultures (1085, 1190), and as described previously, so do inhibitors of NOS, which is very probably activated by Ca\(^{2+}\)/calmodulin.

Taken together, these studies present a good case for the involvement of cytosolic Ca\(^{2+}\) in cell death in cultures, although rigorous studies in which intracellular buffers were used to show that cytosolic Ca\(^{2+}\) and damage were causally related would be most persuasive.

B) IN VIVO: GLOBAL ISCHEMIA. I) NMDA. The Ca\(^{2+}\) measurements show that NMDA-mediated Ca\(^{2+}\) entry accounts for the early increase in cytosolic Ca\(^{2+}\) in rat; measurements have not been made in the gerbil. The actions of NMDA antagonists on cell death are considered in some detail in section \(\text{C}\) because there are major artifact problems, in particular a very large and prolonged (12–48 h) hypothermic effect. When these are taken into account, as they have been in very careful studies on gerbil (454, 768) and rat (99, 135, 819, 1089), it appears that MK-801 partially protects (~50%) against 5-min ischemia in the gerbil, providing the temperature is 37°C or below. It does not protect if temperature is 38.5°C or above (136, 454). However, even here, as discussed (454), it is quite possible that protection is not due to block of Ca\(^{2+}\) entry because very high doses of MK-801 (10 mg/kg) had to be used to get reliable protection. A dose of 3 mg/kg generally gives maximal MK-801 effects on Ca\(^{2+}\). Thus the protection may be mediated by blockade of Na\(^{+}\) channels (414), leaving no positive evidence that Ca\(^{2+}\) entry is important in damage.

MK-801 does not appear to protect against 5- or 15-min ischemia in rat (99, 819, 1158), except in a 2-VO model in which hippocampal blood flow is only reduced to 20% (rather than the usual 1–3%) (1089).

The lack of protection in no way eliminates the possibility that Ca\(^{2+}\) is important in damage; NMDA-mediated Ca\(^{2+}\) entry does not contribute very much to cell Ca\(^{2+}\) increases, especially after 2–3 min. Other sources of Ca\(^{2+}\) may be leading to damage when the NMDA receptors are blocked (see sect. \(\text{V.B}\)). Alternatively, because MK-801 and other blockers of NMDA actually cause neuronal damage in normoxia (299) and ischemia (1037), there could be a damaging effect that is countering a protective effect. However, the lack of protection does prevent using these data as evidence that Ca\(^{2+}\) is important.

II) Voltage-dependent Ca\(^{2+}\) channels. Voltage-dependent Ca\(^{2+}\) channel blockers offer significant protection against global damage, without affecting posts ischemic blood flow. Nimodipine (781, 842) and flunarizine (252) protected against damage in the gerbil, with optimal protection occurring when the drug was present before or right after the ischemia and for a long period after the ischemia. Fifty to one hundred percent protection was achieved, the latter when treatment was continued for 24 h. Although these results are impressive, they are somewhat flawed because in those early studies temperature was not controlled, nor were effects of the blockers on temperature measured. The N-channel blocker SNX-111 (\(\omega\)-conotoxin MVIIA) (139, 1158) was protective when added up to 24 h after 10–15 min 4-VO in the rat, preventing ~50% of the damage. This was very probably not due to prevention of glutamate release, since a conotoxin that was much more potent against glutamate release did not protect against damage. Core temperature was controlled for 6 h after ischemia or drug treatment, which was far better than in most studies, and was very probably adequate.

If temperature depression was not a basis for the protection with the channel blockers, then the data suggest that either there is a posts ischemic opening or upregulation of L (and (postsynaptic) N channels or that sensitivities of intracellular processes are altered so that normal Ca\(^{2+}\) entry via the L or N channels is damaging. The apparent involvement of both channels suggests the latter may be the case. Alternatively, prolonged opening of both channels could help account for the increased cell Ca\(^{2+}\) in the posts ischemic period. The issue is not yet resolved, but in either case, using L and N channel blockers together should enhance protection; this should be tried.

III) Ca\(^{2+}\) from intracellular sources. As described previously, dantrolene provided significant protection against cell death in gerbil global ischemia, in studies where temperature was very well controlled (1196). There are no known sites of dantrolene action aside from blocking Ca\(^{2+}\) release from the ER (1196), so this result is reasonable evidence that such Ca\(^{2+}\) is damaging during global ischemia.

As discussed in section \(\text{V.C}\), blockade of Na\(^{+}\) fluxes is very protective in global ischemia. This might reflect damage caused by Na\(^{+}\)-induced release of Ca\(^{2+}\) from the mitochondria as seen in the hippocampal slice. There is currently no evidence for this, but it could be tested by...
examing effects of the mitochondrial 2Na\(^+\)/Ca\(^{2+}\) exchange blocker CGP37157 on global ischemic damage.

IV) Summary. There is equivocal evidence, because of the high MK-801 dose needed, that NMDA-mediated Ca\(^{2+}\) entry contributes to damage in gerbil (but no evidence in rat) and reasonable evidence that this may be mediated by resulting CICR from ER. It is speculated that Na\(^+\)-induced Ca\(^{2+}\) release from mitochondria might be damaging and would explain the very potent effects of Na\(^+\) channel blockers. The most persuasive evidence in favor of Ca\(^{2+}\) is that postschismic Ca\(^{2+}\) entry via voltage-dependent channels contributes to damage in both rat and gerbil, providing effects of blockers were not contaminated by temperature artifact.

Reasonably good evidence for the importance of Ca\(^{2+}\) comes from the role of Ca\(^{2+}\)-dependent processes in cell death. The only calpain inhibitor studied has been leupeptin, which is not at all specific for calpain over cathepsins or proteasomes. Damage was strongly attenuated in the mouse NOS knockout, and when a specific nNOS inhibitor was used in rat, implicating NOS and hence Ca\(^{2+}\). As discussed in sections ivB and ivA, respectively, both calpain and NOS could be activated by events other than increased Ca\(^{2+}\).

c) In vivo: Focal Ischemia. The evidence that Ca\(^{2+}\) is important in damage is stronger for focal insults than it is for global insults.

I) NMDA. Protective effects of NMDA blockade are clear in both temporary and permanent ischemia (334, 782, 1102), providing temperature is maintained at 37°C or below (753). This effect, which is limited to reducing the size of the penumbra, is very robust, and both competitive and noncompetitive antagonists produce a 30–50% reduction in total infarct size. Unlike in global ischemia, NMDA antagonists do not cause a postischemic temperature fall. If antagonists are protecting by blocking Ca\(^{2+}\) entry, the results strongly implicate increased cell Ca\(^{2+}\) in damage.

There are, though, other possible modes of action, which are considered further in section viC. The most serious artifact is that NMDA antagonists may be protective by increasing blood flow rather than by reducing Ca\(^{2+}\) entry (141, 1102), although the weight of evidence argues against this.

In addition, there are focal models (thrombotic lesions or spontaneously hypertensive rats) in which NMDA blockade is not effective at all (141, 970, 1102, 1234). Perhaps ATP levels fall further in these cases, making the situation akin to global ischemia. At the least, these data fail to support a role for Ca\(^{2+}\) in damage in this model. The absence of protection by MK-801 is rather important clinically because so many human strokes are thrombotic. It may help account for the poor performance of NMDA antagonists in clinical trials, to date.

II) Effects of Ca\(^{2+}\) channel blockers. L-channel blockers are protective against transient (156, 1150) and permanent (96, 500, 1166) focal lesions, reducing measured infarct sizes by ~50% in all cases. A critical issue here is whether protection results from increased blood flow. The results are not clear cut. There were no effects of nimodipine on blood flow during temporary ischemia (1150), but the drug caused a 50% hyperperfusion during the postischemic period. The latter is probably not important because blood flow does not appear to be limiting in the postischemic phase, but the possibility cannot be ruled out. Nimodipine does not affect blood flow for at least several hours if it is injected right after the onset of ischemia (87, 262). However, it did strongly increase blood flow to the core when it was preperfused for 2 h (501), and this may account for the protection seen in the three reported studies of permanent focal ischemia (96, 500, 1166).

The N-channel Ca\(^{2+}\) blocker SNX-111 reduced infarct volume, after 4-h permanent focal ischemia, by ~65% (1103) and also reduced infarct by 45% after temporary ischemia. This is a strong effect that is not mediated by effects on blood flow, and it may well reflect blockade of damaging Ca\(^{2+}\) entry into the neurons as receptors are present at significant concentrations on dendrites (761). However, protection may also have resulted from reducing glutamate release (1103).

III) Summary. The protective effects of NMDA antagonists and Ca\(^{2+}\) channel blockers are the only direct tests of the role of Ca\(^{2+}\) in damage. Thus they are very important. The results are consistent with damaging Ca\(^{2+}\) entry via NMDA receptors as well as L and N channels, and hence, the conclusion that Ca\(^{2+}\) is damaging. However, there are alternate explanations for the results that cannot be discounted as well as instances where NMDA antagonists are not protective. As discussed in section viC, the weight of the data indicates that the protective effect of NMDA antagonists is not due to increased blood flow, suggesting that Ca\(^{2+}\) entering cells via NMDA receptors is indeed damaging. However, this is not completely certain.

The involvement of NOS activation and calpain in focal ischemic damage evidenced by mutational and pharmacological studies are both good evidence for the involvement of Ca\(^{2+}\), although here too there are alternate explanations. Calpain can be activated at normal cell Ca\(^{2+}\) levels by phospholipid breakdown and by activator proteins. Although no Ca\(^{2+}\)-independent mechanisms for nNOS activation have been demonstrated to date (505, 690), the enzyme is induced within 4 h of the onset of focal ischemia (1294), and this could account for its toxicity. There are other possible mechanisms such as regulation by palmitoylation (505) and by removal of an endogenous inhibitor (506), but there is no serious evidence for these.

The calmodulin antagonist trifluoperazine reduced the lesion size in Wistar rats by 87% 24 h after 2-h temporary focal ischemia. This is the largest protective effect of any drug that has been reported. The drug had no effects on blood flow in the postischemic period (626). Unfortu-
nately, the drug is not specific to calmodulin; it affects Ca$^{2+}$ channels for example. However, calmodulin or another Ca$^{2+}$-dependent process is a very likely site of action, so the result does provide strong evidence for the importance of Ca$^{2+}$ in damage.

5. Possible mechanisms by which increased cytosolic Ca$^{2+}$ causes cell death

These have been discussed in the earlier sections of the review and so are just be noted here. They include NOS activation, calpain activation, generation of free radicals via phospholipid metabolism or by transforming XD to XO (in global ischemia), damaging mitochondria (282) or of course in ways that have not yet been studied, particularly those mediated by calmodulin that could well involve effects on cytoskeletal integrity (517). Calcium may also well be involved in upregulation of damaging genes during and after ischemia, although this has not been shown yet.

None of the inhibitor studies has addressed the question of whether Ca$^{2+}$-mediated damage (NMDA or L-channel dependent, for example) preferentially causes apoptosis or necrosis. However, an important study suggests that apoptotic death may be Ca$^{2+}$ independent. When in vitro ischemia in cell culture was prolonged to 90 min, neuronal death developed even in the presence of NMDA blockade (405). Unlike the normal cell death in the cultures, this cell death was associated with increased TUNEL labeling and was prevented by protein synthesis inhibitors (405), giving it apoptotic-like qualities. Death may have been triggered by intracellular Ca$^{2+}$ release, or by non-NMDA-mediated entry processes that are not manifested during 45-min ischemia (Ca$^{2+}$ entry during 45 min is almost completely blocked by NMDA receptors, Ref. 376), but a serious alternative is that it may be Ca$^{2+}$ independent. Unfortunately, Ca$^{2+}$ levels were not measured in the studies. The result raises the possibility that apoptotic-like death in focal ischemia and hypoxia/ischemia may occur in cells where Ca$^{2+}$ levels do not rise enough to activate other processes.

6. Conclusions

A) Evaluation of Evidence. Although the conclusion that Ca$^{2+}$ is critical to ischemic cell damage is widely accepted (615), one of the aims of this section was to highlight the quite large uncertainties that remain as to whether this is indeed so for in vivo ischemia.

There is undoubtedly Ca$^{2+}$ accumulation in brain cells during global and focal ischemia. Certainly, it is likely that this will lead to increased cytosolic Ca$^{2+}$ as it does in slices. However, there are only two studies showing an increase in cytosolic Ca$^{2+}$ during global ischemia and only one, indirectly, indicating an increase in cytosolic Ca$^{2+}$ during focal ischemia. Furthermore, only one of the three studies is quantitative. There are several studies showing increased total cell Ca$^{2+}$ during the 24 h after global and focal ischemia, and it is reasonable to infer that there is also increased cytosolic Ca$^{2+}$, despite the lack of measurements.

A major way to demonstrate involvement of increased cytosolic Ca$^{2+}$ or total Ca$^{2+}$ in damage is to test effects of blocking the increase. Unfortunately, most drugs that should block Ca$^{2+}$ buildup either have very small effects (NMDA blockers in global ischemia) or have other potentially interfering effects. At this stage, the most reliable pharmacological evidence is the attenuation of damage by L- and N-type Ca$^{2+}$ channel blockers in the period after global ischemia, indicating the importance of postischemic Ca$^{2+}$, and also the prevention of damage by NMDA blockers added during or after focal ischemia, indicating the importance of Ca$^{2+}$ during permanent focal ischemia and after temporary focal ischemia. There is, though, a small but real possibility that these blockers act by increasing blood flow (see sect. v.C). Another apparently discrepant result is that calbindin knockout mice show much reduced cell damage after 12-min global ischemia. Thus eliminating a principal cytoplasmic Ca$^{2+}$ binding protein improves outcome. No other common Ca$^{2+}$-binding proteins were upregulated in the knockouts so that it seems that reducing immediate Ca$^{2+}$ buffering reduces damage (589). This could be explained by more rapid clearance of cytosolic Ca$^{2+}$ following the ischemia, but it also may indicate that increased free Ca$^{2+}$ during ischemia is not harmful. More detailed studies need to be done.

The other way to demonstrate the importance of Ca$^{2+}$ is to show that processes activated by increased cytosolic Ca$^{2+}$ are involved in cell death. In this regard, the very clear dependence of damage on NOS and the almost certain dependency on calpain in focal ischemia and global ischemia are very strong indicators of the importance of Ca$^{2+}$. However, there are alternate explanations for the activation of these enzymes that do not rely on activation by cytosolic Ca$^{2+}$. Inhibition of damage by the calmodulin antagonist trifluoperazine is strong evidence for Ca$^{2+}$-mediated damage, although the processes affected are not known. The concern here is the specificity of the drug and the fact that protection was so strong that it would be well to see the study repeated. Evidence was discussed in section v.B that mitochondrial Ca$^{2+}$ accumulation was damaging, but although this is quite likely for glutamate toxicity, it is still only speculation for ischemia. There is a suggestion that Ca$^{2+}$ may not be involved in the apoptotic-like processes, from work on ischemia in cell culture.

Putting the skepticism of the preceding paragraphs in perspective, all the data are consistent with the conclusion that increased cytosolic Ca$^{2+}$ is critical in causing damage. Although this variable has only been measured well in one case, that is really because of the difficulty of making such measurements, not because cytosolic Ca$^{2+}$...
does not rise. The one direct measurement showed an impressive increase in cytosolic Ca\(^{2+}\). The pharmacological studies are not definitive because of potential artifacts and because cytosolic Ca\(^{2+}\) could not be measured.

Results that bring the damaging role of Ca\(^{2+}\) into question are the quite minimal effects of NMDA blockers in global ischemia and in some models of focal ischemia. These may be explained by the very transient nature of NMDA-mediated Ca\(^{2+}\) accumulation during global ischemia. In the focal ischemia cases, other sources of Ca\(^{2+}\) may overwhelm that from NMDA receptors, although there is no evidence.

Calcium-dependent processes seem to play major roles in cell death, providing strong evidence that Ca\(^{2+}\) is damaging. However, the results are not completely conclusive because there are other methods of activating these processes. These are less likely than Ca\(^{2+}\) activation but cannot be entirely dismissed.

Feasible studies that would help confirm or negate the role of Ca\(^{2+}\) include correlating measured cell Ca\(^{2+}\) levels with cell death in the face of different manipulations, demonstrating that NOS activation and calpain activation really result from increased cytosolic Ca\(^{2+}\) and, if possible, showing that preventing the rise in Ca\(^{2+}\) using intracellular buffers prevents cell death. All of these are very difficult. At present, the role of Ca\(^{2+}\) in in vivo cell death must be considered likely, but it remains to be demonstrated more convincingly.

b) Importance of Ca\(^{2+}\) in the Postischemic Period. The timing of Ca\(^{2+}\)-dependent damage is somewhat unexpected and important. Drugs that inhibit Ca\(^{2+}\) entry are protective in the postischemic period in both global and focal ischemia indicating that elevated Ca\(^{2+}\) in this period is key to production of damage. This is consistent with the efficacies of delayed application of calpain and NOS blockers. These findings do not mean that Ca\(^{2+}\) changes during ischemia are unimportant. They may well set the stage for the later alterations in Ca\(^{2+}\) metabolism. Also, they are certainly important during permanent focal ischemia because there is no reperfusion. If postischemic Ca\(^{2+}\) metabolism is indeed a key to damage, then determining why it is elevated becomes a very important goal. Little is known of this at present.

B. Zinc

It has been known for several years that elevated levels of extracellular zinc, leading to elevated intracellular levels, are toxic to cultured neurons and other cell types (summarized in Refs. 195, 1138). Furthermore, during kainate-induced seizures, there is an accumulation of zinc in CA3 cells that are destined for death (335). An important recent study took a significant step in linking these earlier results to ischemic cell death by implying that accumulation of zinc in vulnerable cells is an important component of damage (595). There was a measurable movement of zinc into vulnerable neurons within 30 min after 10-min global ischemia in rat and a clear accumulation at 24 h. Zinc was present in all degenerating neurons after 2 or more days. When looked at in detail, there was a very strong overlap between zinc-containing neurons (measured by fluorescence of TSOQ and eosinophilia, denoting ICC. Most importantly, it was shown that CaEDTA, which is able to chelate zinc (but not Ca\(^{2+}\)), reduced zinc accumulation by the neurons and protected against ischemic damage. Protection was significant after 3 days of ischemia, but less substantial after 14 days, indicating that the protection may not be permanent; zinc may be involved in increasing the rate of cell death by protease activation, while not affecting the final outcome. Another study demonstrated the same selective accumulation of zinc, following 5-min ischemia in the gerbil, using another fluorescent indicator, zincin (1138). In this case, there was an upregulation of the mRNA for the plasma membrane zinc transporter protein, involved in zinc extrusion (867), which is a general response to zinc overload (1138), but no apparent synthesis of the protein. Thus, similar to the expression of several other putatively protective proteins discussed throughout the text, and particularly in section VIII, CA1 shows mRNA expression but no protein expression. This inability to express protective proteins seems to be a lethal pattern that besets the CA1 pyramidal cells. Overexpression of this protein protects cultured cells from zinc neurotoxicity (1138).

The way zinc might cause, or help cause, neurotoxicity is not yet clear (195). It may activate proteases, many of which are zinc dependent (1194).

It is somewhat surprising that CA1 cells, and not CA3 cells, accumulate zinc after ischemia. The zinc is thought to be released presynaptically, and indeed, this has been demonstrated after epilepsy (335). Mossy fiber terminals have far more zinc in them than CA3 pyramidal cell terminals, and indeed, it is this zinc that is notably released in epilepsy, with accumulation in CA3 cells and (possibly) resulting damage (195, 335). Presumably, release of zinc during ischemia is different from its release during neural firing, and so is restricted to CA3 cell terminals in hippocampus. This provides a very interesting and unexpected insight into selective vulnerability. It is possible that, rather than this property residing solely in the postsynaptic cell (which is actually damaged), it resides to some extent in the presynaptic terminal’s ability to release zinc.

C. Glutamate

Because of the likely importance of both Ca\(^{2+}\) and Na\(^{+}\) and protein kinases in cell death, and its own large release during ischemia, the role of glutamate in ischemic cell death has been intensively studied. The work began...
with the seminal finding by Meldrum and co-workers in 1984 (1052) that NMDA blockade attenuated the damage following vessel occlusion in the rat. The question addressed in this section is the role that glutamate binding to its receptors plays in ischemic cell death.

1. Release of glutamate

Normal levels of extracellular glutamate measured by microdialysis are 1–5 μM (1183) and could themselves activate NMDA receptors during the massive anoxic depolarization that accompanies ischemia (1074). However, there is a substantial increase of extracellular glutamate during ischemia in all systems, and this is likely to contribute strongly to any glutamate-mediated damage.

A) Release of glutamate in vivo. As first shown by Benveniste and co-workers using microdialysis (84), there is a severalfold increase in extracellular glutamate during global ischemia, beginning within 1–2 min (84, 766). Glutamate rises to between 16 and 30 μM by 10–15 min (51, 84, 766).

There is a similar rise during focal ischemia, beginning within 2 min of MCA occlusion (1183). Because of the microdialysis technique itself and different probe placements, there is a lot of variation between studies. However, except for one case where levels were extraordinarily high, levels in the striatal core rise to between 50 and 90 μM and in the cortical core and/or penumbra rise to between 30 and 50 μM; levels in the peri-infarct region rise to only ~6 μM (52, 388, 778, 1028, 1183). Distinguishing between core and penumbral regions is very difficult in these microprobe studies and leads to some variability. During prolonged focal ischemia, the high extracellular glutamate levels are maintained in the focus for many hours; however, penumbral and peri-infarct glutamate fall back to baseline after ~1 h (389, 778).

B) Posts ischemic glutamate. After transient global ischemia, glutamate recovers to baseline in 5–20 min; thus there is excess glutamate during the early phase of oxygen reperfusion (51, 84, 772). This is important because glutamate itself is far more toxic in the presence of oxygen than in anoxia (277). There are scattered reports of delayed increases in glutamate, which might be important. There was a twofold elevation of glutamate some 3 h after a global insult (23), and there was a threefold increase in extracellular glutamate in the penumbra after 2-h focal ischemia, which remained for several hours (727). The latter is consistent with the ability of NMDA blockers to protect against focal ischemia when added after the insult.

2. Mechanism of glutamate release during ischemia

There is some conflict over whether glutamate release is via standard exocytosis, cytosolic Ca²⁺-dependent spontaneous vesicular release, or via reversed operation of the high-affinity Na⁺-dependent glutamate uptake system. There is strong evidence for both spontaneous and transporter-mediated release in brain slices, and some evidence for classical exocytosis in vivo, although predominantly during focal ischemia. The other two mechanisms have not been studied in vivo.

When the mechanism is considered, it is relevant that a wide variety of neurotransmitters show the same magnitude and kinetics of extracellular increase during in vivo ischemia, including ACh, which has no transmembrane transport system (40, 51, 621).

A) In vivo. The microdialysate in which glutamate was collected during global ischemia in gerbils was altered to contain either zero Ca²⁺ or zero Ca²⁺-Co²⁺. Removing the Ca²⁺ alone did not inhibit glutamate release (486), suggesting release was not via exocytosis. However, removing Ca²⁺ and adding 10 mM Co²⁺ did delay the glutamate release by ~5 min, and attenuated the later release (274, 536). One explanation for these results is that there is exocytotic release in global ischemia and the zero Ca²⁺ dialysate did not effectively remove extracellular Ca²⁺. The other explanation is that the absence of inhibition by zero Ca²⁺ shows that there is no exocytotic release; Co²⁺ could have blocked release by its ability to delay anoxic depolarization for several minutes (188). There is quite strong indirect evidence for exocytosis or spontaneous vesicle release in that the accumulation of zinc in pyramidal cells shortly after 10-min global ischemia probably arises from vesicles in the presynaptic terminals synapsing near the pyramidal cells (595).

Direct evidence for an early exocytotic release is much stronger for focal ischemia, where removal of Ca²⁺ from the dialysate did slow down the earliest (3–6 min) release of glutamate by 50%, while having no effect on final release levels (1183). Also, the N-type Ca²⁺ channel blocker SNX-111 (1103) reduced maximal glutamate release during focal ischemia by ~50%. The occurrence of exocytosis during focal ischemia, but not global, would be consistent with the much smaller reduction in ATP during focal ischemia (Table 1), since exocytosis requires ATP (828). These studies did not differentiate between release in core and penumbra, and there is likely to be a major difference because there is no anoxic depolarization in the penumbra. There may be exocytotic release in the penumbra during the intras ischemic depolarizations. As discussed below, core release, but not penumbral, is greatly inhibited in nNOS knockout mice (1031), again indicating a major difference between the two regions.

B) In vitro. Accessibility allows more detailed studies of release in vitro. There is absolutely no inhibition of glutamate release from hippocampal slices during ischemia when extracellular Ca²⁺ is removed (685, 888, 1109, 1306), even when 1 mM EGTA is included in the medium (888). This eliminates the possibility that there is classical exocytosis. There is, though, good evidence for both spontaneous vesicular release and release via reversal of the Na⁺-glutamate transporter.
There is an increase in miniature excitatory postsynaptic potential frequency during the first 5 min of anoxia. It is blocked by dantrolene, suggesting it is activated by Ca\(^{2+}\) exiting from the ER via CICR (538). It is also blocked by PLA\(_2\) and cyclooxygenase inhibitors (539). Glutamate release during 2-VO in vivo is also blocked by PLA\(_2\) inhibition (856, 905), indicating there may be spontaneous vesicular release in vivo. However, this is not certain because PLA\(_2\) also blocks glutamate uptake in hippocampal tissue (267). Thus inhibiting the enzyme could reduce glutamate release by enhancing its uptake.

Although outward movement on the Na\(^+\)-glutamate transporter is widely discussed as a major mechanism for ischemic glutamate release (827, 1096), there is only one reported experiment that addresses the issue. The release of glutamate from hippocampal slices between 5 and 15 min after the onset of ischemia was inhibited 75\% by preloading the slices (688) with two competitive inhibitors of the high-affinity glutamate transporter, \(t\)-PDC and threo-hydroxy aspartate (957). Because inhibition is competitive, and because the competitive inhibitors only accumulate in the cell to about the same concentration as the glutamate, this is undoubtedly an underestimate of the total transporter-mediated release. Thus, at least in slices, reversal of the transporter does represent a major efflux pathway for glutamate.

C) SOURCES OF GLUTAMATE RELEASE. Cutting afferent axons in vivo strongly attenuates ischemic glutamate release in CA1 of hippocampus, indicating that the release is very probably from presynaptic rather than postsynaptic neuronal elements or glia (86, 764). In slices, the carrier-mediated ischemic glutamate release was not affected by dihydrokainate (957), the transport inhibitor that is specific for the GLT-1 isoform of the transporter (39). Because this isoform is almost exclusively on glia, the result strongly supports the conclusion that release is from neurons and not glia (957). Cultured glial cells do release glutamate during in vitro ischemia (688), but changes in transporters may occur in culture.

D) SUMMARY. There appears to be a strong exocytotic component to release during focal ischemia, possibly because ATP levels do not fall terribly low, although this may well be limited to the core of the lesion. The situation is less well resolved for global ischemia where evidence for exocytosis is not as strong. Neither reversed transport on the Na\(^+\)-glutamate carrier nor spontaneous vesicular release has been tested in vivo, although there is strong evidence that both occur in hippocampal slices. Current evidence ascribes the major source of glutamate to presynaptic terminals.

3. Modulators of ischemic glutamate release

A) INWARD Na\(^{+}\) FLUX. There is a large and early accumulation of Na\(^{+}\) in brain cells during anoxia in slices (338) and during ischemia in vivo (417). Blockade of this influx by Na\(^+\) channel blockers in slices (148, 1109) and in vivo (245, 343, 388) decreases glutamate release by 50–75\%. This is consistent with release by exocytosis or by reversal of the Na\(^+\)-glutamate transporter. Exocytosis would be blocked because depolarization is attenuated while reversal of the transporter would be blocked because intracellular Na\(^+\) rises less.

B) FREE RADICALS. Free radical scavengers, mannitol and SOD, reduced the ischemic release of glutamate from hippocampal slices by \(-60\%\) (888) and, in another study, SOD almost completely blocked the extracellular glutamate accumulation measured after 5-h hypoxia in cultures (168). In vivo, combined SOD and catalase inhibited glutamate (and GABA) release during 4-VO by 80\% (857). Although limited in number, these results suggest that free radicals play a major role in activating the net glutamate release during ischemia.

The basis for this is unknown. Most attention has focused on the strong inhibition by free radicals of glutamate uptake on the Na\(^{+}\)-glutamate transporter in glia (1177) and also on peroxynitrite inhibition of the three major cloned transporter species GLT1, GLAST1 and EAAC1 (1131). This has led to the suggestion that inhibition of uptake by free radicals accounts for the bulk of the net ischemic glutamate accumulation (1177). There is no direct evidence for this, but if it does, then the ischemic glutamate release in vivo cannot be via the reversed transporter because this too would be inhibited by the free radicals. Thus enhanced exocytosis or spontaneous release coupled with decreased uptake would be the mechanism of ischemic glutamate accumulation.

The other possible site of free radical action is enhanced glutamate release. Activation of spontaneous release by NO/peroxynitrite is not occurring in global ischemia because inhibition of NOS did not affect ischemic release of glutamate in that model (1279). However, in focal ischemia, NOS knockout mice release far less glutamate from the ischemic core than do normal mice (1031), implying that NO (and very possibly peroxynitrite) plays a major role in the ischemic release of glutamate. Although the effect may be via enhanced exocytosis, it may be by decreasing reuptake as discussed above.

C) TEMPERATURE. Ischemic glutamate release is remarkably sensitive to the temperature in focal and global ischemia and in cultures (52, 153, 373, 766, 885, 1308). It is much more sensitive than, for example, dopamine release (373). In both striatum and hippocampus, reductions of 2–4°C, between 37 and 33°C, reduced glutamate release from 4- to 10-fold. Even the smaller reduction bespeaks a huge Q\(10\), of \(-20\). The basis for this remarkable temperature dependency is not known, but it must be incorporated into any model of ischemic glutamate release. Oxygen-derived free radicals are much reduced during hypothermic ischemia, lending some support to the idea that free radical action is important (1300).

D) KINASE SYSTEMS. As discussed previously, inhibition
of tyrosine kinase with genistein almost completely blocked glutamate release during global ischemia (907). The mechanism may well be phosphorylation of synapsin I via the MEK-MAP kinase system, as the ischemic activation of release in cultures is prevented by a MEK inhibitor (924). Another possibility is that MAP kinase activates PLA2 and hence glutamate release (907).

4. Correlative evidence that extracellular glutamate is damaging during and after ischemia

A) Na+ channel blockade. Na+ channel blockers lidocaine, tetrodotoxin, BW-1003C87, BW-619C89, and lamotrigine all inhibit glutamate release and provide very strong protection against focal and global ischemic damage (245, 343, 388, 693, 939). In at least one study, temperature was rigorously controlled (693). The blockers also strongly attenuate damage to brain slices (338, 1195). These results do not necessarily implicate glutamate release in damage because there are many other effects of decreased Na+ entry that might be protective.

B) Ca2+ channel blockade. The specific N-channel Ca2+ blocker SNX-111 (conotoxin MVIIA) reduced glutamate release during focal ischemia by 50% and infarct volume after 4 h by ~65% (1103). Again no conclusion can be drawn; blockade of N channels may protect by preventing postsynaptic Ca2+ entry.

C) Reduced temperature. Both glutamate release and neuronal damage are dramatically reduced by lowering temperature 3–4°C during ischemia (52, 77, 92, 766). In a very nice study it was shown that there was a strong correlation between the reductions in glutamate release and damage to CA1 cells in gerbil hippocampus, when temperature was varied between 39 and 31°C (766). However, as with Na+, there are many other effects of temperature that could reduce damage, so this cannot be taken to show that glutamate release is important in damage.

5. Lesion studies showing that glutamate is damaging during ischemia

Lesions of afferent glutamatergic pathways protect against ischemic damage. In CA1, damage during global ischemia is reduced by lesioning the CA3-CA1 pathway (84), and also by obliterating the dentate granule cells, which attenuates activation of the CA3 cells (509).

Even more persuasive, and very unexpectedly, blocking activity in the deep prepyriform cortex (area tempesta) with a local injection of 6-nitro-7-sulfamobenzoxinoxaline-2,3-dione (NBQX) protects CA1 of the rat against 4-VO global ischemic damage by ~70% (558). The area tempesta is very important in seizure generation; during locally generated seizures, it strongly activates pathways to the dentate gyrus and CA3 cells of the hippocampus (557). This rather startling result received further support when the area tempesta was lesioned. This strongly protected against CA1 damage while also nearly eliminating the ischemic increase in extracellular glutamate in CA1 (557). The implication of these results is that glutamate release in CA1 contributes strongly to damage (although MK-801 does not protect against damage in this model). The way in which area tempesta activity during 4-VO would lead to the release of glutamate is not yet apparent. The simplest explanation is that seizurelike activity is generated in that region and spreads to CA3 neurons that release glutamate in CA1. Whether this can occur during 10 min of 4-VO is a puzzle; the EEG generally becomes silent within ~40 s of starting the ischemia (935). Thus how steady glutamate release would be affected is unknown. An initial burst of release might affect enzyme activities in CA1. Despite the difficulty in explaining them, the data are imposing and strongly suggest an involvement of glutamate in damage.

6. Involvement of NMDA receptors in damage

A) NMDA antagonists in global ischemia. The excitement following early studies that showed protective effects of NMDA blockers (838, 1052) was soon dissipated by conflicting results in both gerbil, where profound and prolonged temperature reduction by NMDA antagonists (136) was seen to be a major artifact, and in rat, where the early results of Meldrum and co-workers (1052) showing protection could not be repeated by others (135). These issues have now been largely resolved.

Variable results in gerbil, even with good temperature maintenance (135, 136, 363), have been explained by a careful study (454) in which it was shown that the ability of MK-801 to protect depends on the temperature that is being maintained. MK-801 was unable to protect when temperature during ischemia was maintained at 38.5°C, but it did protect when temperature was maintained at 36.5°C, although very high doses of MK-801 were necessary (10 mg/kg) and damage was still substantial. This result was supported by a very thorough study in which telemetry was used to monitor and regulate brain temperature for days after the insult. MK-801 (10 mg/kg) was clearly protective at 37°C (1282). The requirement for such high doses of MK-801 suggests that the effect may be on Na+ channels, so evidence for action of glutamate from these studies is weak (454).

Even without temperature control, MK-801 does not usually protect against 4-VO (5 or 15 min) or 2-VO in rats (99, 135, 819), although doses as high as 10 mg/kg, which were needed to protect in gerbil, have not been used. The same lack of protection after 10-min 2-VO was seen with a novel NMDA/glycine receptor antagonist, which significantly protected against focal ischemic lesions (1192).

The group led by Meldrum (1089), which has consistently reported moderate protective effects of competitive antagonists D-APV or D-APH, use a 2-VO model in which blood flow in hippocampus is only depressed to
20%. This is a much milder insult than usual global ischemic events, which probably explains the discrepancy. Protection has been shown in other studies also. Three episodes of 5-min ischemia in rat were quite well protected by MK-801, with 50% recovery of damaged neurons (629). This is consistent with the role of NMDA receptors in the early increase in cytosolic Ca²⁺ (1048), but unfortunately, temperature was only controlled for 30 min after the last insult. The glycine-site blocker 7-chlorokynureate, injected intraventricularly, provided reasonable (50%) protection against 20-min 2-VO, but core temperature was only regulated until 30 min after ischemia (1222).

Overall, the evidence that NMDA blockade is protective during global ischemia is not at all compelling. The protective effects that are seen involve very high levels of MK-801, or the use of halothane, or do not adequately control temperature. There is, though, an important caveat, that NMDA antagonists can be damaging.

b) Damaging Effects of NMDA Antagonists. MK-801 actually causes neuronal damage when administered during normoxia, at the doses that are used for protection (15, 299, 853). Although frank damage has not been seen in hippocampal CA1 (135), there may be minor damage that sensitizes tissues to ischemia. More compellingly, Buchan and co-workers have described actual damage enhancement by MK-801 during global ischemia in CA1, rather than protection (135, 647), indicating that damaging actions may indeed compromise the interpretation of results with this inhibitor.

This possibility was strongly reinforced in studies where NMDA blockade was combined with hypothermia (334, 1037). Hypothermia blocks glutamate release so that only minimal protective effects of NMDA blockade would be expected, because there is little glutamate to cause damage. Thus an NMDA blocker during hypothermic ischemia would better reveal any damaging effect of the blocker itself. Indeed, adding the competitive blocker CGS-19775 did actually reverse the protection offered by hypothermia during global ischemia (1037), showing quite clearly that the blockade does enhance damage by some mechanism.

These considerations mean that the absence of protection by NMDA blockers during ischemia in rats, and in gerbils, must be interpreted with caution. In particular, NMDA receptors may be involved in damage; the possibility cannot be excluded.

c) NMDA Antagonists in Focal Ischemia. MK-801 (141, 874, 1149), NMDA glycine-site antagonists (361), and competitive NMDA antagonists (144, 833, 991, 1102) all reduce infarct size, or the area of significant cell necrosis, caused by permanent (334) and transient (141, 833) focal ischemia. Antisense oligonucleotide to NMDA-R1, when injected at a level that reduced NMDA antagonist binding sites by ~40%, strongly protected against 24-h MCA occlusion in the rat. It reduced infarct size somewhat more than MK-801, by ~40% (1184). A similar protection was afforded in mice in which the NR2A was knocked out; there was no effect of the knockout on blood flow during ischemia (776). In this study, there was no protection against permanent focal ischemia, only against transient, 2-h ischemia. In all these cases, only the penumbra was protected. The core of the lesion was not protected even if the drugs were injected before the insult or, like the antisense or knockout, had exerted their effect before the insult. Although very careful temporal studies have not been done, NMDA antagonists are very effective when added as much as 2 h after the start of permanent occlusion (873), or 15 min before the end of 1-h or 90-min temporary occlusion (771, 833).

Focal ischemia does not produce nearly as large a temperature fall as global ischemia, and protection by NMDA antagonists is not due to temperature effects. When skull muscle temperature was maintained constant throughout 24 h, MK-801 reduced infarct size by almost 50% (334), and CGS-19755, the competitive blocker, reduced infarct size without affecting core temperature (1102).

In contrast to these protective effects, MK-801 was not able to reduce infarct size in spontaneously hypertensive rats (141, 970, 1234) and in a thrombotic stroke model (1253). The drug was able to protect against selective neuronal loss (peri-infarct region) in the latter case. The contradictory results are considered below.

d) Effects of NMDA Antagonists on Blood Flow During Focal Ischemia. The simplest interpretation of the protection by NMDA antagonists in focal ischemia is that they block NMDA receptor-mediated Ca²⁺ entry into the neurons and/or intraschismic depolarizations (see sect. uH). However, MK-801 and other noncompetitive NMDA blockers (891), as well as competitive NMDA blockers (1102), enhance blood flow in control conditions, and two studies demonstrated major increases in blood flow during ischemia, to the core (3-fold) (141) and to the penumbra/core (1102).

Although the above results suggest protection may be due to effects on blood flow, they certainly do not show it. In contrast, at least two studies show significant (30–50%) protection by MK-801 with no measurable changes in blood flow (253, 875, 1149), and one study shows similar protection when the NR2A receptor is knocked out, again with no change in blood flow (776). Furthermore, one careful study showed that MK-801 actually decreased blood flow to collaterals during vessel occlusion (954).

e) Postsischemic Involvement of NMDA Receptors. N-methyl-D-aspartate receptor antagonists are fully protective when added 15 min before the end of a transient focal ischemic insult, and they are also fully protective against permanent ischemia when the antagonist is added 2 h after the onset of ischemia. The protection during reperfusion is consistent with the secondary increase in extracellular glutamate at that time (727) and suggests that
significant damage occurs during this time. It tends to
dissociate protection from prevention of intraischemic
depolarizations because the latter do not occur after the
transient insult is over. The prolonged glutamate toxicity
during permanent ischemia is somewhat surprising as
glutamate levels in penumbra fall back to baseline after
\(\sim 1\) h (389, 778); however, residual glutamate plus com-
promised energy metabolism (356, 839) probably make the
system very sensitive. The delayed protection by
NMDA antagonists in both models of focal ischemia sug-
gests that preventing the action of cytosolic \(\text{Ca}^{2+}\) at this
time is effective. That is consistent with the efficacies
of delayed inhibition of calpain and NOS.

Most groups have found no protection by early post-
ischemic application of NMDA antagonists in global isch-
emia (327, 819, 1023) despite implications that the recep-
tor system is abnormally activated in that period (22, 457,
890). One study did show good protection when NMDA
antagonists were added 15 min and 5 h after 2-VO in rat
(167), but this was done by the group that has consistently
observed a protective effect of NMDA antagonists during
the ischemia and probably reflects the much milder, focal
ischemic-like insult. As discussed previously, it would be
interesting to test very delayed NMDA blockade (12–24 h)
in global ischemia as cell \(\text{Ca}^{2+}\) is elevated in this period.

F) SUMMARY. The effects of NMDA antagonists are
clearly difficult to interpret unambiguously. In global isch-
emia, the antagonists may actually be protective; the
observed lack of protection may result from antagonist-
induced damage masking a protective effect that would
otherwise result from blockade of \(\text{Ca}^{2+}\) entry. Although
this seems somewhat unlikely, it cannot be dismissed. At
the other pole, the protection by high levels of MK-801 in
gerbil may well reflect \(\text{Na}^{+}\) channel opening rather than
NMDA channel opening.

In focal ischemia, the lack of protection in the SHR
and thrombotic models has to be explained, as do the
conflicting data on the effects of antagonists on blood
flow.

A possible reconciliation of the first issue is that
NMDA blockade does indeed protect because it attenu-
ates \(\text{Ca}^{2+}\) entry and that it did not protect in SHR, and the
thrombotic model because the insult intensities were too
great (or, possibly that damaging effects of the antago-
nists were more significant in these models, Ref. 689).
N-methyl-D-aspartate blockade can fail to protect in vitro
systems when the insult intensity is very high (685).

It would be very useful to resolve the quite conflicting
data on blood flow changes. Some very good studies do
demonstrate protection by NMDA antagonists in the ab-
ence of blood flow changes, strongly suggesting that
NMDA damage in focal ischemia is via \(\text{Ca}^{2+}\) entry into
neurons. However, the discrepancies between blood flow
changes measured in different studies need to be satis-
factorily explained.

7. Effects of AMPA/kainate and metabotropic
glutamate receptors on ischemic damage

A) AMPA RECEPTOR ANTAGONISTS DURING ISCHEMIA. At least
five studies very clearly show strong protective effects of
AMPA antagonists during permanent focal ischemia, in
cat (145) and rat or mouse, using NBQX and also newer
water-soluble AMPA antagonists YM90K, YM872, and
ZK200775 (759, 776, 1032, 1255). Temperature was well
controlled throughout the early ischemia in most of the
studies in rat. No studies have been described in which
AMPA receptor blockers have been applied before global
ischemia, nor have effects on temporary focal ischemia
been described.

The protection in focal ischemia might result from
attenuation of the intraischemic depolarizations (47, 759),
or from blockade of \(\text{Na}^{+}\) entry itself. The latter is consis-
tent with effects in hippocampal slices where AMPA
blockade reduced the increase in cytosolic \(\text{Ca}^{2+}\) by pre-
venting \(\text{Na}^{+}/\text{Ca}^{2+}\) exchange at the mitochondrial mem-
brane (1289). The protection by AMPA blockade may also
result from temperature reduction; temperature was not
explicitly maintained throughout the ischemia and, as
discussed below, at least some AMPA blockers do lower
temperature in the postischemic period.

B) POSTISCHEMIC INVOLVEMENT OF AMPA/KAINATE RECEP-
TORS IN GLOBAL AND FOCAL ISCHEMIC DAMAGE. NBQX attenu-
ated damage when added at different times up to 12 h
after either 2-VO or 4-VO ischemia (140, 647, 819, 1176),
suggesting that delayed activation of AMPA receptors
was damaging. However, it has now been shown that
NBQX lowers core temperature and that this hypother-
mia quantitatively accounts for almost all the protec-
tion in gerbils, and very probably in rats (844). When
gerbils were treated three times with 30 mg/kg NBQX
between 50 and 90 min after 3-min ischemia, almost all
CA1 neurons were protected from the normally severe
neuronal loss (75%), measured 4 days later. However,
the drug caused a 1.5°C drop in temperature, relative to
control, that persisted at least 2 days. When the tem-
perature drop was prevented for 24 h, the drug pro-
vided no protection. Furthermore, maintaining the tem-
perature 1–1.5°C below normal for 24 h gave about the
same protection as afforded by NBQX (the latter main-
tained the lower temperature for 1–2 days longer). This
study is very persuasive, and it is impossible to con-
clude, at this time, that posts ischemic activation of
AMPA receptors is involved in promulgating the
damage.

The water-soluble quinoxalinedione ZK-200775 atten-
uated the infarct size by 45% when added 2 h after 90-min
temporary focal ischemia (1144) so that focal ischemic
damage is also able to be rescued by posts ischemic block-
ade of AMPA receptors. In this case, temperature was not
well monitored, so it is impossible to know whether the
They may be quite important.

Another mechanism, for very delayed damaging effects of AMPA/kainate receptors, soon before delayed death, is suggested by recent studies from Zukin and colleagues (384). They showed that the ratio of GluR2 to GluR1 receptor mRNA was selectively decreased in the CA1 pyramidal cell layer beginning 24 h after 5-min global ischemia in the gerbil and that this became exaggerated in the next 2 days. There were no ratio changes in CA3 and DG. If followed by the same change in receptor protein (which appears to be the case; R. S. Zukin, Princeton Conference, 1998), this receptor change would increase Ca\(^{2+}\) permeability of the AMPA receptors. There is explicit evidence for the latter as the cytosolic Ca\(^{2+}\) increase elicited by AMPA (in the presence of cyclothiazide, which prevents inactivation) is far greater in CA1 neurons 3 days after ischemia than it is in control CA1 neurons or even those taken 48 h after ischemia (384). It remains to be shown that these currents are actually damaging. If they are, this might help explain the delayed death; it might also account for the increased cell Ca\(^{2+}\) measured 24 h after global ischemia (285, 1272).

c) METABOTROPIC RECEPTORS. There are no reported studies of the actions of metabotropic glutamate antagonists in vivo. The combined group I/II agonist t-ACPD is protective in focal ischemia (193) as is the II/III agonist (S)-4C3HPG in global ischemia (442), suggesting that the net effect of metabotropic activation will be protective via group II type receptors. However, this is difficult to assess as prior addition of metabotropic agonists causes long-term changes that are probably not produced immediately upon glutamate release. Antagonists are better suited to this issue.

8. Other transmitters

Several other neurotransmitters including monoamines acetylcholine and GABA show quite massive extracellular increases during ischemia, with similar time courses to that of glutamate (51, 497, 621). Inhibition of GABA reuptake greatly improves recovery from 5-min global ischemia in gerbil, indicating a protective effect of this transmitter. However, enhanced GABA activity is strongly hypothermic (1010), and the protective effect seen during ischemia can be completely explained by hypothermia induced by the uptake block (497). There are reports of damaging effects of some transmitters, particularly serotonin acting at 5-HT\(_2\) receptors (374) and dopamine acting at D\(_2\) receptors (433). Because little at present is known about the possible mechanisms of these effects, they will not be considered further in this review. The multiple receptor types for these transmitters means that much work is required to sort out their true effects during ischemia. They may be quite important.

9. Relationship of glutamate involvement in ischemia to excitotoxicity: mechanisms of glutamate damage in ischemia

a) GLUTAMATE LEVELS. The previous discussion establishes that glutamate is likely to play roles in focal damage and may well play a role in global ischemic damage. The question is the extent to which the action of glutamate in ischemia mimics its action as an excitotoxic agent. That is, is the role of ischemia simply to cause release of glutamate, which then causes excitotoxic cell damage?

Concentrations of glutamate reached during global ischemia and in the penumbra of a focal lesion are \(~30\) \(\mu\)M. These are of the order that will cause excitotoxic damage in cultures during short exposures, equivalent to global ischemia, when energy metabolism is compromised (356, 839). They also cause damage in cultures during longer exposures when energy metabolism is normal (395), as in the penumbra of focal lesions.

b) GLOBAL ISCHEMIA. While conditions in the focal penumbra are compatible with the occurrence of excitotoxicity, conditions during global ischemia are not. In cultures, short exposures to glutamate are only toxic in the presence of oxygen (277); this is probably for at least two reasons. Glutamate toxicity depends on free radical (282, 631, 883) and NO (236, 237, 1074) formation, and it also appears to depend on mitochondrial uptake of entering Ca\(^{2+}\) and resulting mitochondrial damage (26, 142, 994, 1206). The former two require oxygen, and the latter requires very active oxidative metabolism. Thus, although glutamate may well contribute to global ischemia damage, as evidenced by the studies in which area tempesta in the endopiriform cortex was lesioned, it probably does not do so by a purely excitotoxic mechanism. Either it contributes to damage because it enhances Na\(^+\) or Ca\(^{2+}\) entry or it acts via metabotropic receptors in some way.

c) FOCAL ISCHEMIA. In the focal ischemic penumbra, oxygen levels are quite high, both during and certainly after the insult, as are ATP levels (Table 1). Glutamate is at levels that cause excitotoxic damage in cultures; unfortunately, there are no measurements of excitotoxic levels in vivo. Thus conditions are appropriate for glutamate excitotoxicity. Furthermore, ischemic damage and excitotoxic damage share many characteristics. Glutamate will cause both apoptosis and necrosis (919), as is seen after focal ischemia. Also, excitotoxic damage in cultures has very similar dependencies to those of focal ischemic damage, including the time required for damage development (12–24 h). It is dependent on NO formation (236, 237, 1074), on free radical formation (282, 631, 883), on calpain activation in most (125, 126, 158) but not all (709) cell types, on calcineurin as defined by dependence on both cyclosporin and FK-506 (26), and on PARP activation (298). Thus it seems quite likely that very similar mechanisms are followed in the focal ischemic penumbra and in excitotoxicity. If so, then mitochondrial damage...
may be important in focal ischemic damage, as discussed in section ivB.

This being said, there are still major differences between glutamate toxicity and ischemic damage that necessitate great caution in moving from one to the other. In particular, PARP activation does not seem to be involved in damage following permanent focal ischemia; this differs from glutamate toxicity. Furthermore, focal ischemia is a more complex insult than excitotoxicity in cultures. Not only is there a primary inhibition of oxidative phosphorylation, but there is induction of important enzymes and cytokines in focal ischemia (see sect. viii), and there is also the involvement of the vasculature and, in particular, leukocyte accumulation. Blocking cytokine production and blocking leukocyte production both greatly alleviate focal damage, the latter only in temporary ischemia.

In permanent focal ischemia, MK-801 reduces the production of the cytokine TNF-\(\alpha\) by 60%. Both MK-801 and treatments that independently neutralize TNF-\(\alpha\) action reduce infarct size by \(\sim 50\%\) (90, 636). This activation of cytokine production may be a major basis for the NMDA-mediated damage in permanent focal ischemia. This mechanism has not yet been tested in temporary ischemia; it is almost undoubtedly not operative in excitotoxicity.

A very important question that emerges from these considerations is why focal damage is so dependent on factors such as white blood cells and cytokines when glutamate toxicity occurs in cultures without these. Possibly glutamate levels themselves are not elevated enough, or for long enough in the penumbra, to cause toxicity in vivo. As mentioned above, there are no existing measures of the concentrations of glutamate that are lethally excitotoxic in vivo.

### 10. Conclusions

#### A) GLUTAMATE INVOLVEMENT IN GLOBAL ISCHEMIC DAMAGE.

Blocking glutamate release in CA1 by inactivating the area tempesta of the endopyriform cortex is strongly protective against 4-VO. If those results stand up, and no artefactual explanations are found, they are extremely strong evidence that glutamate release is a major effector of cell damage in global ischemia. This creates a quite paradoxical situation, because pharmacological evidence for the involvement of glutamate in global ischemic damage is very weak. As discussed, there are no studies on metabotropic or AMPA/kainate receptors, and NMDA antagonism gives no protection in the rat and very little or none in the gerbil.

There seem to be four reasonable resolutions of this paradox. The first is that good pharmacological studies will reveal that AMPA/kainate or metabotropic receptors do contribute to cell death. At least the former certainly do contribute during intense insults in vitro, and they do increase cytosolic Ca\(^{2+}\) by releasing the ion from mitochondria during ischemia in slices. The second possibility is that the absence of protection by NMDA antagonists, principally MK-801, is misleading because the antagonists also cause damage, which offsets their protective effects. This was discussed at length, and there is reasonable evidence that it is so. Indeed, the fact that NMDA antagonists are completely useless above 37°C may be because they cause greater damage at higher temperatures. The third possible explanation is that, because the intensity of the insult is great, combined NMDA, AMPA (and perhaps metabotropic) blockade is necessary to see any marked reduction in damage. A final, and important, possibility is that the effects of AT lesions on glutamate release are not the basis for their protective effects so that glutamate is, in fact, not important in damage. Resolution of this important issue awaits further studies.

A delayed role for glutamate is suggested by the findings that AMPA receptors become far more Ca\(^{2+}\) permeant in vulnerable regions between 1 and 3 days after global ischemia (384). It remains to be shown that this contributes to damage. In the limit, it is possible that this is actually the key toxic event in global ischemia and that preceding events are damaging because they eventually cause this change in AMPA receptors, which then leads to classical excitotoxicity. There are no pertinent studies in focal ischemia.

#### B) GLUTAMATE INVOLVEMENT IN FOCAL ISCHEMIC DAMAGE.

Both NMDA receptor antagonists and AMPA receptor antagonists strongly attenuate damage in the penumbra in permanent and temporary ischemia in most, but not all, cases. The weight of evidence indicates that NMDA protection is independent of actions on blood flow, and it is certainly independent of effects on temperature. These possible artifactual effects of AMPA antagonists have not been as well controlled or tested, so there is more ambiguity in the conclusion that AMPA receptors contribute to damage.

Glutamate-mediated damage may result from the intraschismic depolarizations, which activate damaging ion fluxes through voltage-dependent and receptor-mediated channels, as has been argued by Hossmann (463). Alternatively, damage may be independent of the depolarizations and due to receptor effects on Ca\(^{2+}\) and Na\(^{+}\) fluxes. It is likely that both mechanisms are operative and that the depolarizations enhance receptor-mediated influx. The fact that NMDA antagonists are effective when added near the end of temporary focal ischemia, after which there are no intraschismic depolarizations, shows that major glutamate damage continues without them. Ion-mediated mechanisms of glutamate action very probably resemble those in classical excitotoxicity because conditions are similar, but the importance of NMDA-mediated TNF-\(\alpha\) activation shows that there are additional effects in ischemia.

The core of the infarct is immune to protection by glutamate antagonists (although a combined treatment...
has not been tested), which highlights the role that glutamate plays in cerebral ischemic damage. All tissues suffer ischemic damage, so ischemic brain cell death would certainly occur in the absence of glutamate. Glutamate appears to sensitize brain tissue to ischemia, making short exposures (global ischemia), or prolonged but only mildly hypoxic exposures (penumbra of focal ischemia) lethal. The core of the focal lesion is presumably so metabolically compromised for so long in all the paradigms that are used that damage occurs without glutamate.

D. pH

As illustrated in Table 3, intracellular pH (pHi) falls by 0.5–1 unit during cerebral ischemia. This fall is very rapid, and the question is the extent to which this contributes to ischemic damage (1042, 1045, 1120). Unlike glutamate, these pH changes alone do not damage neurons, as discussed below, so that the question becomes whether they exacerbate the damage.

Although the weight of evidence suggests that the pHi fall is damaging, the issue is not completely resolved. One difficulty is the absence of drugs that alter the pH change during ischemia. Thus its role can only be tested by metabolic manipulations that also alter other variables. Another difficulty is that pHi and extracellular pH (pHo) are very closely linked (748, 815, 1057) so that manipulations generally affect both these variables very similarly. Because the two pH changes may have opposite effects (see below), interpretations have to be made cautiously.

I. pH fall during ischemia

A) GLOBAL ISCHEMIA. The extracellular space begins to go acid within 20 s of beginning anoxia or global ischemia (845, 1057), well before the anoxic depolarization (717, 845). Intracellular pH follows a similar time course, beginning to fall within ~1 min and reaching a minimal value after ~2 min (297, 419, 1086).

The magnitude of the fall in pHo during global ischemia is ~1 unit. As seen in Table 3, the average pHo in rat brains falls from ~7.0 to 6.4, although in the one study of gerbils it fell further. Tissue lactate rises from ~4 mM (793) to between 8 and 15 mM (550, 551, 793).

B) FOCAL ISCHEMIA. At the core, where blood flow was reduced to 25% at 10 min and to 10% by 3 and 4 h, pHo fell to 6.6, 6.2, and 6.1 at those times. In the penumbra, where flow was reduced to 40%, there was no significant pHo drop at 3 h and a slight drop (to pH 6.75) at 4 h (755, 943).

C) CELLULAR LOCUS OF PHi CHANGES. This is an important issue that has not been well resolved since few of the studies measure changes in different compartments. In the limit, all pH changes could be in glia, which would greatly affect interpretations. Several groups have noted multiple pH profiles during ischemia using 31P-NMR (108, 326, 1086), but relevant compartments have not been able to be determined. In one study, where the pHi fall was measured in neurons with microelectrodes (1048), pHi fell to 6.3 in both CA1 (vulnerable) and CA3 (ischemia-resistant) cells. This is similar to pHi falls measured in whole tissue (Table 3), suggesting strongly that the latter do reflect neuronal changes.

2. Postischemic recovery of pH

Because acidity exacerbates free radical damage, the pH profile during reperfusion is a very important parameter. Unfortunately, there is a lot of variability in reported recovery times, possibly because little attention has been paid to this quantity. In most cases, 50 and 95% recovery times for pH range between 2 and 8 min and 5 and 30 min, respectively (419, 633, 1057, 1086, 1128, 1210), but there are several cases where measured recovery is more prolonged (297, 1048). In one case, pHi remained 0.3 units below normal for several hours (1128), and in another, when localized pHi was measured with umbelliferone after 5-min ischemia in gerbil, there was a rapid recovery to pH 6.8, but a rather slow (2 h) recovery from 6.8 to 7.1 that was peculiar to CA1, the most vulnerable region (784). Whether these are functionally important is not known.

3. pH changes in brain slices and cell cultures

Resting pHi is high, ~7.4, in hippocampal and cortical slices when they are in bicarbonate buffer (124, 531, 634). When extracellular glucose is 10 mM, pHi falls to between 6.7 and 6.5 at the end of 10-min anoxia (531, 913). No studies have been reported for other glucose concentra-
Mechanism of fall in pH

The early decrease in pH (553) probably results from the immediate blockade of oxidative phosphorylation and continued flux through the glycolytic pathway (358). The mechanism for the maintained decrease in pH has not been studied fully but has generally been assumed to be the activation of glycolysis stemming from the lowered ATP. Certainly this is an important determinant; the pH fall usually correlates with increased lactate concentration as blood glucose is increased (550). However, there are indications that the regulation is more complex (1045). Although there is controversy (550), several groups have reported that moderate hyperglycemia has little or no effect on the pH change during ischemia, even though lactate production is increased about twofold (214, 465, 610). This suggests a homeostatic mechanism is in play. A second consideration is that the inhibitor of NOS, NG-nitro-L-arginine methyl ester, prevented the fall in pH in the penumbra of a focal ischemia lesion at a concentration that did not affect cortical blood flow, or mitochondrial redox state (943). There is no good explanation for this quite surprising but strong effect, although it may well be relevant that free radicals lead to decreased pH in cardiac tissue (1225). These anomalies in pH regulation suggest that transporter activities, or unknown parameters, are altered by the ischemic insults and modulate the pH change.

Role of pH fall during ischemia

The major strategy to get at this critical question has been to vary the pH change during ischemia by manipulating the blood glucose levels. The simple idea is that hyperglycemia will attenuate the decrease in pH, whereas hyperglycemia will enhance it so that if the normal pH decrease is contributing to damage, then hypoglycemia should be protective. Hyperglycemia might enhance damage if the normal fall in pH does not saturate the system. Showing the protective effects of hypoglycemia is the most critical to the hypothesis that the normal fall in pH is damaging; unfortunately, these results are the least conclusive.

Another strategy, which has only been used to date in focal ischemia, is to test the effects of buffering the normal decrease in pH. These approaches will be described in the following sections.

Buffering pH change during focal ischemia. An alkaline trisaminomethane buffer was infused into the ventricles during focal ischemia in cats. The concentration was adjusted to almost completely prevent the extracellular acidification during the insult. The treatment reduced the infarct volume by 40% (796). Although only pH, was explicitly maintained, and pH was not measured, the buffer readily crosses the cell membrane (796) so very probably buffered pH also. The simple conclusion from this is that the reduction in pH during focal ischemia makes a major contribution to development of damage. This needs to be repeated by other groups and examined further. It represents, in principle, a very useful approach. A caveat is that the buffer may have been acting elsewhere (for example on NMDA receptors or free radicals), although there is no evidence that this is so.

Effects of hypoglycemia. Hypoglycemia has been induced either by fasting or by insulin. The results with insulin, which attenuated the pH fall from 0.7 to 0.3 units (550), are too complex to interpret because of effects of insulin itself (346, 643, 1076, 1175).

Forty-eight hours of fasting decreased blood glucose by 40% and decreased lactate production during 4-VO by 50%, suggesting a smaller fall in pH (711). Twenty-four hours of fasting reduced blood glucose by only 20% but sharply reduced the increase normally seen during and at the end of ischemia (260). The pH nadir during 16-min ischemia in cats was 6.3 rather than 6.0 (198).

These fasting regimens did reduce the neuronal damage following global and hypoxic/ischemic insults (260, 375, 711). Food-deprived animals were very strongly protected against 30-min 4-VO, a fairly profound insult (711), and were also almost completely protected against early infarct after 25-min hypoxia/ischemia, when compared with controls which suffered major damage (375). These protections correlated with the attenuation of the pH fall, but protection may have been due to other effects of starvation. [The low insulin caused by starvation would, if anything, exacerbate damage, so it is not a complication (1174).] Attempts were made to distinguish possible protective effects from the effects of hypoglycemia at the time of the insult by injecting glucose just before the insult (375). Unfortunately, the results were ambiguous. However, it was shown that the protective effects of hypoglycemia were not due to ketosis (375), nor to reduced release of glutamate. Fasted rats showed much less severe striatal infarction and penumbral damage 24 h after 15-min hypoxia/ischemia, despite actually releasing more glutamate during ischemia (260). As expected, ATP levels fell more rapidly in hypoglycemic conditions (550), and protection occurred in spite of this. These results are consistent with the fall in pH being damaging, but other possible effects of the starvation have not yet been satisfactorily excluded.

Effects of hyperglycemia. Since the early serendipitous findings of Myers and Yamaguchi (795), and follow-up work by that group (792–794), a large number of studies have very clearly established that moderate hyperglycemia, in which glucose levels are elevated to 16–20 mM, greatly enhances damage after global ischemia (255, 522, 552, 651, 932) and both permanent and temporary focal ischemia (221, 240, 1178, 1256). Individual cellular necrosis (255, 649, 650, 932) as well as infarct develop-
opment (522, 794, 932) are both enhanced. The most straightforward and consistent interpretation is that the increased damage results from a larger than normal decrease in pH.

Supporting this conclusion, profound hypercapnia during normoglycemic global ischemia, with CO₂ levels chosen so that pHᵢ fell to about the same level as during the hyperglycemic ischemia, increased cell damage to about the same degree as hyperglycemic ischemia (552).

D) Relationship between degree of pH reduction and extent of damage during hyperglycemic ischemia: Is there a threshold for damage? This is a tricky but very important question because if there is a threshold, it argues that the pH fall in normoglycemic conditions is not enhancing damage, contradicting the inference from the hypoglycemic studies. Results from several groups agree that elevating blood glucose to 20 mM or above enhances damage at the same time as it accentuates the rise in lactate and the fall in pH (609, 793, 1057). In general, the rapid falls in pHᵢ during ischemia in normoglycemic conditions are enhanced by 0.3–0.6 units at 20 mM glucose. Intracellular pH falls to between 5.9 and 6.2 (633, 1057, 1210). Larger increases in glucose produce larger average decreases in pHᵢ during ischemia (1210); microelectrodes identified a profound decrease, to 5.3, in astrocytes, when glucose was high enough (50 mM) to lower pHᵢ to below 6.2. Neuronal pH fell to ~6.0 (607).

The increased damage due to 20 mM glucose is thus associated with pHᵢ decreases that are 0.3–0.6 units greater than normal. If the normal drop in pHᵢ is causing damage, as suggested by the fasting studies, then smaller increments in this drop, produced by smaller increments in blood glucose, should also increase damage. However, Siesjo and co-workers (651) found that damage was only measurably enhanced when blood glucose was elevated to ~15 mM (651); pHᵢ at this point fell ~0.3 units more than normal (550). If pHᵢ during ischemia is a continuous function of blood glucose, these results indicate that there is a pHᵢ threshold for damage and thus that the normal decrease in pHᵢ is not enough to be enhancing damage, opposing the conclusions from the studies of hypoglycemia.

Indirect measurements of pHᵢ by Siesjo and co-workers (550, 1057) suggested pHᵢ was indeed a continuous function of glucose and thus supported the threshold concept. However, other studies suggested that pHᵢ is a discontinuous function of glucose (and lactate) levels. Kraig et al. (610) noted a nonlinear relationship in which pHᵢ fell to 6.8 when blood glucose was anywhere below ~15 mM, and fell to 6.1 when blood glucose rose to 18 mM or greater (610), about the level at which damage begins to be manifested. Furthermore, NMR studies of pHᵢ in rat (465) and gerbil (214) showed that glucose levels had to be elevated to 15–20 mM before pHᵢ fell more than normal. These quite direct studies are persuasive and are consistent with the conclusion that there is not a threshold pHᵢ for damage and thus that damage is a continuous function of the pHᵢ fall during ischemia.

E) Other explanations for enhanced hyperglycemic damage. In principle, there are several ways by which hyperglycemia might be damaging other than exaggerating the fall in neuronal pH. These need to be considered.

1) Seizures. There are almost always seizures following hyperglycemic insults and they could, in principle, be the reason that damage is exacerbated. Although cell death has been anecdotally dissociated from the occurrence of seizures (651), the glucose threshold for inducing seizure activity after global ischemia (12–16 mM) is about the same as the glucose threshold for significant aggravation of necrotic cell damage (650). The strongest experimental argument against this explanation for the enhanced damage is that the damage enhancement caused by elevated CO₂ was not associated with an increase in seizure activity (552). To the extent that effect of hypercapnia represents the effect of lowered pH, as it very probably does, these studies show convincingly that seizures do not account for the enhancement of damage by reduced pH.

2) Effects on vasculature and infarct formation. Damaging effects of hyperglycemia, and lowered pH, may, in principle, be mediated by effects on the vasculature or on glia. This remains a possibility particularly because no in vitro models have been found in which hyperglycemia enhances damage. In brain slices, increased glucose in the bathing medium always improves recovery of synaptic transmission (60, 532, 686, 1119), probably because it maintains ATP levels and retards anoxic depolarization. Although there are no data directly demonstrating a vascular or glial site of damage, severe hyperglycemia does lead to infarct formation even after global insults (522). This suggests that processes may be occurring that are qualitatively different from those occurring during normoglycemic ischemia. On the other hand, in a careful study of effects of hyperglycemic ischemia on small vessel patency, Siesjo and co-workers (648) found no effect of the hyperglycemia, eliminating the possibility that decreased pH caused postischemic obstruction. The broader vascular issue needs to be well resolved before it can be concluded that hyperglycemia is damaging by decreasing pH in neurons.

3) Other metabolic changes. Other metabolic changes caused by hyperglycemia and hypercapnia would seemingly protect against, rather than enhance, ischemic damage. Hypercapnia in vivo reduces net Ca²⁺ entry into cells during ischemia and delays anoxic depolarization (613). The ATP fall and anoxic depolarization are retarded by hyperglycemia; these slowings should be protective (1178). However, it has been argued by one group that it is precisely the attenuation of the ATP fall that makes hyperglycemia damaging (465), because it decreases formation of adenosine, which it very probably does. Adenosine is protective in several ischemic models (21, 904),
probably via action on the A1 receptor (904). Although unexpected, this idea cannot really be dismissed at this stage, as is discussed in section viA.

IV) Immediate effects of glucose infusion. Recent studies by Schurr and colleagues (A. Schurr, R. S. Payne, M. Tseng, and J. Miller, personal communication) cast a novel perspective on the effects of hyperglycemia. In a cardiac arrest global model, they find that although hyperglycemia greatly exacerbates cell damage when ischemia is induced within 15 min of the glucose injection, it actually reduces damage when the ischemia occurs 2 h after the glucose injection. Blood glucose levels are actually higher at the later time point (22–35 mM). If this holds up it is very important. It would indicate that damage effects of hyperglycemia may result from a rapid (hormonal?) response to the injection rather than from the elevated glucose levels.

f) Conclusion. The effects of hypoglycemia and hyperglycemia, as well as of hypercapnia and of pHo buffers, are consistent with the conclusion that the fall in pHi (or pHo) during ischemia is a significant determinant of the degree of cell death that follows global and focal ischemia. However, there are alternate explanations of the effects of all these agents, except perhaps elevated CO2. None of these alternate explanations negates the conclusion that the pH drop is important at this stage, but they show that the question is not yet resolved. All the manipulations used to show the importance of pHi very probably affect pHo as well as pHi, so that the former could be the damaging change. However, as seen below, extracellular acidity is likely to be protective so that the damaging change is very likely to be pHi.

6. Protective effects of decreased pHo in vitro

Although evidence that the pH fall is damaging in vivo is quite strong, artificially reducing pHi is protective against both glutamate and ischemia (518, 1118, 1119) in vitro. The basis for the protection is not completely known.

Reduced pHo attenuates NMDA-mediated Ca2+ entry (290, 1097, 1107, 1120). This is almost undoubtedly an extracellular action as it occurs in excised patches (1107). It would be very protective in cell culture where NMDA-mediated Ca2+ entry is the major source of damage. However, this cannot be the whole story as MK-801 combined with acidity is far more protective than MK-801 alone (518). Furthermore, low pH buffers are also protective against ischemia in nonneural tissues (109, 229, 1021), where there are no NMDA receptors. In liver cells, protective effects of extracellular acidosis are due to the resulting intracellular acidity (123) which, at pH 6.4, very strongly inhibits the protease activity induced by anoxia in isolated hepatocytes (123).

If these protective effects are operative in vivo, then normal damage is occurring despite them, and they must be outweighed by other, damaging, effects of decreased pH. This issue has been discussed from several points of view, in some cases maintaining that the fall in pH must be protective (1045, 1120). This is very difficult to accept based on the evidence discussed in the previous sections, and at this stage, it seems most likely that the net roles of pH in vitro and in vivo are quite different. This is readily explicable if in vitro effects are primarily on NMDA-mediated entry because the latter is more important in culture than in vivo. Indeed, it is particularly unimportant in global ischemia, where effects of pH are most profound. Another obvious possibility is that damaging effects in vivo result from effects on the vasculature, which would not be significant in vitro. This does not seem likely, as discussed above. Certainly, the protective effects of lowered pH in vitro indicate multiple effects of pH during ischemia. However, at this stage, the weight of the evidence is that lowered pH is damaging in vivo.

Unlike NMDA, AMPA/kainate receptor-mediated cell death is actually greatly accentuated by reducing the pH in cortical cultures from 7.4 to 6.6 (741). Furthermore, the AMPA/kainate component of death resulting in vitro ischemia is also enhanced by the lowered pH (741). To the extent that AMPA/kainate receptors mediate cell death in vivo, these studies provide further evidence that the decreased pH is likely to be damaging.

7. Possible mechanisms of pH-mediated damage

A) Effects of low pHo alone. The pH values reached during global and focal ischemia, even in the presence of 20 mM glucose, are not adequate to kill neurons so that reduced pH certainly cannot be considered to be the lethal activator of damage. Lowering pHo to 6.5 for 75 min in vivo, using hypercapnia, produced no histological damage measured 1–4 wk after the incident (209); it required several hours of exposure to pH 6.2 to produce delayed damage (817). The pH had to be lowered to ~4.5 to get a delayed damage from short (10 min) exposures (608, 815, 817).

In vitro systems are more sensitive to lowered pH than the in vivo systems described above; however, this is probably not relevant to in vivo ischemia. Lowering pHo to 6.7 for only 30 min caused delayed degeneration in hippocampal slice cultures, probably as a result of free radical production (1024). Just this condition could arise during recovery from global ischemia, and in the penumbra of focal ischemic damage, but at present, because of the measurements showing the far smaller sensitivity in vivo, it has to be assumed that this phenomenon is restricted to culture systems.

B) Low pH combined with ischemia. There are at least four reasonable explanations for the damaging effect of the lowered pHo during ischemia, but there is little evidence at present for any of these.

Lowered pHo could increase intracellular Na+ by ac-
tivation of Na⁺/H⁺ exchange, as apparently occurs in the heart after ischemia when extracellular H⁺ is washed out during reperfusion. Blockers of Na⁺/H⁺ exchange markedly reduce Na⁺ accumulation and infarct size (225, 911). This has not been well tested in brain.

Another possible mechanism is enhancement of free radical formation, which is likely to result from lowered pH via iron delocalization and the Fenton reaction (725, 930). There is some evidence for such a mechanism. Focal ischemic damage was exacerbated by preexisting hyperglycemia and, concomitantly, there was more than a two-fold enhancement of brain free radical production in the hyperglycemic animals. Free radical production was measured by salicylate microdialysis (1197). This is a profound effect. It is also quite possible that protonated ONOO⁻ is more damaging than ONOO⁻ itself if so lowered pH would enhance damage in this way (1013). There is one study testing whether free radical scavengers abolish the enhanced damage seen in hyperglycemia. The free radical scavenger from Upjohn, Tirilizad, markedly improved the acidity-induced decrement in metabolic function measured 3 h after 30-min ischemia, without affecting the large fall in pH, to 5.6, that occurred during this insult (569). Although far too few in number, these studies indicate that pH action may be mediated by enhancement of free radical production.

Another possible mechanism of damage is suppression of neurotrophin synthesis. Glucose (20 mM) completely suppresses the activation of the immediate early gene, c-fos (213), and both hyperglycemia and hypercapnia almost completely block the increased transcription of BDNF mRNA that normally occurs in CA3 and the dentate gyrus after 20-min global ischemia (1146). Those regions are normally not vulnerable and synthesize BDNF. It is possible that pH-induced vulnerability results from failure to synthesize BDNF or other neurotrophins. It would be of interest to see whether hypoglycemia allowed BDNF to be synthesized in CA1 of hippocampus after ischemia; it usually is not.

Another mechanism, suggested by studies on cortical cultures, is that the lowered pH may decrease the ability of cells to lower cytosolic Ca²⁺ after ischemia (741). This effect is extremely strong. When pHo was adjusted to 6.6, cytosolic Ca²⁺ was maintained at ~50% over baseline values for at least 100 min after a short exposure to kainate or (AMPA-cyclothiazide), with no sign of decay at that time (741). At pH 7.4, it returned within a few minutes. The basis for this is unknown at present, and it promises to be difficult to work out as, for example, the same phenomenon does not occur when cytosolic Ca²⁺ is elevated by NMDA. However, the effect may well occur in vivo, and it would clearly maintain a potentially toxic situation for a very prolonged period.

Finally, less specifically at this stage, pH may act by altering key enzymes or structural proteins at critical times, by transient denaturation or other means. For example, endonucleases are activated by a pH of ~6.6 (288).

Of these possible mechanisms, the most likely to cause damage is effects on free radical production. This could well lead to a continuous relationship between pH and damage, as appears to be the case. Maintaining cytosolic Ca²⁺ at elevated levels after ischemia also constitutes a viable mechanism for damage enhancement and needs to be considered.

8. Conclusions

Most of the existing data are consistent with the hypothesis that the fall in pH during ischemia is damaging. There are, though, points of contention in all three of the major experimental approaches: use of hypoglycemia and hyperglycemia in global ischemia and use of hyperglycemia in focal ischemia. These have to be resolved, but until that time, it is prudent to conclude that the fall in pH probably (strongly) exacerbates damage during ischemia. This is despite specific protective effects of small decreases in pHo (and possibly pHᵢ).

The cellular site of action of low pH is not completely resolved, and the apparently opposite effects in culture and in vivo suggest that the vasculature may be important, either because of breakdown of the blood-brain barrier or activation of production of free radical or other toxic substance. This being said, at this stage a site of action on neurons, or glia, is in no way ruled out.

The mechanism by which decreased pH exacerbates damage is not known. At this stage, effects on free radical production, which appear to exist but have not yet been extensively investigated, and effects on BDNF production, appear the most likely and are quite reasonable. Certainly, the latter might explain the difference between in vitro and in vivo results, since there is no particular evidence that endogenous neurotrophin production exerts a protective effect in cultures, whereas there is good correlative evidence for effects in vivo (see sect. VIII C).

At this stage, it should be assumed that if the pH change during ischemia in vivo was prevented, damage might well be very severely attenuated. This very strong effect of pH is suggested by the fact that hyperglycemia and elevated CO₂ enhance damage, despite delaying anoxic depolarization and attenuating the normal fall in ATP, two events that are generally considered to exacerbate damage. A caveat here is the possibility that damage after glucose injection is due to another, immediate effect of the hyperglycemia, rather than the decreased pH; this was discussed above. Hypoglycemia attenuates damage despite enhancing the fall in ATP.

VII. INITIATORS OF ISCHEMIC DAMAGE

The primary event during ischemia is inhibition of the electron transport chain and hence oxidative phosphory-
2. Evidence that decreased ATP is important in cell death

The decrease in ATP has many consequences that might activate cell death, primary of which is inhibition of the Na\(^{+}\)-K\(^{+}\)-ATPase with subsequent effects on intracellular Na\(^{+}\)/K\(^{+}\) and membrane potential. Other important consequences of the ATP fall may include activation of glycolysis and the resulting fall in pH, activation of free radical formation by increasing xanthine levels, and also possible changes in phosphorylation states of different enzymes and structural proteins. Transient inhibition of the Ca\(^{2+}\)-ATPase in ER and in the plasma membrane may also be important, although there is no direct evidence that this is so in vivo. In addition, several key transporters, including Na\(^{+}\)-H\(^{+}\) and the Na\(^{+}\)-K\(^{+}\)-Cl\(^{-}\) cotransporter, require low levels of ATP for their activity (17, 385), probably due to the importance of phosphorylation (17, 406) or to other events in the case of the Na\(^{+}\)-H\(^{+}\) transporter (249). These might be adversely affected.

There are also protective effects. There is a 30-fold increase in cAMP levels during global ischemia (783) which is likely to come, at least in part, from adenosine produced by the ATP breakdown (1215). There is little work relating to how this increase affects damage and that which exists is ambiguous. In the one direct study, the phosphodiesterase inhibitor rolipram alleviated damage after 3-min global ischemia in the gerbil (540), indicating that cAMP may be beneficial, at least in this mild insult. The only other relevant data concern type II metabotropic glutamate agonists, which affect cAMP metabolism. These are protective when added before a global ischemic insult (193, 910). Unfortunately, these results are difficult to interpret. Type II receptors generally depress activation of adenyl cyclase (436, 988), and if this is the case, the results suggest cAMP is damaging, contradicting the studies with rolipram. However, type II-like metabotropic receptors also accentuate the production of cAMP that normally accompanies β-adrenergic or adenosine receptor activation (1215). The very large release of norepinephrine during ischemia (401), as well as the released adenosine, may thus lead to a situation wherein the type II receptors enhance cAMP accumulation. If so, then the above studies taken together would indicate that cAMP production is protective in ischemia (133). The situation remains unresolved at present, but the weight of the evidence indicates a protective effect. If so, this marks a protective effect of the ATP reduction.

Other more certainly protective effects of the ATP decrease include dephosphorylation and inactivation of NMDA (686), and probably L-type Ca\(^{2+}\) (529, 616) channels, opening of ATP-sensitive K\(^{+}\) channels (444), and production of adenosine (21).

Despite the strong likelihood that the fall in ATP is, overall, damaging, there are only two reported direct tests. In both of these, the PCr buffer system for ATP was

A. Decreased ATP

1. Decrease in ATP and PCr

Decreases in ATP during anoxic/ischemic insults are in Table 1. Not illustrated in Table 1 is the fact that the ATP fall in global ischemia and, to an extent, in slices is quite precipitous. Levels fall from ~90% to close to their lowest values within about a 1- to 2-min time interval (553, 676, 717). This fall follows a significant depletion of the PCr (PCr) stores; for example, the fall in ATP during global ischemia only begins after PCr has declined to between 10 and 20% of control values (553). This precipitous decline in ATP is predicted by analysis of the PCr kinase-catalyzed equilibrium (1170)

\[
\text{ADP} + \text{PCr} + H^+ \leftrightarrow \text{ATP} + \text{Cr}
\]

where Cr is creatine, and the fairly long delay is due to the quite robust nature of the PCr kinase system in brain tissue. Enzyme levels are high, particularly in synaptic regions (339), and resting PCr levels are about twice as high as ATP levels (294, 676).
fortified by preexposure to creatine. Early studies were done in the hippocampal slice preparation, where preincubation with 25 mM creatine built up a store of creatine phosphate (676) that retarded the fall in ATP during anoxia (531, 532, 676). This in turn retarded the fall in transmission (676), the anoxic depolarization (59; D. Lober and P. Lipton, unpublished data) and strongly attenuated the Ca$^{2+}$ entry into the cells during 10-min anoxia (532). In terms of long-term damage, the creatine preincubation quite dramatically reduced the damage to synaptic transmission (531) and protected against anoxic damage to protein synthesis (165), measured 1–3 h after the anoxic insult. It did not attenuate the anoxic decrease in pHi, measured using the creatine kinase equilibrium (531). Thus attenuating the fall in ATP significantly delayed the appearance of acute forms of damage. More recent studies indicate the protective effects are achieved at much lower creatine concentrations, of 1 mM (59).

In vivo, animals were kept on a regime of dietary creatine for 2 wk and then exposed, not to ischemia, but to levels of the mitochondrial inhibitors malonate or 3-nitropropionic acid, which lowered ATP by $\sim$50% for several hours. The creatine diet elevated PCr by $\sim$60%, far less than in the slice studies, but it dramatically reduced the size of the infarct produced by each of the mitochondrial inhibitors (736). This strongly suggests that maintaining ATP levels during the insult is protective. Measured effects of creatine showed it maintained ATP levels relative to control and also decreased free radical and peroxynitrite production. Unfortunately, those measurements were made at the time the infarct had developed in the untreated tissue so that ATP and other metabolite levels in healthy tissue were being compared with levels in damaged tissue. Thus the differences may not reflect effects of creatine during the time when damage was developing. Measurements before infarct formation would show whether the creatine pretreatment did, in fact, attenuate the ATP fall and free radical production.

In contrast to these results, hyperglycemia increases damage, despite maintaining ATP for a longer period during the ischemia. This is thought to be because the damaging effects of decreased pH overwhelm the marginal protection from the delayed fall in ATP, but this has not been proven. Another explanation, based on the fact that in some instances mild hyperglycemia was damaging although it did not increase the fall in pH, is that ATP maintenance is actually damaging (465). Although this seems unlikely, there are several ways this might occur, as discussed above.

3. Regeneration of ATP after ischemia

It is possible that the recovery rate of ATP after short lethal durations of global ischemia may be important in development of damage, since a slow recovery rate will significantly extend the short period of energy deprivation. The rate in vivo has not been measured carefully, but in slices, levels are back to their maximal levels after 10 min (533). The rate of recovery may be accelerated by the lactic acid buildup during ischemia. Although this has not been tested directly, brain slices recover far better from in vitro ischemia when lactate is the postischemic substrate than when glucose is the substrate (1009). The basis for this may well be that glucose metabolism requires phosphorylation by hexokinase and so may be slowed during conditions of low ATP. Measurements on ATP recoveries have not yet been made, but it seems probable that the resistance to damage is due to more rapid recovery of ATP in the lactate-exposed slices. It is not known whether this phenomenon occurs in vivo.

4. Conclusion

Although experimentally verifying the importance of the ATP fall in damage may seem “academic,” it is not. It is possible to envision a damage scenario in which the fall in ATP is irrelevant (or protective) and where damage stems from the blockade of the mitochondrial respiratory chain. The latter could produce increases in free radicals and in cytosolic Ca$^{2+}$ and, via opening of the MTP, might also trigger apoptosis. The increased cytosolic Ca$^{2+}$ could increase the spontaneous vesicular release of glutamate, leading to further toxicity. The decreased oxidative phosphorylation would lead to a lowering of pH. The single set of data opposing this possibility in vivo is that where creatine preincubation was protective against metabolic inhibitors (736). Although limited, these data are quite persuasive in confirming that the fall in ATP is damaging. However, they were carried out on chemical insults, not ischemia. Further studies are very much necessary to verify the very fundamental hypothesis that the fall in ATP is a key initiator of ischemic damage.

B. Anoxic Depolarization

A very important review of early ion changes and anoxic depolarization was published some 12 years ago by Hansen (417), and there are more recent excellent reviews (554, 717).

1. Characteristics of anoxic depolarization

Anoxic depolarization is a cardinal feature of anoxia/ischemia in vivo (537, 604, 635, 1231) and in slices (58, 60, 611, 1038). It has not been observed in cell cultures. It occurs between 60 and 180 s after onset of global ischemia in situ, and between 1 and 12 min after the onset in slices. In the latter it is very dependent on glucose levels in the incubation medium (950) and on the level of anoxia. Anoxic depolarization is observed in the core of focal ischemic lesions (188) but does not occur in the penumbra.

The phenomenon is characterized by a large (10–30
mV) extracellular negative direct-current shift that occurs in both neuropil and cell body regions of the ischemic tissue. This is invariably accompanied by precipitous decreases in extracellular Na\(^+\) (to \(\sim\) 50 mM), Cl\(^-\), and Ca\(^{2+}\) (to \(\sim\) 0.1 mM), and by a rise in K\(^+\) (to \(\sim\) 60 mM) (611, 635, 717, 1231). There is a rise in extracellular impedance, or extracellular marker concentration (537, 604), indicating significant cellular swelling that is almost undoubtedly due to isotonic entry of Na\(^+\) and Cl\(^-\) (417).

Although intracellular ion changes cannot be measured with the same temporal precision as extracellular ion changes using electrodes, there are large increases in intracellular Cl\(^-\) in hypoglossal neurons at about the time of depolarization (508), and there is an approximate doubling in the level of intracellular Na\(^+\) after 5 min of anoxia in the hippocampal slice (338). These almost certainly reflect the acute changes seen extracellularly.

There is a large depolarization of pyramidal cells (to near 0 mV) at the time of the anoxic depolarization in slices (934) and in situ (1233). This is associated with a slight increase in membrane resistance in situ and a 50% or more decrease in resistance in the slice (858, 934). The basis for this difference is not known (1233), but it is clear that no huge membrane resistance change can account for the massive depolarization. Thus it probably results from the ion changes rather than membrane permeability changes. The concomitant failure of transmission in the presynaptic terminals of the Schaffer collaterals in the hippocampal slice (see below) shows that the depolarization affects neuropil elements also.

2. Mechanism of anoxic depolarization

The basis for the anoxic depolarization has not been determined. There are three reasons for thinking that it is largely due to inhibition of the Na\(^+\)-K\(^+\) pump.

First, there is a consistent, and strong, correlation between cell levels of ATP and the occurrence of the anoxic depolarization. Conditions that increase the rate of ATP depletion, such as hypoglycemia (613) and 0 mM Ca\(^{2+}\) buffer, which increases the rate of depletion of ATP in hippocampal slices (686), shorten the time to anoxic depolarization, whereas conditions that slow the rate of ATP depletion, such as hyperglycemia (613, 950, 1040), creatine preincubation (59), hypothermia and barbiturate anesthetics (1108, 1233), all increase the time to anoxic depolarization.

Several studies have established the correlation quite quantitatively. In rats maintained at different core temperatures (38 and 28°C), or given different doses of anesthesia, ATP levels were between 13 and 18% of control at the time of anoxic depolarization whether this was 73 s (normothermia), 125 s (high levels of anesthesia), or 242 s (hypothermia) (1168). In another study, the very rapid kinetics of ATP depletion and anoxic depolarization in cortex were measured during cardiac arrest. In different rats, the anoxic depolarization always occurred within 5 s of the time at which the ATP suffered a precipitous fall (between 60 and 90 s) (553). Average ATP values were 40% at the time of anoxic depolarization. In slices, there is a good correlation between the buildup of PCr in creatine-fortified buffers and the duration of anoxia required for anoxic depolarization (59).

The other reason for ascribing a major role to pump inhibition is that ouabain, the specific inhibitor of the pump, leads to a massive depolarization that has the appearance of anoxic depolarization. In fact, low concentrations of ouabain, which mimicked the levels of Na\(^+\)-K\(^+\)-ATPase inhibition seen during anoxia, led to a large depolarization at almost the same time as the anoxic depolarization (R. Rader, T. Lanthorn, and P. Lipton, unpublished data). A similar but more rapid effect of a higher (100 \(\mu\)M) dose of ouabain was observed by Balestrino (57). A third reason to conclude that the anoxic depolarization is due to pump inhibition is that it is essentially independent of extracellular Ca\(^{2+}\) (1264), and NMDA receptor activation (394, 611, 635, 934, 1231) and only very slightly (1231) or not at all (635) dependent on non-NMDA glutamate receptors. The ability of pump inhibition to cause the rapid depolarization is thought to be due to the very high surface-to-volume ratios of neuronal elements, which means that pump inhibition produces rapid changes in ion concentrations (675, 677).

3. Ion fluxes and transmitters during anoxic depolarization

If pump inhibition is indeed the basis for the anoxic depolarization, the shift in K\(^+\) as well as any specific permeability changes are likely to be responsible for the majority of the depolarization (378). The electrogenic contribution to membrane potential in vivo is not known but is not generally very large (1115).

Glibenclamide, which blocks the ATP-sensitive K\(^+\) channels, moderately slows the increase in extracellular K\(^+\), suggesting some of the K\(^+\) efflux is via this channel. Excitabile Na\(^+\) channel blockers such as tetrodotoxin, lidocaine, and phenytoin both in situ (1229, 1231) and in slices (1109, 1195) delay the anoxic depolarization from 2 to 6 min in situ and from 6 to 9 min during a relatively mild insult in slices. The delay is probably a direct result of blocking Na\(^+\) influx, and hence Na\(^+\)/K\(^+\) exchange after inhibition of the pump. By virtue of blocking the Na\(^+\) entry, the drugs also reduce the rate of fall in ATP (105, 338), and this will add to the delay in anoxic depolarization.

Exaggerating the ischemic reduction of pH, either by hypercapnia in situ (552) or by extracellular H\(^+\) in slices (1119), delays the anoxic depolarization dramatically. The mechanism of this effect is not known. Presumably, a critical ion channel is inhibited so that Na\(^+\)/K\(^+\) exchange is delayed.
4. Role of anoxic depolarization in cell death

This is very difficult to determine. Damage is strongly correlated with the anoxic depolarization, in that artificially delaying the anoxic depolarization in one of several ways almost always increases the duration of anoxia or ischemia that is necessary for damage. This is so for pharmacological agents (60, 1109), temperature variations (805), age (950), osmolarity of anoxic solution (472), and comparisons between different brain regions (58). Furthermore, there are massive biochemical changes and ionic changes at the time of the anoxic depolarization, including the very large increase in FFA during global ischemia (553). The reason it is hard to conclude that the anoxic depolarization itself contributes to the damage is because the anoxic depolarization is coincident with, and very probably dependent on, the decrease in ATP levels, and increase in Na\(^+\) entry, two factors which almost undoubtedly strongly activate damaging events. There is currently no way to prevent the anoxic depolarization without affecting the fall in ATP and rise in Na\(^+\) also. The main argument that the anoxic depolarization is important is that blockers of voltage-dependent Na\(^+\) channels are protective, suggesting that depolarization-induced opening of these channels is important. However, as seen immediately below, this protection is manifested when the Na\(^+\) channel blockers are added several hours after the anoxic depolarization is terminated. To the extent that the anoxic depolarization is critical for glutamate release, it is very probably important in damage. However, the involvement of the anoxic depolarization in this release is not clear.

The fact that \(CO_2\) exacerbates damage while greatly slowing the anoxic depolarization shows that the anoxic depolarization is not essential for damage. It is clearly a marker for profound metabolic change but may not itself contribute to the damage; that is, damage might be just as great if there were no anoxic depolarization.

C. Increased Cell Na\(^+\)

1. Evidence for a role in cell damage

There is a large increase in cell Na\(^+\) during the first few minutes of anoxia or ischemia that coincides with the anoxic depolarization.

Drugs that block Na\(^+\) fluxes and prevent these ion changes are always strongly protective in both permanent focal (388, 939, 1058) and global (226, 245, 343, 693, 1036) ischemia. Most of the drugs used are quite specific for Na\(^+\) channels, although they, of course, have other undocumented effects. They include lamotrigine and related compounds (245, 388, 1058), tetrodotoxin (693), and lidocaine (343). Temperature appeared to be very well controlled throughout the permanent focal ischemia studies (388, 1058) and was well controlled, or shown to be unaffected by the drug during, and for \(\sim\)15 min after the global insults (226, 343, 693). It was not maintained or measured after this so that it is possible that drugs, which are potent inhibitors of Na\(^+\) channels and hence neural activity, may have affected temperature during the hours after ischemia. There is no strong reason to think this is the case. Thus Na\(^+\) entry is very likely to be a major cause of damage.

Because of the large Na\(^+\) entry during ischemia, and the glutamate release at that time, one would expect the effects of Na\(^+\) entry to be exerted early during the insult. This may be true. However, the Na\(^+\) channel blockers discussed above are very effective in preventing damage from global ischemia when applied 15 min (226) after the end of global ischemia and even 2 h after the insult (245). This quite surprising result suggests that Na\(^+\) entry is (also) causing damage in the postischemic period. Drug effects cannot be due to seizure blockade since postischemic seizures are not present in rats. It has not been possible to isolate the action of the drugs to the beginning of the insults, which would allow determination of whether the early entry of Na\(^+\) is also damaging. It may well be. However, it is clear that delayed Na\(^+\) entry is damaging.

2. Possible mechanisms of Na\(^+\)-induced damage

There are several ways that Na\(^+\) might be damaging during the acute phase of the insult. These have been discussed previously and include causing glutamate release, increasing cytosolic Ca\(^{2+}\), and helping to deplete ATP levels via activation of the Na\(^+\)-K\(^+\) pump. The latter is well-illustrated by the fact that tetrodotoxin and lidocaine slow the rate of the ATP fall during anoxia (105, 338). Entry of Na\(^+\) (and Cl\(^-\)) will cause intracellular edema, but there is no particular reason to think this is damaging.

The postischemic involvement of Na\(^+\) channels in global insults means that a very important effect of ischemia is to enhance the damaging efficacy of Na\(^+\) channels for a prolonged period. One possibility is that their open probability increases either due to a change in channel properties or to a small maintained depolarization. A similar effect is indicated in studies of glutamate toxicity. In cultured striatal neurons, a continual cycle of Na\(^+\) entry, glutamate release, and NMDA-mediated activation of NOS persists for many hours after an initial 5-min glutamate exposure and accounts for the development of damage. Free radical damage from NO elevation is the actual damaging step (1074). This “vicious cycle” (1074) may result from damage to any one of the components, but a reasonable target is the Na\(^+\) channel, and indeed, damage is prevented with tetrodotoxin. Such a mechanism might well be operative in the penumbra of focal ischemia because damage there is sensitive to delayed NMDA blockade (771, 833)” (see sect. \(\alpha D\)). However, global
ischemic damage is generally not attenuated by postsischemic NMDA blockade (except when the ischemia is mild, Ref. 167) so that the same mechanism cannot be operating there. However, if Na\(^+\) channels are altered, then continual Na\(^+\) elevation with resultant cytosolic Ca\(^{2+}\) elevation might well lead to damage in the same way. This seems the most likely mechanism for the Na\(^+\)-dependent damage.

This does not obviate the possibility that the early massive entry of Na\(^+\) is also damaging. There are clearly many mechanisms by which it might help set in motion events that lead to the delayed Na\(^+\) entry. However, absent the delayed entry, it would not cause cell death, because blocking the delayed entry later strongly attenuates cell death.

D. Events in Focal Ischemia

The core of the focal insult, which experiences the large changes in ATP, Na\(^+\), and membrane potential, is not protected by any manipulations affecting Na\(^+\) entry so that it is not possible to determine whether these events are damaging, although it seems very likely that the fall in ATP and membrane depolarization are important. It is not clear why Na\(^+\) entry blockers do not ameliorate damage.

The penumbra does not suffer the massive ATP loss, anoxic depolarization, and Na\(^+\) entry, so the role of these changes in damage is moot. The minor loss in ATP may well be critical, but to show this explicitly, it would need to be prevented, and this is extremely difficult over such a long duration. The Na\(^+\) channel blockers prevent damage in the penumbra of permanent focal ischemia when added 1 h after the start of ischemia; no studies are reported for temporary ischemia (388, 939, 1058). Damage in the penumbra is very sensitive to glutamate, and the channel blockers do prevent glutamate release, so this could account for their effects. Alternatively, or in addition, it is quite possible that the vicious cycle described in section VaC might well be responsible for Na\(^+\)-dependent damage; damage in the penumbra is prevented by NMDA blockade, both early during permanent ischemia and soon after temporary ischemia (771, 833). Neither the importance of Na\(^+\) entry nor the timing of any effect has been studied for temporary focal ischemia.

E. Summary

The fall in ATP, the anoxic depolarization, and the initial rise in cytosolic Na\(^+\) are three very early ischemic changes in global ischemia and in the core of focal ischemia that are closely interrelated. They are all quite strongly correlated with damage in that they occur early, and when they are blocked, damage is almost invariably attenuated. (The only time this is not true is during hyperglycemia or with elevated CO\(_2\) where damage is enhanced despite the fact that all these processes are slowed. These results are very probably an indication of the importance of the pH change in damage.)

It is difficult to know with any certainty whether the ATP fall and the anoxic depolarization are indeed contributing to the damage in global ischemia. Blockade of the fall in ATP with creatine incubation is very protective against early functional damage in slices and in a chemical in vivo model of energy deprivation, but this has not yet been shown in an in vivo global ischemic model. If it were it would show the importance of the fall in ATP. There is no independent evidence that the anoxic depolarization is critical either; the correlations between anoxic depolarization onset and damage may well be explained by the effects of the different conditions on the fall in ATP or on entry of Na\(^+\). There is no explicit demonstration of delaying anoxic depolarization without delaying the fall in ATP or the rise in Na\(^+\).

There is strong evidence that Na\(^+\) entry is important. However, here too, the results are not simple. If Na\(^+\) entry during the insult was damaging, this would strongly implicate the pathway comprising ATP decrease, membrane depolarization, and massive Na\(^+\) entry. However, the identified damaging Na\(^+\) entry appears to be after the initial dramatic changes so that its direct cause is neither the large decrease in ATP nor the anoxic depolarization. The early Na\(^+\) entry may also be damaging, but this has not been directly demonstrated.

Thus experimental evidence for the sequence of ATP fall, membrane depolarization, and massive Na\(^+\) entry as a principle way by which ischemic damage is initiated in global ischemia is woefully lacking. Certainly many conditions that slow the fall in ATP and the anoxic depolarization are protective. However, they also doubtless slow other sequelae of energy metabolism inhibition, such as the pH fall and free radical generation.

At this stage, it remains possible that mitochondrial changes are sufficient, without mediation by ATP, as discussed above. Certainly, it is difficult to accept that the fall in ATP and the anoxic depolarization may simply be epiphenomena; however, experiments need to be done to show otherwise.

Sodium channel blocker studies do show an extremely important role for Na\(^+\) entry in damage, both in global ischemia and in the penumbra of focal ischemia. Damage is strongly attenuated by several channel blockers, and effects of temperature have been reasonably well controlled. However, this damaging Na\(^+\) entry occurs at a relatively late stage, well after the massive Na\(^+\) influx in global ischemia has subsided. It most likely results from a long-term membrane change, possibly in the Na\(^+\) channels themselves. The origin of this change is not known, but its apparent importance in damage makes it extremely important to study.
VIII. GENE ACTIVATION AND TOLERANCE

Ischemia activates synthesis of potentially protective and potentially damaging proteins. The functional significance of some of these proteins is considered in this section.

An underlying pattern, to which there are important exceptions, is that the protective proteins are synthesized in relatively nonvulnerable cells and are not synthesized in vulnerable cells, or cells that will die (see Table 5). This raises the possibility that the absence of these proteins may be important in development of cell death. This is supported by the phenomenon of tolerance, in which normally vulnerable cell populations become resistant to damage. Evidence suggests that tolerance results from synthesis of protein that is normally not synthesized in the vulnerable cells but that is synthesized in cells that do not go on to die.

In contrast to the protective responses, at least four putatively damaging proteins, COX-2, nNOS, iNOS, and Bax, are synthesized, as are at least two cytokines, IL-1β and TNF-α, which are largely damaging, and there is good evidence that they greatly enhance cell death (185, 420, 477, 837). These damaging proteins are synthesized in both global and focal insults.

Mechanisms of gene activation are extremely complex in that they involve many signals and transcription factors. In general, the mechanisms by which genes are activated by ischemic conditions, and in postischemic periods, have not been worked out, so I do not consider them in any detail. Nevertheless, it is important to point out that Ca²⁺, neurotrophins, PAF, as well as neurotransmitters acting at metabotropic receptors are all capable of activating genes via the MAP kinase system or other pathways. Much of this is very nicely summarized elsewhere (345). Mitogen-activated protein kinase is hyperphosphorylated soon after ischemia in most regions (468) and so may well mediate early effects; increased Ca²⁺ may also do so by the MAP kinase or by alternate pathways (510), as may free radicals (864), for example as a result of NFκB (206, 597) or AP1 activation (1018).

A. Immediate Early Genes

1. Gene activation

There is a rapid transcription of a large number of immediate early genes including c-fos, c-jun, junB, krox-20, and zif-268 within the first 30 min after global ischemia, during or after focal ischemic insults (19, 212, 456, 485, 556, 567, 575, 1061, 1223), and after hypoxia/ischemia (270). There are good recent reviews that detail these increases and their localizations (8, 323, 593), and there is much discussion as to whether they play protective, destructive, or no roles (8, 566, 1259). Overall, this issue has not been completely resolved, but certainly the nature of the proteins that are eventually changed suggests some of the early genes are important. The mechanisms of these early gene activations are not known. Synthesis is significantly blocked by MK-801 in gerbil global (1223) and rat focal (212) ischemia, suggesting that Ca²⁺ entry is important. Other factors have not been identified, although free radicals are certainly able to activate immediate early genes (864), as is PAF (70).

2. Localization of protein increases

Far more important than the mRNA synthesis is whether proteins are synthesized. In gerbils, transcription of several early genes increases equally in all hippocampal regions (567, 1148, 1223); however, although the proteins are upregulated in several regions, there is almost no expression of the proteins in the vulnerable CA1 cells. Levels sometimes fall below baseline (567). There are no studies that directly address whether there is a causal relationship between the lack of synthesis of the proteins and the ensuing cell death. Certainly, there are situations in which cells go on to die despite immediate early gene expression. For example, in the young rat exposed to 15-min carotid occlusion and 8% O₂, there is an expression of several immediate early gene proteins within an hour, throughout a region of the brain in which there is massive delayed cell death (270). Still, this does not obviate the possibility that synthesis of one or more immediate early gene products is critical for recovery, at least in global ischemia. Studies with controlled gene knockouts are necessary to determine the roles of these early genes.

B. Heat Shock Proteins

1. Pattern of heat shock protein expression after ischemia

The pattern of heat shock protein expression after global ischemia in the gerbil is very similar to that of the immediate early genes. Messenger RNA for heat shock protein (HSP) 70, HSC70 and HSP90 begin to rise within a few minutes of the insult in all regions, and persist (485, 556, 1223). The protein is upregulated in several early genes increases equally in all hippocampal regions (567, 1148, 1223); however, although the proteins are upregulated in several regions, there is almost no expression of the proteins in the vulnerable CA1 cells. Levels sometimes fall below baseline (567). There are no studies that directly address whether there is a causal relationship between the lack of synthesis of the proteins and the ensuing cell death. Certainly, there are situations in which cells go on to die despite immediate early gene expression. For example, in the young rat exposed to 15-min carotid occlusion and 8% O₂, there is an expression of several immediate early gene proteins within an hour, throughout a region of the brain in which there is massive delayed cell death (270). Still, this does not obviate the possibility that synthesis of one or more immediate early gene products is critical for recovery, at least in global ischemia. Studies with controlled gene knockouts are necessary to determine the roles of these early genes.
occlusion never show an HSP70 immunocytochemical response and show only a very small mRNA response even though they are morphologically intact for the first 6 h after the 2-h exposure. Neurons in the penumbra, many of which recover, show a marked HSP70 production (576, 655). Thus the correlation between HSP and resistance to damage is present in focal ischemia.

Ubiquitin, a low-molecular-weight HSP involved in conjugation and breakdown of damaged proteins is dramatically decreased in both gerbil and rat hippocampus within hours after ischemia and then recovers over the next 1–3 days in all cell types except CA1 cells that are destined for death (251, 703, 1241). This phenomenon may be involved in damage, possibly by allowing buildup of partially denatured proteins (703), but unfortunately, there is no further evidence to this effect.

In summary, in the gerbil, the anatomical association between HSP expression and recovery is consistent with the conclusion that HSP is necessary for survival and is involved in survival. Although HSP may be necessary, it is certainly not sufficient in the rat, at least when expressed 1 day after the insult. CA1 cells die despite expressing HSP. Perhaps the absence of expression in CA1 earlier than 1 day after ischemia is critical, but there is no particular reason to think so. At the present time, experiments in which HSP are knocked out are necessary to test their role in protection of nonvulnerable neurons. As is shown in section VIII, their upregulation in vulnerable cells partially overlaps the rescue of these cells.

2. Mechanism of HSP increase

As for immediate early genes, induction of HSP by ischemia is dependent on activation of NMDA receptors. MK-801 blocked its production in carefully temperature-controlled studies in gerbil (546, 1148). Free radicals may well play a part in the induction of the HSP; they do after ischemia in liver (998). In that sense, free radicals may be protective. Similarly, the inhibition of HSP (and immediate early gene) formation by MK-801 will tend to make NMDA blockade damaging rather than beneficial.

3. Protection by HSP

Overexpression of HSP70 protects cultured neurons against ischemic damage (2-h in vitro ischemia, 4-h recovery) (18) and also protects astroglia (871). The amount of LDH release was reduced from 80 to ~18% of available LDH. Thus HSP certainly has the potential to protect against ischemic damage.

There is no well-accepted mechanism for how protection occurs. Expression of the protein inhibits the activation of the transcription factor NFκB (317), which is activated in ischemia and appears to be associated with toxicity. This might well be the protective mechanism; it has not been studied explicitly. The 70-kDa HSP also protects against apoptosis, by blocking the downstream actions of caspases on activation of PLA2, the cell nucleus, and other processes (499). This would certainly be a protective mechanism in ischemia. The major biophysical action of the HSP70 and HSP90 classes of proteins with their coactivator proteins is to bind to unfolded or partially unfolded peptide chains, largely by binding to hydrophobic regions of the protein, and to ensure that proper folding occurs (440). To the extent that they are protective against ischemia in this way, it suggests that loss of protein structure is an important component of ischemic cell damage. If so, this is a very important conclusion. Nothing is known about possible molecular targets of these protective actions.

C. Neurotrophins and Neurotrophic Factors

1. Pattern of expression after ischemia

Basal levels of neurotrophins are lower in CA1 than in other hippocampal regions. This is consistent with their importance in damage (599). There are several somewhat conflicting reports of increases in mRNA for BDNF, trkB receptors, and NT-3 in hippocampus that differ in their descriptions of onset times and durations. However, message levels are certainly increased by global ischemia in all regions (667, 754, 1100, 1139). However, as with other putatively protective proteins that have been discussed, protein levels do not rise in vulnerable regions. After global ischemia in the rat, there is a small increase of BDNF in dentate gyrus at 24 h, little change in CA3, and a large decrease of BDNF in vulnerable CA1 and neocortex (599). This appears to occur largely in neuropil, perhaps in the dendrites (1239). This same large decrease is seen in the gerbil at 24 h (321). Thus, considering the combination of low basal levels, and changes during ischemia, the vulnerable CA1 pyramidal cells have a much lower level of this neurotrophin than other regions at critical times. Probably of equal importance, there is only a very low density of trkB receptors on CA1 pyramidal cell somata compared with other regions of the hippocampus (321). This contrasts with the population of parvalbumin-staining GABAergic neurons in the CA1 pyramidal cell layer (321) which strongly express trkB. This may be the basis for the survivability of these neurons. Receptors for another protective factor, epidermal growth factor (889), are reduced in CA1 pyramidal cells 24 h after ischemia (321). Thus, to the extent that growth factors offer protection, CA1 pyramidal neurons are certainly vulnerable.

2. Protective effects of exogenous growth factors

Neurotrophic factors mitigate ischemic cell damage in several different preparations. They are usually added 24–48 h before the insult to allow full expression of their induced proteins and are maintained throughout the insult and recovery phase. However, such a preincubation is
not necessary. BDNF protected against ischemic cell death in CA1 pyramidal cells of the rat (73, 1139) and in the GABAergic reticular nuclear cells of the thalamus (559) after global ischemia. In one of the first two cases, the BDNF was added only 90 s before the insult and was maintained throughout recovery (73). The neurotrophin also provided very strong protection against infarct in young rats 7 days after 2.5-h hypoxia-ischemia, and in this case it was administered right before the insult. It was quite protective even when added immediately after the insult (192). The degree of protection depends very strongly on the presence of trkB receptors, and almost all neurons containing these at high densities are protected by the neurotrophin (192, 320). A causal relationship has not been established however.

Acidic fibroblast growth factor protects CA1 cells of the gerbil against global ischemia (702, 990), as does a mixture of basic fibroblast growth factor and NGF (1026).

Troponin factors block apoptotic death (664), excitotoxic cell death (737), and ischemic cell death (112) in vitro.

3. Damaging effects of exogenous growth factors

Unlike the in vivo cases described above, preexposure to exogenous BDNF actually exacerbates damage from ischemia and from high levels of NMDA in mouse cortical cultures (594). This is associated with increased Ca\(^{2+}\) influx (594). Free radical-induced necrosis is also enhanced by BDNF in these cells (403), and the growth factor may exacerbate mitochondrial free radical production (D. Lobner, personal communication). At least a partial explanation for this toxicity is that the BDNF preincubation leads to an increased number of nNOS-positive neurons in the culture (986). Other possible mechanisms of damage include activation of MAP kinase and subsequent activation of cytosolic PLA\(_2\) (345). At the moment, this effect does not appear to play a role in in vivo ischemia.

4. Mechanism of protection by growth factors

There is little known about how endogenous, or exogenous, growth factors might protect against ischemic damage. Some growth factors may act by inhibiting Ca\(^{2+}\) increases. Acidic fibroblast growth factor administered soon before the insult delayed the early rise in cytosolic Ca\(^{2+}\) during in vitro ischemia in gerbil hippocampal slices by ~8 min (767), certainly enough to afford protection against the 5-min (or 10-min) insult in gerbils. It is not known if this effect pertains in vivo. The quite rapid effectiveness in this case, and when BDNF was administered just before focal ischemia, suggest that (some of the) protection by growth factors in ischemia results directly from rapid effects of the factors such as phosphorylation (281), rather than from resultant gene activation. Protection against NMDA toxicity by BDNF occurs rapidly, within 4–8 h. The protection against submaximal doses is very profound and is associated with preventing the decrease in PKC that normally occurs, and that has been shown to be instrumental in damage (1130). The mechanism is not known, because the mechanism for the downregulation of PKC is not known. However, the fact that PKC is also downregulated in ischemia suggests that BDNF may protect in this way against ischemic damage.

Neurotrophins also exert a concerted set of changes that reduce the increase in free radicals (737). This appears to be dependent on protein synthesis. Twenty-four-hour preexposure to BDNF increased SOD activity and glutathione reductase activity in rat hippocampal cultures. This led to a marked decrease (~50%) in free radical generation 30 min after exposure to glutamate, as well as protection against glutamate toxicity (737). Such a mechanism might help rescue postischemic neurons, although this effect has not been shown in vivo. There is a maintenance and upregulation of SOD in nonvulnerable neurons and a loss of SOD in CA1 pyramidal cells 1 day after global ischemia (680, 734). This is likely to be important in differential sensitivity of the neuronal populations and may well result from the differences in BDNF metabolism.

Maintenance of protein synthesis is mediated in part by tyrosine kinase systems that are activated by neurotrophins or growth factors. As discussed previously, the absence of protein synthesis in vulnerable neurons has been suggested to result from the absence of neurotrophin actions (468).

5. Conclusions

Basal neurotrophin levels and changes of levels in different cell populations, as well as known protective effects of neurotrophins in ischemia and other challenges, suggest that they may act as endogenous neuroprotections. (There is no evidence for a relevant damaging role in vivo.) If so, there are many possible sites, most dominant of which are prevention of free radical accumulation, prevention of PKC degradation, and, possibly, reestablishment of protein synthesis. They may also attenuate delayed Ca\(^{2+}\) increases, but this is very speculative. A major gap at this stage is a demonstration that knocking out BDNF (or other neurotrophin) or pharmacological neutralization of BDNF (525) leads to increased sensitivity to ischemia in vivo.

D. Bcl-2/Bax System

1. Protein expression after ischemia

As with HSP, The Bcl-2 group of proteins is selectively upregulated in nonvulnerable regions of hippocampus after global ischemia. This occurs in both rat and gerbil and in this respect is a more viable candidate than HSP70 or HSP90 as a major protectant molecule. In-
increased Bcl-2 and Bcl-x long were expressed in CA3 and dentate granule cells of rat hippocampus within 2–4 h of 15-min global ischemia but were not expressed in CA1. This was despite the induction of mRNA in all regions (181). A similar pattern was observed in gerbil after 5-min ischemia (420). Six hours of focal ischemia led to a decrease in expression of Bcl-2 and Bcl-x in neurons of the penumbra, the great majority of which go on to die (364).

In contrast to the protective members of the Bcl-family, the “killer” protein Bax was upregulated in CA1 of both rat and gerbil after the global ischemic paradigm (185, 420) as was the Bax-like protein Bcl-short (264), and both were expressed during development of ischemic damage. Bax was not increased in CA3 but was increased, along with the Bcl-2, in nonvulnerable dentate granule cells. Bax was also upregulated in the penumbra of the focal lesion (364).

Thus there is a dramatic downregulation of Bcl-2-like proteins and upregulation of Bax-like proteins in vulnerable tissues. In dentate gyrus, which is nonvulnerable, the increase in Bax is accompanied by an increase in BCL-2, which probably neutralizes it. There is every reason to believe that elevated Bax/Bcl-2 will be damaging and that the elevation of Bcl-2 in nonvulnerable regions may well be protective. Overexpression of Bcl-2 strongly protects against anoxic/ischemic damage in focal ischemia (721), global ischemia (27), and cultures (791). Overexpression of Bax leads to apoptotic cell death in many systems.

2. Mechanism of protection against damage

Bcl-2 strongly protects against free radical-mediated cell death that is probably necrotic in a way that is not yet known (524). It also has profound protective effects on mitochondrial function, greatly enhancing maximal Ca\(^{2+}\) uptake and reducing the ability of increased mitochondrial Ca\(^{2+}\) to impair respiration, presumably by preventing opening of the MTP (787). These effects are likely to protect against necrotic cell death. Bcl-2 also strongly protects against apoptosis. One way it does this is to prevent cytochrome c release and thus prevent activation of the ICE-like proteases (591, 774, 1066, 1250). It also protects downstream from the mitochondrial release of cytochrome c (940).

E. Gene Products That Enhance Damage

As discussed in previous sections, gene products that should enhance damage are also expressed in the post-ischemic period, after transcription of their mRNA. These are COX-2, iNOS, nNOS, and cytokines, particularly IL-1β and TNF-α. c-Jun expression may also enhance damage. Other proteins, discussed more fully elsewhere, include Bax, Bcl-x short, and cell adhesion molecules.
2. Cytokines

Two major proinflammatory cytokines, IL-1β and TNF-α, appear to play very important roles in focal ischemic damage and may play a role in global damage (241).

A) INTERLEUKIN. Immunoreactive IL-1β increases very rapidly, within 15 min of the onset of focal ischemia, maximizing by 2 h. It is seen mainly in endothelial cells and microglia (1295). Messenger RNA for IL-1β is elevated in all cell types within 2–8 h of global ischemia in rats, but the protein is mainly expressed in glial cells. All cells showed increased mRNA for the receptor for IL, but the protein was largely expressed in neurons, with some in endothelial cells (974). Thus IL and its receptors are upregulated in a timely fashion.

There is good evidence implicating IL-1β in permanent focal ischemic damage. The natural peptide antagonist to the IL-1b receptor, IL-1ra, decreased neuronal death in the peri-infarct zone of a permanent focal lesion (352) and reduced infarct size after 24-h permanent ischemia by 50% (946). Furthermore, an antibody to the IL-1β receptor antagonist, which is upregulated by ischemia, exacer-

bated damage (687). This argues that this antagonist is generally helping to reduce damage, presumably by attenu-

ating the IL-1β damaging action. In transgenic mice over-

expressing the receptor antagonist, the lesion size after 24-

h permanent ischemia was reduced by ~50% (1249).

These quite dramatic savings were not mediated by re-

duced temperatures. This effect is not mediated by block-

ing white cell accumulation, since the latter is only pro-

ective in temporary, and not permanent, focal ischemia (203).

Surprisingly, when injected directly into brain, the antagonist had to be put into the striatal core, even to protect the cortex (1075). This suggests that IL-1β effects are being exerted in or near the core of the lesion and that resulting damage moves out into cortex. One such mecha-

anism would be via spread of glutamate or K⁺ (and pos-

ibly resulting intraischemic depolarization). Interleu-

kin-1 does lead to glutamate release in cerebral tissue (968). Thus the antagonist may block core release of glutamate; this should be measured. Core release is blocked in nNOS knockouts (1031), and these two events may be related.

B) TNF-α. Tumor necrosis factor-α appears in the circulation within 20 min of initiating focal ischemia and persists for at least 2 h of reperfusion (636). This may be independent of its synthesis. It is upregulated in microglia and macrophages within 30 min of initiating focal ischemia, and its levels peak by 8 h (154). The receptor, TNFR1, which mediates toxic effects and possesses the “death domain,” is upregulated by ~6 h (116). Tumor necrosis factor-α is also upregulated ~6 h after 5- to 10-min global ischemia in the gerbil (975) and within 1.5 h after global ischemia in the mouse, largely in microglia (1155). Thus the time course of expression is similar to that of IL-1β, although the cell types in which the two cytokines are expressed are somewhat different.

Tumor necrosis factor-α has actually been shown to be protective in vitro, and when added many hours before ischemia in vivo (190), suggesting it may engender longer-term protective effects, as discussed in the section on ischemic tolerance. Indeed, TNF-α receptor null mice have increased susceptibility to ischemic injury (131). This may well reflect the protein synthesis-dependent anti-apoptotic protective effect that is induced by TNF-α in many of its target cells (43, 71, 1160, 1186). This effect may be sustained in basal conditions by circulating TNF-α. In contrast to these artifactual effects, there is a large body of evidence indicating that cytokine that is endogenously released during ischemia is toxic in both transient and permanent focal ischemia. Damage is strongly attenuated by antibodies in the circulation (636), or in the cortex/ventricles (746, 1248), or by binding proteins in cortex (812) and also by a drug that prevents TNF-α synthesis in brain by inhibiting its translation quite specifically (746). The level of protection reached a 75% reduction in infarct volume, which is extremely large.

Thus, as for IL-1β, TNF-α is upregulated quite soon after the onset of focal ischemia and within hours of starting global ischemia. It is shown to be damaging in both temporary and permanent focal ischemia. Unfortunately, there are no reports on whether or not cytokines are damaging in global ischemia.

C) MECHANISMS OF UPREGULATION OF CYTOKINES. Preexisting cytokines are either released from cells, such as macro-

phages, or are synthesized in response to the ischemic insult. Their synthesis is strongly coupled so that each activates synthesis of the other, and this could account for the appearance of both cytokines. Alternatively, their synthesis could be activated independently. Sequelae of ischemia that are known to activate cytokine synthesis (and release) include free radicals, possibly via activating NFκB and activation of MAP kinase (747). At this stage, however, the trigger for synthesis or release is not known (747). More distally MK-801, at levels which decreased infarct size by ~50% (3 mg/kg), reduced the TNF-α production by 60% measured after 12 h of permanent MCA occlusion (90). This suggests that NMDA receptor activation, presumably via increased cell Ca²⁺, is a major trigger for TNF-α production. Thus much of the damaging action of NMDA receptor activation may be mediated by activation of TNF-α synthesis. This may well be very important, and it would be interesting to know whether IL-1β synthesis is also modulated in this way.

D) MECHANISMS OF DAMAGE BY THE CYTOKINES. These mol-

ecules strongly interact with each other and influence a very large number of signal transduction mechanisms including those affecting gene activation (83). Thus deter-

mining how the cytokines enhance damage is a formidable task that has only just begun to be tackled. It is, of
course, a very important issue because it should give insight into basic mechanisms of ischemic cell damage.

I) Vascular effects. One well-worked out effect of the cytokines is upregulation of endothelial cell adhesion molecules, in response to ischemia (1035), and the (resulting) accumulation of neutrophils in ischemia (352). This may mediate the action of TNF-α in temporary ischemia, where there is good evidence that neutrophil accumulation is damaging, but it cannot mediate the effect in permanent ischemia where the evidence strongly indicates that neutrophil accumulation does not exacerbate damage. The same is true for IL-1β, which is very effective against permanent ischemia. Other effects on the vasculature are certainly possible, particularly because circulating antibodies to TNF-α very effectively prevented damage in temporary focal ischemia. However, intracerebrally injected antibodies or inhibitors are protective in many studies, and it seems likely that circulating TNF-α is damaging after uptake into the brain; there is significant pinocytosis after focal ischemia (895, 898).

II) Generation of free radicals. Cyclooxygenase-2 synthesis and iNOS synthesis (477) are induced by both cytokines, and might certainly mediate their effects, by enhancing free radical production. Interleukin-1β is particularly effective in activating iNOS in cultured vascular endothelial cells (113).

Tumor necrosis factor-α toxicity is strongly attenuated by overexpression of Mn-SOD in some tumor cells (1221), as is ischemic damage. This indicates that mitochondrial generation of free radicals may be a mechanism of damage. This idea is supported by a good study, again in tumor cells (1003). Tumor necrosis factor-α affected complex I of the mitochondrial electron transport chain and enhanced free radical production via ubiquinone. Toxicity could be strongly alleviated by blocking electron transport through that site, and also by free radical scavengers. Toxicity was enhanced by blocking electron transport at a more distal site which should enhance free radical production. These results are compatible with what is known about mitochondria in ischemic damage. It is not known why the TNF-α affects the mitochondria.

III) Apoptosis. Tumor necrosis factor-α is a potent activator of apoptosis via the TNF1 receptor, which is upregulated after ischemia. Although TNF-α activation of apoptosis requires inhibition of protein synthesis in several systems (1160), this is not the case for neurons, at least in culture (311), so that this is a viable mechanism for TNF-α action after ischemia. The putative apoptotic death after ischemia is blocked by protein synthesis inhibitors.

E) Summary. The profound protection by the antagonists, particularly those to TNF-α, suggests that the cytokines are responsible for a great deal of the damage in focal ischemia. Studies of effects on global ischemic damage are still needed. Further work is necessary to determine how early cytokine release and later synthesis are activated and, in determining the mechanism of their action. There is a great need for studies in which TNF-α and IL-1β actions are blocked and where measurements of free radical production, NO production, and glutamate release are then made.

3. c-Jun

There is reasonable evidence that c-Jun protein expression well after the ischemia might initiate damage, perhaps by apoptosis. There is a massive expression of c-Jun 24 h after 15-min hypoxia/ischemia in adult rats that is restricted to cell populations that are destined for death (270, 271). This synthesis also occurs after status epilepticus (271). A less detailed study reports a delayed induction of mRNA for c-Jun and c-fos in CA1 pyramidal cells of the rat 24–48 h after 20 min of 4-VO (1203). The basis for the late increase in c-Jun is not known but certainly could be delayed free radical production, possibly via COX-2 and/or NOS upregulation.

Evidence that this could be toxic comes from sympathetic neuron cultures. c-Jun was activated after NGF withdrawal, and when a c-Jun dominant negative mutant was introduced into these cells it prevented the cell death that normally occurred after the NGF withdrawal. Constitutive expression of c-Jun caused apoptosis in these cells even in the presence of NGF (415). Although clearly not definitive, these results indicate the potential importance of delayed expression of a gene that regulates transcription factors. The c-Jun target(s) is not known.

F. Ischemic Tolerance

1. Definition of phenomenon

Nonlethal short exposures to ischemia in both rats and gerbils are extremely neuroprotective. When the animals are challenged between 2 and 4 days later with ischemic insults that are usually lethal, there is almost no damage in global ischemia and much-reduced damage in focal ischemia (64, 445, 584, 586, 681, 846, 847). Similar protection is provided by short periods of thermal stress (196, 587), cortical spreading depression (733), and mechanical manipulation (372). Tolerance induction cuts across ischemic models in that 5 min of 4-VO global ischemia in rat provided very substantial protection against subsequent focal ischemia, 4 days later. Infarct volume after 3 h of temporary MCA occlusion was reduced, from 69 to 23 mm3 (732).

In an important study it was shown that induced tolerance in CA1 did not alter the initial profound response to ischemia, which included dissolution of microtubules, blockade of protein synthesis, and disaggregation of polysomes (348); rather, it allowed recovery from these changes in the CA1 pyramidal cells. Understanding what factor(s) mediates tolerance will provide major insight into what is normally causing damage.
2. Proteins that may be responsible for tolerance

There are four generically different explanations for tolerance: 1) tolerance may provide vulnerable cells with a recovery mechanism that other cells already have, 2) tolerance may provide vulnerable and nonvulnerable cells with a new recovery mechanism, 3) tolerance may reduce the initial insult in vulnerable cells so they can recover using normal mechanisms, and 4) tolerance may remove a normally lethal mechanism such as upregulation of COX.

Because tolerance takes several days to become maximal, and lasts for 7 days (543, 545, 584, 998), it seems very likely that it involves the synthesis of new protein. The importance of synthesis directly after the short pre-conditioning bout has been nicely shown for tolerance in focal ischemia where administration of cycloheximide before the 10-min focal ischemia completely prevented the induction of tolerance to prolonged MCA occlusion (64). Later additions of cycloheximide had no effect. The mechanistic focus has thus been on protective proteins that might be induced by the nonlethal stresses and that might then protect vulnerable tissues during a major insult. The “protective” stress-related proteins discussed earlier in this section thus all become attractive candidates; they are induced after brief insults, show a time course of expression that quite closely parallels the time course of tolerance induction, and are protective when administered artifactualy. Furthermore, all these proteins are expressed in nonvulnerable regions following ischemia so may well be acting protectively when expressed endogenously.

A) HSP. The most widespread hypothesis had been that induction of HSP by the preexposure protected during the later ischemia, since there appeared to be strong correlations between HSP expression before the second ischemia and tolerance in gerbils (547, 584) and rats (681). However, more detailed examination has weakened the correlation.

First, as discussed above, HSP does rise in CA1 of rat hippocampus 1 day after lethal ischemic exposures (681) so that its induction by prior insult seems unnecessary. However, it is possible that HSP is not present early enough to protect CA1, unless it is induced by tolerance. There are more empirical arguments against HSP involvement. The correlation between HSP elevation and the degree of tolerance is very nonlinear (584). One day after the short (2 min) ischemia in gerbils there is substantial tolerance with very little HSP induction. At 4 days, there is better tolerance than at 2 days, despite the fact that HSP levels are about the same as those two times. Along the same lines is a careful study by Abe and Nowack (1). Two-minute preischemia protected 86% of the neurons against 5-min ischemia 4 days later, but HSP mRNA had only increased in 46% of the neurons at the time of the second ischemia. Thus tolerance occurred in the absence of (measurable) HSP mRNA synthesis. In a third study, anisomycin added at the time of the initial short ischemia (and present for ~2 h later) largely inhibited the increased HSP synthesis. However, it did not affect tolerance (546). All these results could be explained by a very nonlinear relationship between HSP and tolerance where small changes in HSP produce the large protective effects, but there is no particular reason to think that such a relationship exists.

Although the above results militate against a role for prior induction of HSP in tolerance, another study shows tolerance may be associated with an increased ability of CA1 pyramidal cells to synthesize HSP in response to the second insult. Heat shock protein was greatly enhanced in CA1 3 h after 3.5-min ischemia in gerbil, when tolerance had been induced by 2-min prior ischemia (28). If this HSP synthesis is, indeed, conferring the tolerance, then the issue becomes what is allowing its synthesis to be activated after ischemia in the tolerant brain.

B) BCL-2. At present, Bcl-2 is the least-flawed candidate for mediating tolerance. There is a good correlation between the time courses of appearance of Bcl-2 and of tolerance. Bcl-2 appeared in CA1 30 h after 2-min ischemia in gerbil. It was not present at 12 h and was maximal at 4 days (1029). Tolerance to ischemic cell death due to 5-min ischemia followed a very similar time course (545). However, more detailed studies might reveal discrepancies, as they did for HSP.

C) NEUROTROPHINS. Although neurotrophins seem reasonable candidates a priori, there is a quite persuasive study that argues strongly that they do not have a role (445). Expression of NGF and BDNF mRNA are very slightly upregulated by (generally lethal) 6-min ischemia. However, if the 6-min ischemia is preceded by a 3-min conditioning pulse that induces tolerance, then the neurotrophin mRNA are drastically downregulated 1 day after the 6-min ischemia. Without measuring the protein, no firm inferences can be drawn, but this result suggests that the neurotrophins are rendered less potent by the tolerance-inducing stimulus.

D) SUMMARY. A very notable feature of these studies is that while ~2 min of ischemia leads to induction of protective proteins in CA1, 5 min does not, although it does in other cell types. This key event may well be a critical feature of damage development in vulnerable cells. Presumably events occur in CA1 after a short duration of ischemia that make it impossible for those cells to synthesize protective proteins. The most likely locale for this is at the level of global inhibition of protein synthesis. Synthesis inhibition in CA1 is certainly more long-lasting than in other regions and may well be more profound at early times; this has not been rigorously tested. If this is the basis for the inability to synthesize protective proteins, then inhibition of global protein synthesis assumes a dominant role in development of cell death after global ischemia.
3. Mechanism of induction of tolerance

There is now evidence that IL-1α or IL-1β (847), or TNF-α (813), elevation may induce, but not mediate, the tolerance. Evidence is more complete for the interleukins.

Interleukin-1α and IL-1β are moderately elevated in blood after 2- to 3-min global ischemia. Maintenance of IL-1ra, the IL receptor antagonist, during the interval between the short ischemia and the usually lethal ischemia completely prevented the induction of tolerance. Furthermore, injection of IL-1β or IL-1α for 3 days, in the absence of an inducing ischemia, led to very strong tolerance against the 5-min ischemia. If this is substantiated, then IL-1α may be acting to induce synthesis of stress-related protein such as Bcl-2, or HSP, or may be acting to allow the synthesis of HSP during ischemia, as discussed above. This should be able to be determined and to provide insight into the species responsible for tolerance.

Tumor necrosis factor-α may also mediate tolerance, in focal ischemia, although the data are not nearly as strong as for the action of IL-1β in global ischemia described above. Injection of TNF-α into the cortex 2 days before 24-h MCA occlusion reduced the size of the infarct (813).

These results highlight the complex role that the cytokines play in ischemic damage. Not only do they enhance damage during a normally damaging insult but, presumably by activating genes, they induce tolerance by increasing synthesis of protective proteins several days after nonlethal insults.

4. Cellular mechanisms of tolerance

The few existing studies show that tolerance appears to be associated with an increased ability to buffer cytosolic Ca²⁺ loads. Ohta et al. (846) showed that mitochondria in tolerant neurons (in vivo) accumulate more Ca²⁺ than normal CA1 pyramidal layer neurons within the first 15 min after global ischemia, whereas cytoplasm has much less total Ca²⁺. Over the next several hours, the cells in tolerant animals lose their mitochondrial and cytoplasmic Ca²⁺, whereas those in normally vulnerable neurons maintain elevated mitochondrial Ca²⁺ and also elevated cytosolic Ca²⁺ (846). Thus mitochondria in tolerant neurons show much better buffering of entering Ca²⁺ and, overall, lower levels of total Ca²⁺ in the cytoplasm during the postischemic period. The basis for the latter may be the early buffering by the tolerant mitochondria, but it may also be because the short, tolerance-inducing, ischemic exposures induce a twofold increase in plasmalemma Ca²⁺-ATPase (846). Interestingly, all the properties associated with the tolerant CA1 neurons are shown by CA3 neurons, whether or not they have been exposed to the short ischemic episodes, confirming that tolerance represents the acquisition of properties shown by less vulnerable cells. In a study by a different group, 2-min ischemia 2 days before the insult reduced the fluo-

rescence increase of the Ca²⁺ dye rhod 2-AM in CA1 cells by 50% when excised slices were exposed to in vitro ischemia (1030). Although the relationship between the two studies was not discussed, it is very probable that the increased buffering by the mitochondria plus the enhanced Ca²⁺-ATPase activity, noted in vivo in the first study, is the cause of the reduced cytosolic Ca²⁺ accumulation noted in both studies.

Thus the ischemia-tolerant neurons appear to be able to buffer entering Ca²⁺ better than normal using the mitochondria, and also to eliminate Ca²⁺ from the mitochondria far more quickly after the ischemia. As mentioned above, at least the first of these effects is similar to those caused by overexpression of Bcl-2 (787). This is consistent with the observed presence of Bcl-2 expression in tolerant cells. It does not prove that the Ca²⁺ effect, or enhanced Bcl–2, is the basis for tolerance.

To the extent that it explains tolerance, this result is consistent with the third generic mechanism, described at the beginning of the section. Such an effect might well reduce the damage due to increased cytosolic Ca²⁺ in vulnerable cells as distinct from targeting the recovery process. The result also highlights the importance of Ca²⁺ in damage. Bcl-2 is not normally upregulated until 2–4 h after a global ischemic insult. Thus the result suggests that effective buffering of cytosolic Ca²⁺, or uptake into mitochondria without causing damage, as late as 4 h after ischemia will protect neurons. The corollary is that elevated cytosolic Ca²⁺, or the inability of mitochondria to appropriately handle Ca²⁺, is damaging well after the ischemia, implying that cytosolic Ca²⁺ levels remain somewhat elevated and damaging when insults are toxic.

G. Summary

A large number of putatively protective genes are expressed after global ischemia, mainly in cells that are not destined to die. Although appealing as a correlation, and teleologically, there is no evidence, yet, that this endogenous production of growth factors, Bcl-2, or HSP (or immediate early genes) actually protects cells from the insults, nor is this known for focal ischemia. Creative studies with knockouts will be necessary to establish this. The possibility will be supported by the tolerance phenomenon if it can be clearly shown that tolerance results from synthesis of one of these classes of molecules.

Vulnerable cells, in global or focal ischemia, suffer from the absence of synthesis of these protective proteins, or in some cases from their downregulation. This might well be contributing to development of cell death, and this would be well demonstrated if tolerance occurs as a result of their synthesis. Why these proteins, as well as immediate early genes, are not synthesized in vulnerable cells is unknown. It could result from the general downregulation of synthesis, although this is not a compelling argument. Certainly in
global ischemia synthesis in all regions is very low for the first 6–12 h, the times at which these proteins are being synthesized. Thus determining the basis for the differential synthesis of protective proteins is an outstanding task. In particular, what is the reason that CA1 cells are unable to synthesize so many of these proteins that are upregulated by other cells (Table 5)? Lack of synthesis of these proteins is not as clearly correlated with cell damage in focal ischemia. This may be simply due to experimental conditions; appearance of the proteins needs to be correlated with cell viability in a very mixed population of dead and living cells.

At the opposite pole, and of equal if not more importance, it now appears very likely that induction of a set of putatively damaging genes is a critical factor in the sensitivity of the penumbra to focal ischemic challenges. It may also be very important in global ischemia, but this has not been shown. Cyclooxygenase-2 and iNOS, which are clearly capable of causing damage, are upregulated, and their inhibition ameliorates damage. Interleukin-1β and TNF-α are upregulated, as well as being released, and although these cytokines can be protective when added before ischemia, they appear to be very damaging when released during or after ischemia. At present, the pharmacological interventions have been limited to focal ischemia and, for interleukin, to only permanent ischemia. However, many of the proteins are activated in global ischemia and, when it is done, pharmacology in that model may well be revealing. The basis for activation of these genes needs to be determined, as does the mechanism by which the cytokines are exerting their damage. It is likely that a vicious cycle may develop in which free radicals activate synthesis of proteins that beget free radicals.

Early observations on the cellular changes involved in tolerance have shed light on a role that the protective proteins might play; they might act to enhance the Ca\(^{2+}\) buffering capabilities of the mitochondria and the cell membrane. If so, then the latter are important sites of vulnerability during ischemia.

An interesting result from this series of studies is that NMDA receptor activation is required for synthesis of immediate early genes and HSP in global ischemia. To the extent that these are beneficial, and there is reason to think they are, this would render NMDA antagonists harmful. This is notable with respect to clinical trials.

IX. FINAL CONCLUSIONS

A. Mechanisms of Ischemic Damage

The major focus of the review has been to evaluate the likelihood that different ion, metabolite, macromolecular, and functional changes participate in causing cell death in the major models of ischemia. This section is devoted to synthesizing the information into reasonable hypotheses for cell death in global and focal ischemia, the two models that have been considered most intensively. There is no attempt to summarize the material in each section because each change was covered very thoroughly and summarized. Here the “bottom lines” are used to try and describe reasonable mechanisms for cell death.

Tables 4 and 5 reflect much of what has been discussed in the review and are very useful in constructing hypotheses for cell damage. Table 4 lists the results of most of the pharmacological, knockout, and transgenic studies that have been used to determine which variables are involved in ischemic cell death. Table 5 lists changes in, and basal levels of, variables that are different in vulnerable (CA1 hippocampal pyramidal cells) and nonvulnerable cells, and thus that might be responsible for the great vulnerability of the CA1 pyramidal cells in global ischemia.

1. Mechanism of cell death after global ischemia

A) Participation of different processes in cell death.

There is reasonably strong causal evidence, derived from pharmacological or other types of intervention, that certain changes are critical for development of damage after global ischemia. Much of this is apparent from Figure 1 and Table 4. These include 1) decreased pH, 2) entry of Na\(^+\) in posts ischemic period, 3) posts ischemic Ca\(^{2+}\) entering via voltage-dependent Ca\(^{2+}\) channels, 4) calpain, 5) free radical action, 6) NO generation, 7) caspase activation, and 8) accumulation of zinc.

The level of confidence in these conclusions was discussed in the main text, but it is great enough to incorporate them into a model for damage with reasonable certainty.

There are other changes in ions and metabolites that are very probably damaging, but for which explicit evidence in ischemia is very weak or lacking. These include 1) decreased ATP during ischemia, 2) increased cytosolic Ca\(^{2+}\) during ischemia, 3) increased cytoplasmic Na\(^+\) during ischemia, 4) increased FFA during ischemia, and 5) increased extracellular glutamate.

There is evidence that certain macromolecular changes are also important. In this case the studies do not establish causality, but they do show appropriate changes in the amounts or activities of the molecules which, combined with what is known of their effects, suggests strongly that they will be important in damage. Many of these are indicated in Table 5. They include 1) increased in Bax/Bcl-2, 2) decrease of SOD 1 day after the insult; 3) increased levels of TNF-α; 4) increased iNOS in glia starting 1 day after the insult; 5) failure to synthesize protective proteins such as growth factors, HSP, and Bcl-2; 6) loss of BDNF; and 7) prolonged diminution in CaMKII and PKC activities.

Another change that is certainly a possible basis for damage, but for which evidence is too restricted at present, is the loss of mitochondrial synthesis of cytochrome aa₃.
Finally, there is recent evidence, which suggests but far from proves, that microglial activation plays a role in global ischemic damage.

All of the above are within the class of damage inducers; they are factors that will ultimately affect macromolecular structure, and hence cell function. Although there is some uncertainty about the time at which these different changes become important, because measurements are often not directed at this, it is reasonably certain that they all play an important role. It is notable how many processes are involved in cell death, and it is quite certain that more will be discovered. It is obviously difficult to construct a unique model for damage when so many variables are involved. However, it is important to try and do this, both for initiation of apoptotic change and for necrotic cell death. In the latter case, the question is how the inducers, and particularly the perpetrators, cause the prolonged macromolecular and functional changes associated with cell death.

I) Early events. Global ischemia is characterized by a short and what surely must be characterized as a tumultuous period during electron transport blockade. The loss of oxygen and glucose initiate a great many intracellular ion and metabolite changes that are both very rapid and very large. These include decreased ATP, increased ion and metabolite changes that are both very rapid and very large. These include decreased ATP, increased cAMP, decreased pH, increased cytosolic Ca2+, increased cytosolic Na+, membrane depolarization, free radical production, release of glutamate (and other transmitters), increases in FFA, and decreases in phospholipids, in particular phosphatidylinositides. Although these clearly set in motion the prolonged development of apoptotic and necrotic damage and cell death, it has been almost im-

### TABLE 4. Agents that mitigate ischemic cell death

<table>
<thead>
<tr>
<th>Agent</th>
<th>Insult</th>
<th>Protection and Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antileukocyte adhesion agents</td>
<td>Temporary focal*</td>
<td></td>
</tr>
<tr>
<td>Matrix metalloproteinase inhibitor</td>
<td>Permanent focal</td>
<td>+ +</td>
</tr>
<tr>
<td>Caspase-3 inhibitors</td>
<td>Temporary focal</td>
<td>+ +</td>
</tr>
<tr>
<td></td>
<td>Global</td>
<td>+ +</td>
</tr>
<tr>
<td></td>
<td>Mild temporary focal</td>
<td>+ +</td>
</tr>
<tr>
<td>Antibody to CD95 ligand</td>
<td>Temporary focal</td>
<td>+ +</td>
</tr>
<tr>
<td>CDP-choline</td>
<td>Temporary focal</td>
<td>+ +</td>
</tr>
<tr>
<td></td>
<td>Global</td>
<td>+ +</td>
</tr>
<tr>
<td></td>
<td>Functional recovery</td>
<td></td>
</tr>
<tr>
<td>Reducing free radical accumulation in various ways</td>
<td>Global (rat and gerbil)</td>
<td>+ to +</td>
</tr>
<tr>
<td></td>
<td>Temporary focal</td>
<td>+ + to + + +</td>
</tr>
<tr>
<td>NADPH oxidase knockout</td>
<td>Temporary focal</td>
<td>+ +</td>
</tr>
<tr>
<td>nNOS inhibitors or knockouts</td>
<td>Temporary focal</td>
<td>+ +</td>
</tr>
<tr>
<td></td>
<td>Permanent focal</td>
<td>+ +</td>
</tr>
<tr>
<td>PARP inhibitors</td>
<td>Temporary focal*</td>
<td>+ +</td>
</tr>
<tr>
<td>COX-2 inhibitor</td>
<td>Temporary focal</td>
<td>+ +</td>
</tr>
<tr>
<td></td>
<td>Global</td>
<td>+ +</td>
</tr>
<tr>
<td>nNOS inhibitors and knockouts</td>
<td>Temporary and permanent focal</td>
<td>+ +</td>
</tr>
<tr>
<td>Calpain inhibitors</td>
<td>Temporary focal</td>
<td>+ +</td>
</tr>
<tr>
<td></td>
<td>Global</td>
<td>+ +</td>
</tr>
<tr>
<td></td>
<td>(Temperature not monitored)</td>
<td></td>
</tr>
<tr>
<td>L- and N-type Ca2+ channel antagonists</td>
<td>Global</td>
<td>+ (Postischemic)</td>
</tr>
<tr>
<td>Calmodulin antagonist (trifluoperazine)</td>
<td>Temporary focal</td>
<td>+ + (+) (Drug specificity poor)</td>
</tr>
<tr>
<td>NMDA antagonists</td>
<td>Temporary focal</td>
<td>+ + (+)</td>
</tr>
<tr>
<td></td>
<td>Permanent focal</td>
<td>+ +</td>
</tr>
<tr>
<td>PAF antagonist</td>
<td>Permanent focal</td>
<td>+ +</td>
</tr>
<tr>
<td></td>
<td>Global</td>
<td>+ +</td>
</tr>
<tr>
<td>AMPA/kainate antagonist</td>
<td>Global</td>
<td>+ + (Very high doses needed)</td>
</tr>
<tr>
<td></td>
<td>(In area tempesta)</td>
<td></td>
</tr>
<tr>
<td>Hypoglycemia</td>
<td>Permanent focal</td>
<td>+ +</td>
</tr>
<tr>
<td></td>
<td>Global</td>
<td>+ +</td>
</tr>
<tr>
<td>Interleukin-1 antagonism</td>
<td>Hypoxia/ischemia</td>
<td>+ +</td>
</tr>
<tr>
<td>TNF-α antagonism</td>
<td>Permanent focal</td>
<td>+ +</td>
</tr>
<tr>
<td></td>
<td>Permanent focal</td>
<td>+ + (+)</td>
</tr>
<tr>
<td>Block of polyamine oxidase; reduces 3-aminopropanal (408)†</td>
<td>Temporary focal</td>
<td>+ + (+)</td>
</tr>
<tr>
<td></td>
<td>Permanent focal</td>
<td>+ +</td>
</tr>
<tr>
<td></td>
<td>(Core protected)</td>
<td></td>
</tr>
<tr>
<td>S20 proteasome inhibition</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Agents selected are those that affect known processes as distinct from those that provide protection in an unknown fashion (e.g., BDNF, adenosine). All the studies were described in text, and antagonists are presented in order they were discussed in text. For focal ischemia: + is 10–15% reduction in infarct; + + is <45% reduction; + + + is >45% reduction; + + + (+) is 75% reduction or greater. For global ischemia: + is <20% reduction in CA1 cell death; + + is <50% reduction, and + + + is >50% reduction. *Indicates agent was tested in other form of focal ischemia and was ineffective. †Reference given because not discussed in text. nNOS, neuronal nitric oxide synthase; PARP, poly(ADP-ribose) polymerase; COX-2, cyclooxygenase-2; iNOS, inducible nitric oxide synthase; PAF, platelet-activating factor; NMDA, N-methyl-D-aspartate; CDP-choline, cytidine diphosphate choline; TNF-α, tumor necrosis factor-α.
TABLE 5. Potential bases for selective vulnerability of CA1 pyramidal cells

<table>
<thead>
<tr>
<th>Detrimental Changes in CA1</th>
<th>Beneficial Changes in Other Cells Which Do Not Occur in CA1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increased Caspase mRNA levels (826)</td>
<td>Synthesis of immediate early gene protein products (567)</td>
</tr>
<tr>
<td>Protein synthesis permanently depressed (700, 1113)</td>
<td>Synthesis of BDNF (599)</td>
</tr>
<tr>
<td>Decreased SOD after 24 h (680, 734, 887)</td>
<td>Preferential synthesis of heat shock protein (gerbil only) (840, 1121)</td>
</tr>
<tr>
<td>Maintained elevation of free fatty acids (4)</td>
<td>Synthesis of Bcl-2 (181)</td>
</tr>
<tr>
<td>Basal calpastatin levels low (344)</td>
<td></td>
</tr>
<tr>
<td>Large sustained decrease in PIP and PIP2</td>
<td></td>
</tr>
<tr>
<td>Persistent decreased activity and translocation of CaMKII (780)</td>
<td></td>
</tr>
<tr>
<td>Sustained decrease in tyrosine kinase and casein kinase (467, 468)</td>
<td></td>
</tr>
<tr>
<td>Elevation of calcium/lowering of ATP at 24 h (452)</td>
<td></td>
</tr>
<tr>
<td>Zinc accumulation (595, 1138)</td>
<td></td>
</tr>
<tr>
<td>Delayed decrease in GluR2/GluR1 receptor ratio (384)</td>
<td></td>
</tr>
<tr>
<td>Slow recovery of pH (784)</td>
<td></td>
</tr>
<tr>
<td>Prolonged elevation of NFkB (206)</td>
<td></td>
</tr>
<tr>
<td>Permanent loss of ubiquitin (251, 703, 1241)</td>
<td></td>
</tr>
<tr>
<td>Loss of BDNF (509)</td>
<td></td>
</tr>
<tr>
<td>c-Jun mRNA elevation 24–48 h after ischemia (1203)</td>
<td></td>
</tr>
<tr>
<td>Selective vulnerability of CA1 cells to superoxide free radical, in organotypic culture (1212)</td>
<td></td>
</tr>
</tbody>
</table>

Differences between responses of vulnerable (CA1 pyramidal) and nonvulnerable (CA3 or dentate granule) cells in hippocampus are shown that might reasonably account for vulnerability of CA1 cells to global ischemia. SOD, superoxide dismutase; PIP, phosphatidylinositol 4-monophosphate; PIP2, phosphatidylinositol 4,5-bisphosphate; CaMKII, Ca2+/calmodulin-dependent protein kinase II; GluR, glutamate receptor. Reference numbers are given in parentheses.

Possible to explicitly demonstrate in a satisfactory way that any one of them is involved. Thus one has to work backwards from what is known about the later processes that are important.

Inhibitor studies strongly suggest that important sequelae of the early maelstrom is maintenance of damaging conditions that include the continued generation of free radicals and the maintained increase in cytosolic Ca2+ and Na+.

Continued free radical generation is postulated to result from mitochondrial damage, which generates free radicals and which is amplified by a cycle involving free radical activation of NFkB, TNF-alfa, and IL-1 synthesis, and further activation of mitochondrial free radical generation. This system, and activation of NFkB in particular, leads to delayed elevation of COX-2 and iNOS, which enhance free radical and peroxynitrite production.

Increased Ca2+ and Na+ are likely to be maintained by long-term changes in voltage-dependent channels or NMDA receptors, based on studies of the efficacies of inhibitors. These could result from calpain-mediated cleavage (439), from free radical attacks, or from other persistent modifications. The increased Na+ might well enhance the increased Ca2+ via the 3Na+/Ca2+ exchanger. The elevated Ca2+ will contribute to ongoing mitochondrial damage, partly by activating the MPT. Other key molecular damages are also maintained, including inactivation of protein kinases and decreases in phosphoinositides. These are likely to be very important in preventing critical cell signaling. The bases for the initial inactivations are not known but could also be proteases or free radicals. The profound inhibition of protein synthesis in vulnerable cells ensures that channels, kinases, and possibly key enzymes of PIP metabolism are not resynthesized after the initial inactivations.

The overall postulate is that these prolonged ionic and macromolecular changes result from the early events, particularly the increased Ca2+ and free radical production (but possibly from other causes also), and lead to the subsequent major functional changes. As summarized below in section I.A3, explicit evidence for much of this is lacking.

II) Apoptosis. Despite the absence of an easily identifiable apoptotic morphology, the biochemical and pharmacological evidence strongly suggests that the apoptotic program plays an important role in cell death. The most likely initiators of the process are one or more of the following: CD-95 ligand, TNF-alfa, the cyclin D1, the prolonged decrease in several protein kinases, and decreased amounts of neurotrophin or ability of neurotrophin to signal. The question then is what causes these changes. A reasonable basis for CD-95 ligand and TNF-alfa is the continual generation of free radicals discussed above with NFkB activating the appropriate genes. The upregulation of cyclins may result from the increased MEK/MAP kinase activity (1129), which in turn results from increased cytosolic Ca2+, or from the upregulation of c-Jun (1218), which may in turn result from free radical action or the MEK/MAP kinase pathway. The basis for the prolonged protein kinase inactivations is not known but may well reflect the inhibition of global protein synthesis as discussed above, and the lowered phosphoinositides would contribute to the “growth factor withdrawal” syndrome.

The way in which the ensuing “post global-ischemic” apoptosis differs from more standard sequences is not known. The protection by caspase inhibitors, although
not great, indicates that proteolytic actions of caspases contribute to the death of the neurons. Assessment of changes in specific protein targets, both intranuclear and extranuclear, is required to get a full idea of the apoptotic contribution. The paucity of apoptotic nuclear morphology may result from activation of other endonucleases or enzymes by ischemia, which prevent the normal chromatin condensation, as indicated by the abnormal cleavage pattern of the DNA.

III) Necrosis. The progression of damage is characterized by a fairly dramatic, delayed, coincidence of changes about a day after the ischemia. These include decreased levels of SOD in the CA1 pyramidal cells, increased superoxide in the same cells, appearance of induced COX-2 in these cells and iNOS in adjoining astroglia, a change in the subunit composition of AMPA receptors which renders them far more permeable to Ca\(^{2+}\), and also a major activation of calpain. The reduced SOD may result simply from reduced global protein synthesis; it is likely to be instrumental in the increase of free radicals. The change in AMPA receptor composition results from action at the genome, but the mechanism is unknown. Calpain activation may result from a delayed rise in Ca\(^{2+}\) but could also result from long-term changes in calpastatin or in activators of the enzyme. All these events appear to be damaging (although there is no explicit evidence for iNOS) so that it is reasonable to assume that this is a key event in the development of necrotic cell death (it may also be important in the initiation of apoptotic processes as both calpain and free radicals are important). Although not explicitly measured at this late time, the prolonged elevation of Ca\(^{2+}\) should also maintain activity of nNOS and, hence, peroxynitrite production. The altered AMPA receptor composition is likely to exacerbate the increase in cytosolic Ca\(^{2+}\). Thus the stage is set for quite massive attacks on different cell functions.

The calpain increase could be responsible for the delayed disorganization of microtubules (in rats), which adds to the damage done by the blockade of transport initiated by damage to motor proteins.

Mitochondrial dysfunction is likely to be exacerbated by the increased production of free radicals/oxynitrite (particularly because it is the mitochondrial SOD that is downregulated). The free radicals will damage electron transport and also sensitize the MTP to Ca\(^{2+}\). If cytosolic Ca\(^{2+}\) levels increase, via the altered AMPA receptors, it will greatly exaggerate these changes. The delayed reduction in cytochrome aa\(_3\) should also inhibit mitochondrial function.

Excess free radical/oxynitrite production, along with diminished protein synthesis and intracellular transport, possible activation of PLA\(_2\) by Ca\(^{2+}\), might well cause membrane dysfunction and possible leakiness, although this has not been tested.

At some stage, possibly aided by a massive proteolysis due to cathepsin upregulation or proteasome activity (which has not been tested), the cell undergoes the phase change that characterizes ICC.

There is reasonably good evidence for autophagocytosis in the gerbil, and this may well represent a parallel process that is continuing along with those described above. It may well be associated with the proliferation of ER, since the autophagic vacuoles appear at the time the ER proliferation falls away. Some suggestions have been made for how it is induced, but much more work is needed to define that process.

b) Summary. The key events in necrotic cell death are postulated to be the persistent generation of free radicals, cytosolic Ca\(^{2+}\), and Na\(^+\) (acting largely to maintain Ca\(^{2+}\) but possibly, also, to maintain extracellular glutamate levels) in the vulnerable cells, brought about by several factors. Over time, these lead to the somewhat explosive increase in free radicals and calpain which, together, act on several cell functions to cause excessive damage and, ultimately, a massive change in cell morphology via a “phase change.” The process is enabled by the persistent inhibition of protein synthesis and (probably as a consequence) the persistent downregulation of signaling systems.

Activation of an apoptotic cascade occurs in parallel with the necrotic processes. The activation results from one of several defined stimuli and leads to caspase activation. The resultant proteolysis adds to the necrotic processes described above to exacerbate damage; normal apoptotic morphology is precluded by the dominance of necrotic processes, evidenced by the relatively small saving effects of caspase inhibitors. Autophagocytotic breakdown may well contribute to the cell death process in the gerbil.

A key feature of ischemic death is that many different inhibitors alleviate much of the cell death, suggesting that death requires the combined effects of several activators and perpetrators. On reflection, this is perhaps not terribly surprising for relatively mild insults such as are being considered here. As discussed in section nB, there is a clear homeostatic force that tends to maintain a cell in a viable state. In order for a mild insult to overcome this, it is reasonable that many consequences of that insult will need to work together.

2. Mechanism of cell death after focal ischemia

Unless otherwise stated, all of the discussion pertains to cell death in the penumbra of the focal lesion. Little is known about the core because attempts to prevent damage have been almost universally unsuccessful.

a) Participation of different processes in cell death. As in global ischemia, there is a set of variables whose involvement in focal ischemic damage has been established by experiments in which causality has been tested. Many
of these variables overlap with those that are important in global ischemia, but there are additional ones.

I) Permanent focal ischemia. Variables involved in permanent focal ischemia include 1) decreased pH, 2) Ca2+ entry via NMDA receptors and/or membrane depolarization, 3) Na+ entry and/or membrane depolarization via AMPA/kainate receptors, 4) Na+ entry, 5) free radical generation, 6) NO generation, 7) PAF, 8) IL-1β, 9) TNF-α, 10) delayed iNOS induction, 11) COX-2 induction, and 12) matrix metalloproteinase.

II) Temporary focal ischemia. Several important variables that have been tested in permanent ischemia have not been studied in temporary ischemia, including decreased pH, interleukins, PAF, matrix metalloproteinases, and AMPA/kainate antagonists. At this stage, there is no reason to think that they are not important in temporary ischemia, since both insults include the same early ischemic period so that all the processes in permanent ischemia are probably active in temporary ischemia. On the other hand, there are two factors that are important in temporary ischemia that do not play a role in permanent ischemia: 1) PARP activation and 2) leukocyte adhesion.

Two other enzymes have not yet been tested in permanent ischemia. They are calpain activation and caspase activation. There is an increased Bax/Bcl-2 in permanent ischemia that is probably related to cell death, but this has not been directly tested.

Several changes that are quite dramatic following global ischemia have not been investigated in focal ischemia and so cannot yet be included with confidence in any model for damage. These include the decreased activity of SOD, the decreased synthesis of cytochromes aa3, and the decreased ratio of GluR2/GluR1 AMPA/kainate receptors.

B) General Features of the Cell Death Process: Comparison with Global Ischemia. The damage process has several features that distinguish it from global ischemia.

Damage generally develops quickly, with significant cell death occurring within 12–24 h. The long lag period does not exist. There is a small amount of more slowly developing cell death, between 2 and 3 days after the onset of ischemia. It may reflect a lag period for cells in the mildly energy-deprived periphery of the penumbra, or it may be relatively rapid cell death occurring in response to the further reduction in blood flow with time in the penumbra. Short periods of focal ischemia (30 min) do lead to very slowly developing cell death. Another major difference is the appearance of a more classical apoptotic morphology in many neurons. Although these differences may reflect the different cell types that are being analyzed (the hippocampal pyramidal neurons are not included in analyses of focal ischemic damage), they are more likely to result from the fact that the insult is more prolonged, but milder (ATP falls only by 25% in the penumbra).

The small drop in ATP in the penumbra means that the penumbral neurons will not be directly affected as strongly as neurons are in global ischemia. One noted manifestation of this is the absence of the anoxic depolarization in the penumbra, but there may well be other differences that arise from the smaller fall. For example, protein kinases may not be as strongly inactivated (this has not yet been measured). On the other hand, the reasonably high flow leads to a large production of free radicals in the region. It is very likely that penumbral damage relies on glutamate released from the core of the lesion; this substitutes for the profound ATP fall by leading to the intraischemic depolarizations and Ca2+ entry.

C) Permanent ischemia: Early events. Unlike global ischemia, where there are drastic short-lived early events, permanent focal ischemia is characterized by changes that last many hours and which, judging from times at which inhibitors are active, do not complete their damaging effects in the penumbra for several hours. These critical events include production of free radicals, release of glutamate largely from the core of the lesion that then diffuses to the penumbra, the resulting increase in cytosolic Ca2+ (which has not been measured but which is a very likely result of glutamate action and the intraischemic depolarizations), and the resulting activation of NO synthesis. The strong dependence of damage on NMDA and AMPA receptors suggests that the elevation of Ca2+ due to glutamate is a major factor in development of penumbral damage, with the glutamate largely emanating from the lesion core. This makes sense because the insult is so mild in the penumbra (25% reduction in ATP) that events there would not, in all probability, be able to initiate major damage. On the other hand, as noted from the anoxic depolarization in the core, major changes in ions including Ca2+ and Na+, and hence glutamate release, will occur there. Free radicals and NO (probably via peroxynitrite) also play a major role in the damage as seen from the very strong protective effects of free radical scavengers in focal ischemia. The major importance of decreased pH and of PAF in focal ischemic damage also speaks to the importance of free radicals.

D) Permanent ischemia: apoptosis. Although caspase inhibitors have not been used, morphology and caspase cleavage show that apoptosis is prevalent in permanent focal ischemia. The potential activators of apoptosis are the same as described for global ischemia, although decreases in protein kinases have not been studied, so they may not be a possibility. Unlike global ischemia, protective effects of cytokine blockade have actually been demonstrated, so there is a larger probability that they are important. The reason that apoptotic nuclei develop in focal ischemia while they are essentially nonexistent in global ischemia is obviously not known. One explanation is that cells take more divergent paths in focal ischemia, because of local flow heterogeneity. Thus the apoptotic program may not be as contaminated by events associated with necrosis.

E) Permanent ischemia: necrosis. The ongoing produc-
tion of free radicals and prolonged elevation of Ca\(^{2+}\) are very probably the critical factors in damage. Inducible NOS is induced in endothelial cells and leukocytes, and COX-2 is induced in neurons; thus, much as in global ischemia, there is self-generating mechanism for maintaining free radicals. It seems very likely that this involves the cytokine/NF\(\kappa\)B system, although this has not been shown explicitly. There is good evidence for mitochondrial damage as well, which should contribute to the free radical generation. On the basis of inhibitor studies, these actions become lethal after \(~6\) h. Although no studies have been done with calpain inhibitors, there does seem to be a maintained elevation of Ca\(^{2+}\) and a continuing breakdown of MAP2 that is most likely to result from calpain action. Thus, as in global ischemia but perhaps accelerated by the continual hypoperfusion and high levels of free radicals, there is likely to be continued attack on proteins and lipids of the mitochondria, cell membrane, and cytoskeleton, and it is these, along with possible direct breakdown or denaturation of cytosolic proteins, that lead to the ICC and homogenizing change that characterize necrosis in this model.

F) TEMPORARY FOCAL ISCHEMIA. Unlike other forms of ischemia, damage in temporary focal ischemia is strongly dependent on the activation of PARP and, also, on accumulation of neutrophils in the ischemic tissue. Early events are clearly the same as during permanent ischemia, and development of damage is dependent on NMDA/AMPA as well as free radical/NO generation; COX-2 and iNOS are increased at later times. However, full development of damage appears to depend on the accumulation of neutrophils in the postischemic period and also on activation of the PARP. Blocking either of these is profoundly protective.

Poly(ADP-ribose) polymerase is activated immediately upon reperfusion by a burst of free radicals that may depend, to an extent, on the presence of leukocytes as discussed previously. The most likely explanation for their requirement is that they are necessary to boost the production of free radicals in the reoxygenation period, when electron transport is no longer blocked by the anoxia. It would be very interesting to assess the effect of the agents that block neutrophil adhesion on the activation of PARP. Poly(ADP-ribose) polymerase may be damaging by reducing mitochondrial oxidative phosphorylation, but this seems unlikely as ATP levels in the postischemic period do not fall greatly. It seems more likely that the large-scale polyadenylation of the nuclear proteins in some way exacerbates either apoptosis or free radical production.

Development of damage depends strongly on calpain in the postischemic period since inhibitors are extremely protective at that time. (There is some concern here about inhibitor specificity as proteasome inhibition is also very protective, although the fact that this protection is restricted to the lesion core indicates that calpain blockers are not protecting via their actions on proteasomes.) Mitochondria are damaged throughout the reperfusion period and accumulate Ca\(^{2+}\). The calmodulin blocker trifluoperazine is also very protective, and although this is not specific, other actions (on Ca\(^{2+}\) channels) are also largely on Ca\(^{2+}\)-mediated events. Thus it is likely that cytosolic Ca\(^{2+}\) is maintained elevated despite the absence of the intrainschemic depolarizations in the postischemic period. As with global ischemia, this is postulated to arise from alterations in Ca\(^{2+}\) channels or, perhaps, from NMDA channel changes; NMDA antagonists are very protective when applied postischemically.

G) TEMPORARY FOCAL ISCHEMIA: APOPTOSIS AND NECROSIS. The candidates for activation of apoptosis are the same as in global and permanent focal ischemia at this stage. As for permanent ischemia, there is currently no evidence that protein kinases are inhibited. They need to be measured. There is direct evidence for the importance of the FAS ligand (CD-95L), but it has not been shown that it explicitly affects apoptosis.

Necrosis is thought to result from the combined, concerted, and self-perpetuating effects of free radicals on mitochondria, cytoskeleton, possibly plasma membrane, and possibly on cytoplasmic protein structure, all leading to the phase change associated with ischemic cell death and homogenizing cell change.

H) CELL DEATH IN THE CORE OF THE FOCAL LESION. Little is known about mechanisms of death in this region, because it is almost impossible to prevent with pharmacological agents. There are large increases in cell Ca\(^{2+}\) and Na\(^+\), a maintained depolarization, and a maintained decrease in ATP for several hours in temporary ischemia, as well as a prolonged large elevation of glutamate. Existing evidence suggests that free radical levels are low, at least early in ischemia. There are features of the process that provide clues as to the mechanism.

1) The degree of apoptotic cell death is much smaller than in the penumbra; this is probably a function of the prolonged maintenance of low ATP levels (25% normal).

2) Core damage is not prevented by the agents that prevent penumbral damage. This could be because damage results from loss of ATP and rundown of ion gradients with subsequent arrest of important processes, organelle and cell dissolution; that is, it is almost exclusively a dissipative process. Alternatively, the reason it cannot be protected may be that the insult is so intense that synergism between different processes may not be necessary for damage as it is in the penumbra and in global ischemia. Thus more than one pharmacological agent may have to be used at once to obtain protection; for example, both calpain and other protease inhibitors or NOS inhibitors may need to be coapplied. Of all the agents used, uric acid, which is a very effective free radical and peroxynitrite scavenger eliminated almost all core damage, whereas PARP and calpain inhibitors certainly prevented some damage there. This suggests that damage is medi-
ated by agents that act in the penumbra but that very effective inhibition is necessary in the core.

3) Core damage does seem to be prevented by a proteasome inhibitor. This is a somewhat surprising result; the inhibitor actually extended penumbral damage somewhat (possibly due to the normally anti-apoptotic effect of NFκB). It seems quite unlikely that the effect in the core is due to blockade of NFκB because damage there seems unlikely to require synthesis of new protein; the insult is so intense. Thus the result suggests that activation of proteasome S20 normally occurs and contributes to cell death by causing breakdown of protein.

3. Unresolved issues: future studies

Overall, the evidence is very strong that free radical and peroxynitrite production are major determinants of ischemic damage and that Ca\(^{2+}\), via activation of calpain and possibly other events such as mitochondrial accumulation and phospholipase activation, is very likely to be important both during and, particularly, after ischemia. A great number of effects of knockouts and pharmacological agents are consistent with the conclusion that these two agents represent the major perpetrators of cell death. However, beyond this there is a very large number of extremely important unresolved issues, some of which are apparent from reading the previous two sections. Some of the major issues are discussed below.

A) BASES FOR MORPHOLOGICAL CHANGES: TARGETS OF PERPETRATORS. As was almost brutally apparent in the previous two sections, there is essentially no knowledge of what the protein changes are that follow the action of the perpetrators and produce the morphological changes associated with the end stages. These will presumably be worked out for apoptotic change by the workers in that field, but it is necessary to determine what they are in ischemia. They will presumably be a mixture of caspase-mediated changes and those mediated by necrotic events.

Not knowing these changes makes it extremely difficult to determine what the critical functional changes are. Thus, although changes in those functions have been made clear through the text, the way they might cause final damage is largely unknown because the nature of that damage is unknown.

Thus it is critical to understand the protein composition and structure of the end stages; this is a very difficult task.

B) ROLE OF PLASMALEMMAL DAMAGE. A priori the plasmalemma seems a very likely target of the perpetrators, and one where damage could readily lead to necrotic cell death. However, there is really no work that has been aimed at assessing plasmalemmal function at critical periods of damage development.

C) THE APOPTOTIC SIGNAL. Despite the morphological quagmire it is clear that caspase activation is likely to play a major role in most types of ischemic cell death. Determining the signal, and its origin, which triggers the apoptotic sequence causing caspase activation is an important goal. The recent findings of cyclin D1 and FAS ligand upregulations are exciting developments along this line. The wide-spectrum kinase inhibitions are also possible initiators of apoptosis. Several apoptotic signals, particularly those controlled by Ca\(^{2+}\) and protein kinase B, are mediated downstream by dephosphorylation of the proapoptotic protein BAD (1251). It would be very informative to know whether ischemic apoptosis is associated with changes in its phosphorylation state, which can be measured.

D) TIMING OF ACTIONS. In trying to describe the mechanism of cell death, it becomes apparent that a much firmer knowledge of when different events are critical for damage is required. This applies to free radical changes, calpain activity, Ca\(^{2+}\) changes, and pH changes. Required studies are very difficult, and they have been started to some extent, but discreet time windows of pharmacological actions need to be established. Simply knowing that calpain is important is not enough to reconstruct the mechanism of damage.

E) DAMAGE IN THE CORE. It is very important to initiate studies aimed at determining the basis for damage in the core of the focal lesion, where energy metabolism is very strongly inhibited for a prolonged period. This does not, in general, respond to treatments that are quite successful in the penumbra, so it constitutes a particular challenge. However, it is a critical clinical issue. The brain slice might present a good model here, if used judiciously, as described by Newman et al. (824). Conditions resembling core can be created quite readily, and damage can be studied in detail.

F) GLIA AND ENDOTHELIUM. The damaging roles of glia and of the endothelium/blood-brain barrier need to be explored more fully. There are hints of importance (synthesis of iNOS by glia in global ischemia, the protective action of metalloproteinase-9 inhibition), but these have not been rigorously explored.

G) OUTSTANDING MECHANISTIC ISSUES. The full explanation of damage is not known for any of the variables. However, certain of these mechanistic questions are both important and intriguing.

These include the basis for the prolonged inhibition of protein kinases in global ischemia (it has not been tested in other models); the basis for maintained inhibition of protein synthesis; the basis for cytokine increases in all forms of ischemia.

H) LACK OF CERTAINTY THAT SPECIFIC EVENTS ARE INVOLVED IN DAMAGE. It is clear throughout the text that there are major uncertainties about whether almost all of the proposed inducers are actually involved in damage. These uncertainties stem from ambiguities in interpretation of pharmacological or metabolic studies or from the difficulties of making appropriate measurements. Examples of the former include pH, glutamate, cytosolic Ca\(^{2+}\), and early
entry of Na+. Measurements can be difficult either because of the technology or because appropriate pharmacological agents are not available. Examples of both these include cytosolic Ca2+, ATP, changes in kinases, and membrane depolarization. Thus a major task is to develop definitive ways of establishing the involvements of these changes.

Evidence for most of the perpetrators of damage, calpain, free radical action, PARP, is much stronger, although there are minor problems with each. Very few studies have been done with calpain inhibitors, and none in permanent ischemia. Further work, with more specific inhibitors, and with more detailed consideration of timing, will be very valuable. The scavengers and blockers of free radicals have, in general, not been used along with measurements of free radical changes; this and the quite large variability in results is somewhat unsettling but, overall, it must be considered that the involvements of free radicals, NO and PARP (for temporary ischemia), are about the best established of all the perpetrators. Despite the high likelihood that the changes are important, evidence for the involvement of phospholipid breakdown is quite weak, due largely to the absence of appropriate inhibitors.

1) IS THERE MORE TO KNOW? As discussed above, there are clearly many major remaining issues that urgently need to be resolved. However, this particular question is directed at the validity of the basic model described here, in which free radicals and Ca2+ along (possibly) with permanent kinase inhibition and apoptosis, are the major effectors of cell death. There is no question but that more molecular changes associated with ischemia will be found and that more protective agents will be found. The issue is whether or not all of these will be able to be fit into this paradigm. It is important to differentiate findings that initiate new insight from those that are consistent with the current paradigm. With regard to the former, it seems possible that intensive study of the basis and importance of functional changes: mitochondria, plasmalemma, cytoskeleton, protein synthesis, and apoptosis initiation, may provide results that allow new paradigms to be created. This will require learning what macromolecular changes are responsible for the key functional changes. Once this is done, then reasonable, detailed, mechanisms for damage can be created, from which new insights will certainly emerge.

New insights might emerge in a different way. There are two protective agents that have profound effects in focal ischemia but for which the actual basis for protection is not easily discerned. These are inhibitors of PARP and polyamine oxidase inhibition, which seems to act by lowering levels of 3-aminopropanol (498). These are different from all other protective effects that have been discussed, because it is not clear at all why the decrease in polyadenylation or in 3-aminopropanal would be protective. Perhaps understanding one or both of these will also provide qualitatively new insights into the damage process.

1) EXPERIMENTAL CAUTIONS AND APPROACHES. In trying to solidify the involvement of different factors, it is essential that studies be done with appropriate temperature controls, and also with very good measurements of local blood flows during ischemia. Uncertainties in the contributions of these quantities to measured effects of drugs or knockouts have contributed to the uncertainty in several of the interpretations.

Appropriate use of in vitro systems can be very valuable in mechanistic studies, but end points have to be very well defined. For example, it is probably not useful to measure effects of agents on loss of synaptic transmission in hippocampal slices, since there is really no understanding of the mechanisms underlying the loss of transmission. Similarly, in cell culture, studying necrotic cell death is not very fruitful as, here again, there is little understanding of how it relates to the process in vivo. It would be much more fruitful in both the in vitro models to concentrate studies on specific processes, for example, cytoskeletal changes, apoptosis etc. In all cases, care should be taken to relate results to in vivo conditions.

B. A Final Viewpoint: Attractors, Cellular Instability, and Cell Death

There are three aspects of ischemic cell death which suggest that a formalism that is applied to dynamical systems, in which attractors constitute stable states in a multidimensional space, will provide a useful framework. The first is that there is a very clear insult threshold. Global ischemia and focal ischemia will only cause cell death if they last a certain length of time (~4 and 30 min, respectively). The second aspect of ischemic damage is that there is an early profound damage, lasting ~12 h, whether the insult is subthreshold or superthreshold. This includes profound inhibition of protein synthesis, cytoskeletal disruption (in gerbil), and vacuolization of normal ER and Golgi structures. If the insult is subthreshold, cells recover, whereas if it is superthreshold, they do not. These observations suggest that ischemic cell death occurs when restorative processes that are in place following an insult are unable to effectively compete with the degenerative processes induced by the insult. The third aspect of ischemic cell death which suggests the formalism is appropriate is that the end stages of ischemic damage are metastable states that are very different from the normal state of the cell.

These properties suggest that ischemic cell death can be treated semiformally, based on the formalism used for describing stable and unstable states of dynamical systems in terms of attractors, as developed by Kauffman (555) and others in which the emergent properties of networks are manifested. This kind of analysis has re-
cently been applied to movements between stable states in very complex networks of protein kinase-driven biochemical reactions (93), rendering it particularly applicable to ischemic perturbations.

The cell state is defined by its position in a multidimensional “cell component space” whose axes are the concentrations of all species within the cell, including small molecules, ions, macromolecules, phosphorylated macromolecules, and macromolecular complexes or polymers. The state of the cell is thus uniquely represented by a point in cell component space. The particular concentrations of the components will lead to a constellation of rates of reaction between, and catalyzed by, the components and hence to movement through the component space as concentrations change. According to this, the cell’s steady state is a stable point in cell component space, where concentrations do not change as a result of the ongoing reactions, or there is a short cycling between different points in the space. The dynamical theory shows that such steady states lie at the nadirs of “attractors” (555). The latter are regions in the space (basins, troughs) within which the cell will always move to that steady state.

Physiologically, the attractors represent the homeostatic mechanisms of the cell. Ischemia moves the cell to a new point in cell component space. Within this formalism, subthreshold ischemic insults are not large enough to move the cell out of its normal attractor. Put more physiologically, homeostatic mechanisms are able to overcome the processes set in motion by the insult. (These homeostatic mechanisms may be complex, involving synthesis of new protein for example). In contrast, a superthreshold insult constitutes a major impact that puts the cell outside the attractor for a normal cell. In this case, the cell will take a new trajectory through component space, involving new sets of reactions, toward other attractors. For a lethal insult, the end stages of cell damage will lie at the nadir of these other attractors. These stages are considered to be within attractors because they are stable enough to be viewed in large numbers of micrographs, so they must persist for a significant time.

In this formalism, the several end stages, including apoptotic cell change, are represented by different attractors, and the particular constellation of component concentrations at the end of a superthreshold insult will determine which pathway through cell component space is followed and hence which attractor is found (which end stage is formed). The fact that different end stages are associated with the same insult can be explained if different cells exit the normal attractor with different concentrations of their components. They would be at a different spot in the space and thus take different trajectories through it. This seems a very reasonable possibility given different initial states of cells and different proximities to glutamate release.

As discussed so far, the formalism tends to treat all variables similarly. This is clearly unacceptable. For example, while an altered level of phosphorylation, or the loss, of a component of the protein synthesis system may be extremely important, similar effects on glycogen phosphorylase are likely to be inconsequential (93). This is reflected in the fact that there is a great asymmetry in cell component space. Large changes in inconsequential parameters will not move the cell out of an attractor, whereas small changes in key parameters will.

It is anticipated that more directed and quantitative application of this formalism to cell biochemistry and physiology (93) will lead to new insights about the relationships between cell stability and ischemic damage.

ADDENDUM

This review was completed in February 1999. Since that time there have been many important publications. In this addendum I include those that appear to add important new information to the ideas that were discussed in the body of the review. The new papers are discussed with regard to the sections of the review to which they are most appropriate.

SECTION II

Section II-L Effects of ischemia on the vasculature. Del Zoppo and co-workers (A9) have continued their work on the roles of the matrix zinc-metalloproteinases (MMP) that degrade laminin and collagen and have now shown that the other principal MMP, MMP2, is hugely activated in the lesion core. There is a fourfold increase in activity within the first hour of MCAO in monkeys, and this persists during 7 days of reperfusion, during which time damage is developing. The mechanisms of activation are not known, although there are complex controls on these enzymes. It is also not established that the activation is causing damage. However, it seems likely that local vascular disturbances, and changes in glial-endothelial relationships, may be very important. In the context of the latter, another recent publication demonstrated that astroglia appear to be severely damaged several hours before neurons in permanent focal ischemia, as judged by loss of immunoreactivity of glial markers within the first few hours of ischemia (A14). It is possible that disruption of endothelial-glial relationships due to MMP action could be significant here, rather than the more obvious suspects such as low pH. The glial changes may influence neuronal damage, but this is not established.

Section II-M Effects of temperature on damage. Among the many possible bases for the protective effects of hypothermia, decreased glutamate release has been prominently considered. A careful study by Yamamoto et al. (A23) strongly negates this possibility. They artifically increased extracellular glutamate levels in CA1 hippocampus during global ischemia in gerbils at 31°C to levels that were actually higher than those that occurred during ischemia at 37°C. Thus they overrode the inhibition of release. Despite this, damage was still just as strongly attenuated by the hypothermia.

SECTION III

Section III-B Apoptotic cell change. Biochemical changes associated with apoptosis. MacManus et al. (A16) have now
published their study showing that the nucleosomal DNA fragments that are produced after either global or focal ischemia are different from those in classical apoptosis or in cardiac ischemia. Rather than being blunt ended, they have a staggered end with recessed 3’-strand. Thus endonuclease activity in ischemia is clearly different from that during classical apoptosis. This suggests alternate methods of activation from the classical caspase 3 activation of the endonuclease CAD (Ref. 301 in main text). This may be one manifestation of “nonclassical” apoptosis occurring during ischemia.

Triggers for apoptosis. The work demonstrating that CD-95L mRNA and protein is upregulated ~12 h after 90-min transient focal ischemia in rats and showing that mutant lpr mice, lacking normal CD-95, have a 60% reduced infarct volume has now been published (A17). In this paper there is quite nice evidence that the CD-95 system is involved in apoptotic death in that the mutant mice showed no cell death in the penumbral region. This death normally has a strongly apoptotic morphology. These authors hypothesized that CD-95L was synthesized after activation of the MAPK-JNK pathway, with c-Jun expression and phosphorylation being a key step. Certainly, c-Jun and Jun phosphorylation were upregulated at the appropriate time, the latter in penumbra. Furthermore, FK-506, the calcineurin inhibitor, blocked c-Jun phosphorylation and upregulation of CD-95L and reduced the infarct volume. Although more work is clearly required in this area to establish causality, these results are really quite exciting in strongly implicating one possible apoptotic pathway following temporary focal ischemia.

Indeed, there are several new reports demonstrating that calcineurin inhibition is protective in transient focal ischemia, reducing infarct sizes by 40–70%. These studies explicitly showed that protection did not arise from inhibition of nitric oxide synthase (A21) and that the FK-506 (in one case) was acting on calcineurin rather than another FKBP (A2). The site(s) of calcineurin action has not been identified but clearly may include blocking the CD-95 system.

Involvement of caspases in apoptosis. Caspase-3 inhibitors are only mildly effective at protecting against many forms of focal ischemia. One reason is suggested by studies of permanent focal ischemia where a large upregulation of caspase-8 by cleavage of the procaspase form hours in the pyramidal cells of layer V was seen after 6 h. Caspase-3 was elevated only in the layer II/III cells (A22). This is interesting from several points of view. One of these is that different populations respond differently; another is that caspase-8 activation is generally associated with the CD95/ligand system (as well as other death domain ligands) and lends further fuel to the idea that this ligand system is involved in apoptosis.

Morphological changes in global ischemia. Two studies from Colbourne and co-workers (A4, A5) reemphasize the lack of apoptotic morphology following very mild to somewhat intense global insults in the gerbil. There were no nuclear condensations as expected in apoptosis, and organelles were systematically dilated until ischemic cell change occurred. This fuels the conflict over the extent of apoptosis in global ischemia, where biochemical studies implicate the process despite lack of good morphological evidence.
these, injected before the 3-h insult, reduced infarct in Wistar rats by \(-70\%\) (A3); temperature effects were excluded.

An elegant study by Moskowitz and co-workers (A6) was aimed at localizing the NO production and the peroxynitrite production within the focal ischemic lesion. They found that NO, measured as citrulline accumulation, was largely produced in the peri-infarct region while peroxynitrite, measured as 3-nitrotyrosine, was increased in the infarct (A6). They thus concluded that the peroxynitrite, rather than the NO, was the major toxic species. Presumably, NO diffuses from where it is formed, as indicated by citrulline deposit, and encounters high levels of superoxide in the infarct region. It reacts to form the peroxynitrite there, and this may well lead to much of the damage in the infarct. This adds to several papers, discussed in the body of the text, that have identified sites of generation of different components of the free radical/peroxynitrite system. The issue is not yet resolved.

Unlike in focal ischemia, there is a paucity of data on the role of NO in global ischemia. A report from Lei et al. (A12) shows that nitric oxide is not elevated after 5-min ischemia in the gerbil but is elevated for at least 3 h after 10- and 15-min ischemia. There are actual decreases in NO during the ischemias. The elevations are completely blocked by either 7-NI or by L-NAME, indicating they do arise from activation of NOS. The results of NOS inhibition by 7-NI are rather paradoxical. There is some protection against 5-min ischemia, where there was no measurable increase in NO, and there was no protection against 10- and 15-min ischemia, where NO rose! These results differ somewhat from those described in the main text (Refs. 1126 and 850) where NO increases were observed during the ischemia and after 5 min. In those cases NO was measured directly, whereas in the current studies it was measured as total nitrites/nitrates and so is somewhat less direct, although is possibly more sensitive. Despite the time course differences, the rather small protection afforded by NOS inhibitors was evident in all the studies; in this case there is almost none. This contrasts with focal ischemia, where NOS inhibition is very protective and also, to some degree, with studies of global ischemia in knock-out mice where protection is more substantive (see sect. Vb in text). The poor pharmacological protection in global ischemia occurs despite the fact that the 7-NI knocked out all NO production, measured as total nitrites/nitrates (A12). Thus the role of NO production in global ischemia remains quite unresolved.

Section Vb: Ca\(^{2+}\)-dependent proteases. Until now there had been only one study of effects of calpain inhibition in global ischemia. Using a tripeptide calpain inhibitor trapped within liposomes, Yokota et al. (A24) demonstrated \(-40\%\) protection against ischemic damage following 5-min global ischemia in the gerbil, although unfortunately temperature was not regulated against ischemic damage following 5-min global ischemia in the mouse (A11). Most excitingly, the IRF \(-/-\) mouse shows a great reduction in infarct volume, which also occurs within the striatal core. The heterozygote shows a 15% infarct volume reduction, while the homozygous knockout shows a 40% decrease in infarct volume, and almost complete protection in the striatum. Thus, with the use knock-out technology, a transcription factor has been shown to be crucial for ischemic cell death. This is an exciting development.

Human studies. Certainly one of the major goals is to apply research on ischemia to human disease. Although therapy has been difficult to achieve, recent studies indicate the relevance of the animal models. In particular, elevated COX-2 has been observed in infarct regions of brains of people who died 1–2 days after stroke. It was observed in invading neutrophils, vascular cells, and neurons in the damaged tissue (A10). In addition to this, another enzyme that is very important in temporary focal ischemic damage in rodents, PARP, appears to be upregulated in brain tissue removed within 1–1.5 days of cardiac arrest. There are greatly elevated levels of poly(ADP-ribose), after these essentially global ischemic insults, in neurons (A15).

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