The term "nerve growth-promoting factor" was introduced in 1954 to designate a nucleoprotein particle isolated from mouse sarcomas 180 and 37 and endowed with the property of enhancing growth and differentiation in sensory and sympathetic nerve cells (31). Two additional and much more potent sources of this factor were discovered in 1956 and in 1958 in snake venom and in mouse submaxillary salivary glands (22, 30, 59, 74). The availability of large quantities of the growth-promoting protein extracted from these two sources made possible a better characterization of this factor, so that since 1956 (30) it has been identified as a protein rather than as a nucleoprotein molecule. The biological properties of this agent, which became known as the "nerve growth factor" (NGF), its range of
action on the target cells, and its sources, metabolic effects, mechanism of action, and biological significance are the subject of this review.

EARLY LITERATURE

In 1948 Bueker reported on the profuse sensory innervation of fragments of mouse sarcoma 180 implanted into the body wall of 3-day-old chick embryos (12). The experiments were part of a more general plan of investigation of the effects elicited by the peripheral end organs on associated nerve structures. The finding that the sensory ganglia providing fibers to the neoplastic tissue were enlarged seemed to fit well in the accepted concept that the size of a given nerve center is dictated by the size of its peripheral field of innervation.

An extensive and detailed inspection of embryos bearing transplants of mouse sarcomas 180 or 37 revealed new facets of this phenomenon and suggested an entirely different interpretation of the effects evoked by the neoplastic tissue. It was found that sympathetic ganglia of embryos bearing transplants of sarcomas 180 or 37 underwent a far more impressive size increase than sensory ganglia. This effect was not restricted to ganglia adjacent to the tumor but was also very evident in sympathetic ganglia remote from the implant and not connected by nerve fibers with the neoplastic tissue (76). The hypertrophic and hyperplastic sympathetic ganglia produced an extraordinary number of nerve fibers that massively invaded the embryonic viscera and even penetrated inside the blood vessels (58). The suggestion was made that both sarcomas 180 and 37 released a diffusible growth factor into the host circulation that was responsible for the excessive overgrowth of the ganglia and the anomalous distribution of sympathetic nerve fibers. This hypothesis received strong support from experiments of extraembryonic tumor implantation. Fragments of mouse sarcomas 180 or 37 were grafted onto the chorioallantoic membrane of 4- to 6-day chick embryos. In these experiments the tumors and the embryo shared the circulation but no direct contact was established between the embryonic and the neoplastic tissues. The effects compared in all respects with the effect of intraembryonic tumor transplants, thus giving additional evidence for the release into the blood stream of a nerve growth factor produced by neoplastic cells (58, 77).

In 1953 a simple in vitro technique was devised to explore the effects elicited by the two mouse sarcomas 180 and 37 on sensory and sympathetic ganglia. Fragments of these tumors were cultured in a semisolid medium consisting of chicken plasma and embryonic extract. Sensory and sympathetic ganglia dissected out from 7- to 9-day chick embryos were incubated in this medium close to the neoplastic tissues. Experimental and control cultures consisting of ganglia combined with sarcomas 180 or 37, or ganglia combined with embryonic mouse or chick tissue, were inspected 10-24 hr later. The production of a dense, fibrillar halo of nerve fibers by ganglia adjacent to the neoplastic tissues, but not in controls, was taken as evidence for the in vitro release of a growth-promoting agent by these tumors (78). No comparable effect was obtained with other tumors. Since this
first experiment was performed, the in vitro production of a fibrillar halo by explanted sensory and sympathetic ganglia has become the most reliable index of the presence of the nerve growth-promoting factor in the culture medium and has been used in all tests performed to detect the presence of this factor in other presumptive sources.

The finding that an extract of mouse sarcomas 180 or 37 retains the growth-promoting activity of intact tumors made possible a chemical study of the growth factor and recognition of its presence in subcellular components. The factor was identified in heat-labile, nondialyzable nucleoprotein particles present in the microsomal fraction of neoplastic cells (31). In order to purify further the growth factor, Cohen made use of snake venom as a source of phosphodiesterase. His experiments, aimed at the enzymatic inactivation of the nucleic acids present in the active fraction, gave an unexpected and unforeseeable result: the snake venom itself was found to possess a nerve growth-promoting activity far more pronounced than that of the tumors under investigation (23).

In 1958 a third and even more potent source of the NGF was discovered in mouse submaxillary salivary glands (24, 59). In vivo and in vitro experiments established the remarkable similarity between the growth effects elicited by this, by the mouse sarcomas, and by snake venom factors.

In 1959 a new facet of the phenomenon came to light with the discovery that a specific antiserum to the salivary NGF destroys the sympathetic ganglia of newborn mammals. The term "immunosympathectomy" was coined to designate this procedure by which animals could be deprived of their sympathetic functions.

CHARACTERISTICS OF THE GROWTH RESPONSE OF SENSORY AND SYMPATHETIC NERVE CELLS IN THE LIVING ORGANISM

Sensory Ganglia

Early studies of the effects elicited by mouse sarcomas 180 and 37 on sensory ganglia of chick embryos were at first interpreted to mean that the growth response is restricted to the ganglia contributing to the innervation of the implant (12, 76). A size increase in these ganglia is apparent on the 6th day of incubation, shortly after their nerve fibers have established contact with the neoplastic cells. From the 6th to the 11th day of incubation, sensory nerve fibers grow vigorously and branch profusely into the rapidly expanding neoplastic tissue. Toward the end of the 11th day of incubation, those sensory ganglia that send nerve fibers into the tumor are three times larger than contralateral ganglia that have no access to the implant. The size increase is due to an intense mitotic activity much more pronounced than in contralateral ganglia, to acceleration of differentiation of early neuroblasts, and to hypertrophy of differentiated nerve cells (76). After the 7th day, a marked difference becomes noticeable between two types of embryonic sensory nerve cells. These two cell types were the object of previous extensive investigations. It was found that they differ from each other in size, developmental pattern, and peripheral field of
distribution. Since they form two groups well segregated from each other, they were designated, on the basis of their position in the sensory ganglia of the chick embryo, as the ventrolateral (V-L) and the mediadorsal (M-D) nerve cells (48, 77).

It was now found that the M-D, but not the V-L, cells are receptive to the growth-stimulating effect elicited by the tumors. The growth response elicited by mouse sarcomas 180 or 37 is maximal between the 7th and the 9th day of incubation. It decreases thereafter and is apparent no more after the 15th day of incubation. An extensive study of the distribution of sensory nerve fibers in the tumor between the 7th and the 13th day of incubation showed that nerve fibers branch profusely into the tumor but do not establish contact with the neoplastic cells. In this respect they differ markedly from embryonic sensory nerve fibers, which soon after they reach their end organs, muscles, or subcutaneous tissue establish provisional contact in the form of end boutons. In the tumor, sensory nerve fibers branch in all directions, building a fiber net far more dense than in any embryonic structures (76, 77).

Soon after the two more potent sources of the nerve growth factor were discovered in snake venom and in the mouse submaxillary salivary glands (22, 30, 59), they were assayed for their growth-promoting activity in chick embryos. At first the purified venom and then the purified salivary factor, when this became available 2 years later in 1958, were injected daily into the yolk sac of chick embryos between the 7th and the 10th day of incubation, in the amount of 50 μg of the purified protein per dose. In both experimental series the M-D sensory nerve cells exhibited a marked increase in number and in size. These growth effects differ in only one respect from those elicited by implanted fragments of mouse sarcomas. In the former, but not in the latter (sarcoma experiments), all sensory spinal and most of the cephalic ganglia of the injected embryos are hyperplastic and hypertrophic; in other words the growth effects are generalized and not, as in experiments of tumor transplantation, restricted to ganglia adjacent to and supplying fibers to the tumor (59, 62). It is likely, though not proved, that quantitative rather than qualitative differences in the NGF from these several sources account for the above results. As is reported in a later section, the NGF is present at a concentration respectively 1000 and 6000 times lower in mouse sarcomas than in snake venom and mouse salivary glands (22, 62).

Injections of a tumor extract in the chick embryos do not elicit any growth effect on sensory ganglia. The hyperplastic and hypertrophic effects evoked by the grafted tumor are conceivably due to the continuous release of minute quantities of NGF from the actively growing neoplastic cells, which would affect only closely adjacent ganglia and result in a local rather than a generalized effect. Further evidence of a gradient in the growth response elicited by mouse sarcomas came from studies on sympathetic nerve cells (reported below). Sensory nerve fibers, produced in excess by hypertrophic and hyperplastic sensory ganglia, collect in thick nerve bundles that branch under the skin of the embryo and even perforate it and appear at the body surface (59). No increase is seen in the enterceptive and proprioceptive innervation of the embryos.
Sympathetic Ganglia

The response of sympathetic ganglia to the NGF from tumors, venom, or submaxillary glands has been the object of more extensive investigation than the response of sensory ganglia to the same factors. Chick embryos were used at first, but when NGF from mouse submaxillary glands became available, the response was studied in newborn and adult mice. (The results are reported later.)

**Growth response of sympathetic ganglia in the chick embryo.** Implantation of fragments of sarcomas 180 or 37 into the body wall of 3-day chick embryos results in a growth response of sympathetic ganglia of the host that far exceeds in magnitude that of sensory ganglia.

The growth effects become apparent in sympathetic ganglia of chick embryos toward the 6th day of incubation and consist of an increase in mitotic activity, acceleration of maturation processes, and hypertrophy of differentiated nerve cells. In these respects, sympathetic nerve cells behave in the same way as the M-D sensory nerve cells. They differ in the following aspects: 1) The whole sympathetic nerve cell population, and not only a percentage of these cells, is receptive to the action of the growth factor released by neoplastic cells. 2) The growth response persists also in sympathetic nerve cells at advanced differentiative stages, until the end of the incubation period. 3) The magnitude of this response far exceeds that of sensory ganglia. The volume of the sympathetic ganglia in an embryo bearing implants of mouse sarcomas 180 or 37 is of the order of six times that of controls at the 11th day of incubation. New sympathetic ganglionic complexes are formed near the implanted tumor and contribute with large nerve bundles to its innervation. 4) Nerve fibers produced in excess by sympathetic ganglia remote from the tumor invade most of the embryonic viscera, building a very dense nerve net in the parenchyma of the mesonephros, metanephros, ovary, testis, thyroid, spleen, liver, pancreas, and feather bulbs. A few organs and embryonic structures are spared such nerve invasion; these are the heart, muscles, and respiratory and digestive tracts (58, 77). No satisfactory explanation is offered for such a differential hyperneurotization of embryonic organs and structures under the action of the tumoral NGF. Two alternative hypotheses have been tentatively suggested: 1) The NGF released by the tumors collects in pools in some but not other organs and structures and exerts a neurotactic effect on sympathetic nerve fibers. In favor of this hypothesis is the exceedingly dense nerve network that forms in the mesonephros between the 6th and the 11th day and in the metanephros only in a subsequent developmental period, when this latter organ takes over the embryonic excretory functions. The formation of large neuromas of sympathetic nerve fibers inside the veins of embryos bearing implants of sarcomas is also suggestive of a similar effect. 2) As an alternative hypothesis, one can conceive of a sort of a built-in barrier against sympathetic nerve fibers in some organs that from early embryonic stages receive a large quota of parasympathetic nerve fibers. These are the heart and the respiratory and digestive tracts (58, 77).

The finding of an exceedingly large number of sympathetic nerve fibers in the
organs and in veins of embryos bearing transplants of mouse sarcomas 180 or 37 first suggested that the tumors release into the circulation of the host a diffusible growth factor responsible for the observed effects. The hypothesis was tested by implanting fragments of sarcomas 180 or 37 onto the chorioallantoic membrane of 4- to 6-day chick embryos. In this position the tumor and the embryo share the circulation but no direct contact is established between the neoplastic and the embryonic tissues. Yet in this experimental series also the sympathetic ganglia underwent marked hypertrophic and hyperplastic changes, and sympathetic nerve fibers produced in excess by these ganglia invaded embryonic viscera and blood vessels (62, 65). These results gave definite evidence in favor of the hypothesis that a diffusible growth-promoting agent is released by these tumors (58).

A systematic comparative study of the volume increase of sympathetic ganglia of embryos bearing intraembryonic or extraembryonic tumor transplants showed that growth effects are more marked in the former than in the latter experimental groups. Furthermore, ganglia adjacent to the tumor in intraembryonic transplants are larger than ganglia remote from the graft. These results, described further in later sections, indicate that the magnitude of the growth effects elicited by the tumoral NGF correlates with the amount of this factor present in the circulation as well as in end organs. Differential NGF release by sarcomas 180 and 37 from different sources and by other mouse sarcomas is considered on p. 545, 546.

Daily injections of the NGF extracted from snake venom and from mouse submaxillary salivary glands into the yolk of developing chick embryos from the 7th to the 10th day of incubation (50 μg of the purified protein per dose) result in a marked increase of sympathetic ganglia of the host, hyperinnervation of the viscera, and penetration of sympathetic nerve fibers into the veins of the host. The biological effects are therefore in all respects similar to the effects elicited by implanted fragments of mouse sarcomas 180 or 37 (74, 75).

NGF effects in newborn and adult mice. Although the implantation of fragments of mouse sarcomas 180 or 37 in newborn and adult mice did not evoke any growth effect in sensory or sympathetic ganglia of the host (Levi-Montalcini, unpublished results), the injection of the purified salivary nerve growth factor in newborn and adult mice called forth a massive enlargement of sympathetic ganglia of treated animals. Lack of any appreciable effect in sensory ganglia of these mice is in line with the results obtained in the chick embryo and shows once more that these cells are receptive to the growth-promoting activity of the NGF only during a restricted period of their early embryonic life (71).

The purified salivary NGF was injected subcutaneously in newborn mice in the amount of 0.05 ml/g of body weight, the injections being repeated daily for periods ranging from 3 to 27 days. The NGF protein is present in this preparation at the concentration of about 300 μg/ml. Since newborn mice weigh at birth between 0.5 and 0.7 g, they received therefore 15–20 μg per dose of the NGF protein. In biological units this would correspond to 1500–2000 units per dose.

The injected and control mice at the end of the experimental period were sacrificed, and the sympathetic para- and prevertebral chain ganglia were dissected out and compared. The superior cervical ganglia and in some instances also
the stellate and thoracic ganglia were sectioned serially at 10 μ, stained, and used for volume determinations, mitotic counts, and counts of the whole nerve cell populations. The mitotic activity as determined between the 3rd and the 9th day after birth is much higher, and the total nerve cell population is between 2 and 3 times larger in NGF-treated than in control mice. At 9 days mitotic activity comes to an end both in normal and NGF-treated animals. In the subsequent developmental periods, the injections of the NGF call forth hypertrophic but no hyperplastic effects (71). The overall size increase of para- and prevertebral sympathetic ganglia varies from one specimen to another and depends on the strength and purity of the injected NGF preparation. In earlier experiments the superior cervical ganglia of mice injected from the day of birth to the 27th day were found to be 6 times larger than the same ganglia of littermates (71). In subsequent experiments performed with an NGF deprived of toxic side effects and further purified, higher doses of the active protein could be injected. The superior cervical ganglia of mice treated with this preparation from birth to the 9th day reached a volume about 12 times as large as those of controls (62, 63). These results bring to light one of most interesting and still unexplained aspects of the phenomenon, namely, the almost unlimited growth potentialities of sympathetic nerve cells of infant mice treated with the NGF.

Newborn mice injected with a non-highly purified NGF exhibited side effects such as stunted growth, lack of hair, opening of the eyelids 6–7 days earlier than littermates, and eruption and calcification of the inferior and superior incisors 3–4 days earlier than untreated mice (64, 71). Subsequent studies confirmed these results (15). Stunted mice recovered when the NGF treatment was discontinued; they resumed growth and 3 months later did not differ noticeably from littermates. Since these prominent side effects did not occur in mice injected with a more highly purified NGF, the conclusion was reached that “obviously we are dealing with two factors, one of which was removed in the process of purification” (71, p. 379). The validity of this hypothesis was proved 2 years later, when Cohen succeeded in isolating the factor responsible for the precocious opening of the eyelids and incisor eruption in another protein molecule that became known as the “epidermal growth factor” (26). Since the chemical nature and the biological properties of this new specific factor resemble in many respects those of the NGF they are briefly considered later.

Extensive cytological examinations showed that the hypertrophic neurons differ from controls not only in their larger size but also in the more intense basophilia and marked increase in size and number of the nucleoli and increase in neurofibrils (71). Determinations showed that the norepinephrine content in the NGF-treated ganglia increases proportionally to the cell volume increase (3–4 times in the cases examined), whereas epinephrine is barely detectable and compares with that of controls (33).

Peripheral distribution of sympathetic nerve fibers in NGF-treated mice. Histological analysis of experimental and control mice stained with specific silver techniques and sectioned serially showed a marked hyperinnervation of the viscera, the external tunica of blood vessels, and the hair bulbs in NGF-treated mice. A detailed
analysis of the peripheral distribution of adrenergic nerve fibers in control and NGF-treated mice, performed with the histochemical technique devised by Falck and others (36), to visualize the adrenergic transmitter in sympathetic post-ganglionic neurons and in their fibers (87), gave an impressive demonstration of the increased density of the adrenergic ground plexus in the iris, submaxillary and parotid glands, and intramural ganglionic plexuses of the intestinal tract in the NGF-treated mice. Furthermore, varicose adrenergic terminals were also detected in areas that are normally devoid of adrenergic innervation. For example, the normally negligible amount of parenchymal adrenergic fibers in the sublingual gland was increased to a considerable number of terminals surrounding the acinar portion of the gland. Similar numerical increase in the adrenergic nerves was found in the extraorbital lacrimal glands, the pancreatic insulae, and the adrenal cortex. One singular exception to the rule that sympathetic adrenergic ganglia and their peripheral fibers undergo size and numerical increase in NGF-treated animals was found in the ganglionic complexes that innervate the vas deferens and the uterus. Cell counts and volume determination of these ganglia showed no appreciable increase in experimental mice (Levi-Montalcini, unpublished observations). The fluorescent histochemical technique utilized by Olson likewise showed no increase in the adrenergic ground plexus in the vas deferens (87). Increased catecholamine content in ganglia and several peripheral organs of mice, rats, and kittens injected with the NGF has been demonstrated with quantitative fluorometric techniques by other authors (34).

CHARACTERISTICS OF THE IN VITRO GROWTH RESPONSE OF SENSORY AND SYMPATHETIC GANGLIA

Fibrillar Halo

As briefly mentioned previously, the in vitro assay of the NGF was first devised to test the effect of mouse sarcomas on sensory and sympathetic ganglia dissected out from 8-day chick embryos and cultured in a semisolid medium near the neoplastic tissue (78). The same test was then used to assay the biological activity of purified NGF added at different concentrations to the culture medium, or of fluids and organ extracts suspected of harboring the NGF. The production of the fibrillar halo is in fact such a consistent and stereotyped phenomenon that the "halo effect" became the unequivocal sign for the presence of the NGF in biological fluids or tissue extracts, even when present only in trace amounts, from the time this test was first devised 15 years ago to the present. Since the size of the halo and the density of nerve fibers that form it vary with the amount of NGF present in the culture medium (although the variations are not proportional to the NGF protein content in the culture medium), it became necessary to grade the response and to establish the biological unit of the NGF activity (60, 62). This is defined as the amount of purified NGF required to produce in a 12- to 18-hr period a fibrillar halo of uniform density around the ganglion,
where each ray is represented by a nerve fiber ending abruptly in the medium and forming with the other fibers a circular or ellipsoidal, perfectly geometrical ring around the explant. The amount of NGF protein required to elicit this effect corresponds to about 1 ng (10^-9) of the NGF purified according to Cohen's procedure (24). There is, however, a certain complexity in the apparently simple problem of establishing a dose-effect relation, as well as much confusion in the literature in this respect, as a result of the fact that with super- or submaximal doses of the NGF these quantitative relationships became obscured. It is therefore necessary to consider the whole spectrum of the NGF's effects from the highest to the lowest concentration of the extract to be assayed, if one wishes to establish the amount of NGF present in the preparation. Unfortunately this criterion has been seldom used and, as a result, many data in the literature on the NGF specific activity in solutions or organ extracts under investigation are of doubtful significance.

An additional reason for conflicting results in the literature may be due to the way the in vitro tests for NGF activity are run in different laboratories. This is quite an important point in evaluating the results of these tests. When we first devised the tissue culture technique and then used it to assay the NGF from snake venom or mouse salivary gland, we found that we could evoke the fibrillar halo characteristic of one biological unit with dilutions as high as 10^{-10} or even 10^{-11} μg NGF/ml of medium if the same pipette was used for subsequent dilutions of the NGF preparation. We then found that if instead the pipette was changed for every successive dilution, only traces of NGF activity were found in dilutions higher than 10^{-8} μg NGF/ml of medium. These results can be explained by the assumption that a few NGF molecules may adhere to the glass pipette and in this way are carried from one dilution to the other, thus giving a very inaccurate evaluation of the activity of the NGF. An extraordinarily high NGF activity found by some laboratories in the crude extract or in the further purified extract from mouse salivary glands may well be due to neglect of this rule of procedure.

Next we briefly consider the characteristics of the in vitro growth response to higher or lower NGF doses.

A two- to fivefold increase in the NGF concentration that corresponds to 1 biological unit, or in other words an increase from 1 up to 2-5 NGF biological units in the culture medium, results in the production of a fibrillar halo much denser and shorter than that elicited by 1 biological unit. A 10- to 100-fold increase in the concentration of the NGF (10-100 biological NGF units in the medium) calls forth, respectively, an exceedingly short fibrillar halo or no fiber outgrowth at all. This effect examined in ganglia in vivo, or stained and studied in toto in the whole mount, was at first taken as evidence of an inhibitory effect elicited by high doses of NGF (60, 64). Closer inspection of hundreds of ganglia cultured for 12-24 hr in the presence of 10-100 NGF biological units, then fixed and stained with specific silver techniques and sectioned at 10 μ, offered a different explanation of the lack of fiber outgrowth from the explanted ganglia. In the presence of a high NGF quantity the nerve fibers, instead of growing in a radial direction around the explant, build a dense fibrillar capsule around the ganglion. The thickness of the capsule increases in width with increased amounts of NGF; the production of
nerofibrillar material, therefore, not only is not inhibited but is further enhanced by the addition to the culture medium of supermaximal NGF doses. The study of sensory ganglia cultured in the presence of one or more NGF biological units and in control media shows an additional feature of the NGF effects. Whereas in media deprived of the NGF the nerve cells undergo an almost total disintegration after 18–24 hr of culture, a high percentage of sensory nerve cells is present in cultures enriched with 1 biological unit of the NGF. Where 100 or 1000 NGF biological units are present in the medium, all sensory nerve cells are well preserved at the end of the 1st day of culture (62, 70). Although it remains to be explained why supermaximal doses of the NGF elicit the formation of a fibrillar capsule rather than a fibrillar halo, these results are against the assumption of inhibitor or toxic effects by high NGF doses. Submaximal NGF quantities, added to the culture medium in amounts corresponding to 0.5, 0.1, or 0.05 NGF biological units, result in the formation of a fibrillar halo of increased length and decreased fiber density. Eventually only a few irregular nerve fibers branch around the explant. Since ganglia in control media may occasionally also produce some nerve fibers in the first 24 hr of culture, it is difficult to evaluate the quantity of NGF present in the medium at these very low concentrations. Reliable data can be obtained, therefore, only by adding the tissue extract or the body fluid to the culture medium at progressively higher dilutions and by then inspecting the entire experimental series. If the undiluted extract or fluid contains less than 0.5 NGF biological units, the results should be considered as negative. The possibility should always be kept in mind, however, that the lack of the fibrillar halo may result from a supermaximal rather than submaximal NGF quantity in the extract to be assayed.

Short- and Long-Term Cultures of Sympathetic Ganglia in Semisolid Media

Short-term cultures of sympathetic ganglia, dissected out from chick embryos between the 7th and the 9th day of incubation and cultured in control media or in media enriched with the NGF, have given results so similar and in fact so nearly identical to the results described for sensory ganglia that further description is unnecessary. It is of interest to consider instead the NGF effects on sympathetic ganglia from newborn mice or chick embryos maintained in vitro for long periods of time in a medium containing chick embryo extract, human placental extract, ox serum ultrafiltrate, and a balanced salt solution. In the experimental cultures the NGF was added in the amount respectively of 1, 10, 100, and 400 biological units (32). The NGF-rich and control cultures were examined every few days up to a month. The best effects were obtained when the NGF was added to the culture medium at the supermaximal doses of 100–400 biological units/ml. Cultures examined in vivo or after fixation and toluidine stain consisted of a sympathetic nerve cell population much larger than in control cultures. The cytoplasm of experimental sympathetic cells showed denser Nissl bodies and nuclei and nucleoli twice as large as in controls. Likewise neurofibrils were markedly increased as shown by treatment with specific silver preparations. The potent stimulating effect of the NGF in these long-term cultures was also shown in electrophysiological
experiments on sympathetic chain ganglia from newborn mice. The nerve impulse propagated for much longer distances in the treated ganglion chains than in controls. These results suggest a more precocious maturation of sympathetic nerve cells in the presence of the NGF than in media deprived of this factor (32).

**NGF Effect on Dissociated Sensory and Sympathetic Nerve Cells in Liquid Media**

Additional evidence for the vital role played by the NGF in the survival and growth of embryonic sensory and sympathetic nerve cells came from experiments on nerve cells dissociated with trypsin and cultured in liquid media consisting of Eagle amino acid solution or Eagle medium supplemented with 10% horse serum. In the experimental cultures the NGF was added in the amount of 5–100 biological units/ml. Neither the Eagle solution alone nor this solution supplemented with 10% horse serum is adequate for survival and further growth of embryonic sensory and sympathetic nerve cells (67). At the end of the first 24 hr of culture most of the dissociated nerve cells have undergone disintegration, and 48 hr later the control cultures consist of fibroblasts and satellite cells with few or no nerve cells. In the presence of NGF, nerve cells survive in excellent condition and undergo vigorous growth, as proved by the very dense nerve net that covers the dish bottom. These cultures can be kept alive for as long as a month. These experiments prove that the requirement of nerve cells for this protein is exceedingly low. Denaturation of the protein by heating abolishes the effect. Both observations rule out the hypothesis that the NGF merely serves as a source of cell proteins and suggest instead that it acts in a catalytic fashion.

**NGF Effects on Sensory Nerve Cells at Ultrastructural Level**

The electron-microscopic analysis by Crain et al. (32) of sensory ganglia cultured in semisolid media for periods ranging from 30 min to 48 hr, showed highly ordered mosaics of cytoplasmic granules in the NGF-treated cells, whereas the same structures were seen only seldom in control sensory cells. The hypothesis was proposed that these paired rows of granules might represent an early stage in the formation of additional ER cisternae or, alternatively, that they might be related to the early formation of neurotubules. Conclusions as to their possible significance, however, should wait for confirmation of these findings.

A more recent study of embryonic sensory cells, cultured in semisolid media for periods ranging from 4 to 12 hr in the presence of 10–100 biological units of the NGF and in control media, showed striking changes in the ultrastructure of the nerve cells. The nuclei developed an irregular contour and a more dense and clumped chromatin in comparison with the controls. In the cytoplasm the Golgi apparatus was dilated; in some cells there was a massive increase in ribosomes associated with an increased number of membranous elements of endoplasmic reticulum. Neurofilaments and neurotubules, sparse in control cultures, were
already prominent after 4 hr of incubation in the presence of the NGF. After 12 hr the cytoplasm of most ganglionic cells was packed with large masses of neurofilaments (73).

**BIOLOGICAL SOURCES OF NGF IN THE ORGANISM**

It is apparent from the brief historical survey already given that a number of tissues, organs, and body fluids share the property of releasing the NGF. A systematic screening of potential sources of this factor, performed during the years from the beginning of this investigation to the present day, has revealed such a large number of sources that one is led to speculate as to the significance of its widespread occurrence and also about the possibility of detecting the site or sites of its production in the organism. [A similar situation obtains for two other specific growth factors, viz. the "erythropoietin-stimulating factor" and the "epidermal growth factor" considered later. More than half a century after the discovery of the erythropoietin-stimulating factor (21), its source of origin in the organism is still a matter of discussion. Likewise the origin of the epidermal growth factor remains to be identified.] Here we list and briefly consider the tissues, organs, secretions, and excretions endowed with a nerve growth-promoting activity, and then consider the problem of its source of origin in the organism.

**Mouse Sarcomas: Characteristics of NGF-Releasing Neoplastic Cells**

Mouse sarcomas 180 and 37 share a common laboratory origin from mouse salivary carcinomas that, through serial transplantations in mice, underwent progressive structural and cytological changes until they had lost all characteristics of their tissue or origin (66). Both tumors consist of a polymorphic cell population with small and large cells closely intermingled; one or the other cell type may prevail in tissue samples dissected out from one or the other mouse sarcoma. In vivo and in vitro experiments indicate that a much higher NGF activity is present in the neoplastic large-cell type (77). Tumors consisting only or mainly of the large cells are highly invasive on implantation into chick embryos. The release of large quantities of the NGF from these transplants is indicated by the massive invasion of the tumor by sensory and sympathetic nerve fibers and by marked hyperplastic and hypertrophic effects on sympathetic ganglia. These changes are seen in ganglia adjacent to the tumor and contributing to its innervation, as well as in those remote from the implant and sending their nerve fibers into the embryonic viscera, veins, and other structures. Implanted fragments of the same tumors consisting only or mainly of small cells evoke a milder growth effect. Here only sensory and sympathetic ganglia innervating the tumor undergo size increase; the effect in these instances is strictly local (76).

Both tumors, sarcomas 180 and 37, retain their nerve growth-promoting activity indefinitely on serial passages in vitro as cell monolayers (66). Differential centrifugation of the tumor homogenate has given evidence that the NGF protein is present in the microsomal fraction of neoplastic cells (31).
NGF Activity in Other Tumors, Tissues, and Body Fluids

The finding of the nerve growth-promoting activity in mouse sarcomas 180 and 37 raised the question of whether the observed effects are due to some specific growth properties of neoplastic cells. A systematic screening of a large number of tumors ruled out this hypothesis. A mild growth effect on sensory and sympathetic ganglia of chick embryos is elicited by mouse sarcoma 1 in experiments either in vitro or in vivo (13, 14, 78), whereas other equally rapidly growing tumors such as a variety of mouse carcinomas, melanomas, and neuroblastomas of mouse or rat origin (14) do not evoke any growth effect. Human neuroblastomas behave in a somewhat different way. Although the tumor's extract has no nerve growth promoting activity, the sera of children bearing the tumor have consistently higher NGF titers than sera of healthy children or children affected with other tumors. A somewhat milder nerve growth effect was obtained from sera of children bearing fibrosarcoma (18). The authors submit the hypothesis that neuroblastoma may elaborate but not store the NGF.

The high NGF activity released by mouse sarcomas 180 and 37 suggested the possibility that any mesenchymal tissue in a phase of active growth might also release this factor. The hypothesis was tested by producing granuloma tissue, explanting it in vitro, and assaying its effects on adjacent sensory and sympathetic ganglia of chick embryos. [A description of the technique used to produce granulomas is given elsewhere (64).] The experiments showed that granulomas from mice, rats, guinea pigs, rabbits, or monkeys elicit in vitro the same growth effects as fragments of mouse sarcomas 180 and 37. Granuloma extracts therefore possess a nerve growth-promoting activity of the same potency as mouse sarcomas.

Screening of normal embryonic and postembryonic tissues and of organs of chicks, mice, rats, and other species revealed the presence of the NGF in trace amounts in the chick embryo (16) and in a number of organs and tissue extracts of mice and rats (64). The NGF is also present in urine and saliva from mice (71). Of particular interest is the discovery of this nerve growth-promoting protein in the serum of all vertebrates assayed for this purpose, man included. Likewise of considerable interest is the finding that the NGF is a normal constituent of embryonic sensory and of embryonic and mature sympathetic nerve cells (64). The rather high NGF content of sympathetic ganglia is revealed by comparing the amounts of extracts of various tissues (measured on the basis of their protein content) that upon addition to 1 ml of the culture medium result in the formation of the fibrillar halo. This amount corresponds (see p. 541) to 1 biological unit of the NGF. Similar fibrillar halos are produced by the addition to the culture medium of 15,000 µg of sarcoma extract or 200 µg of the extract of sympathetic ganglia (22). The much higher NGF activity of snake venom and of mouse salivary glands is considered later.

NGF Activity in Snake Venom

The chemical properties of this potent nerve growth-promoting factor are considered in a later section. A study of the venom gland in vipers is in progress (Levi-
Montalcini and Angeletti, unpublished observations). Here we only mention that this gland has a structural configuration similar to that of mammalian parotid glands. One biological unit of the NGF is present in 3–6 µg of venom when added to 1 ml of tissue culture.

Mouse Submaxillary Salivary Glands and Their Role in NGF Production

One biological unit of NGF is present in 1.5 µg of protein of the crude extract of the submaxillary salivary glands of adult male mice (24). The discovery in 1958 of this new, most potent source of the NGF and its easy availability have channeled almost all subsequent work into the purification of this factor, its chemical characterization, and study of its biological properties. These topics are considered in other sections of this review; here we consider the morphological characteristics of this gland and the role of sex hormones in NGF production.

The mouse submaxillary salivary glands consist of two morphologically and functionally distinct components: the acini and the convoluted tubules. The former contain mucopolysaccharides; their high amylase content is regarded as the result of the in situ production of this enzyme. The convoluted tubules, in contrast, are characterized by their high content in proteolytic and hydrolytic enzymes and their sexual dimorphism (56). The male gland differs from the female in the structural and biochemical configuration of these tubules. In males they undergo a marked size increase at puberty; proteolytic and hydrolytic enzymes begin to be synthesized in these tubules at that time (52). Additional evidence for the role of sex hormones in calling forth these structural and biochemical changes has come from other experimental studies. Testosterone injections in female mice result in a sudden increase in the size of the tubules and in a parallel increment in their protease content, whereas castration sharply decreases the size of the tubules and the enzyme content in the male gland (52). There is extensive direct and indirect evidence for the localization of the NGF protein in the convoluted tubules of these glands (24, 64). The NGF is absent from the glands of newborn mice at a time when the tubules have not yet undergone differentiation; it appears at puberty and increases in subsequent developmental stages to reach a plateau in the adult male. Its concentration is 10 times higher in male than in female glands. Injections of testosterone result in a marked increase in the NGF content in the female gland, whereas castration sharply decreases the content of the same protein in males (20, 68). Further evidence for the localization of the NGF in the gland's tubular components is obtained by treating frozen sections of the gland of adult males with specific fluorescent antiserum to the NGF. The tubular but not the gland's acinar portion shows in these preparations an intense and localized fluorescence (43, 65). Injections of the antiserum to the NGF in the puberal mice result in marked regressive changes in the epithelial cells lining the tubules, and this provides additional evidence for the localization of the NGF in these cells (20). Other similar investigations indicate that the salivary glands play an active role in maintaining the NGF level in the blood of mice (103); but, paradoxically, no proof was obtained in favor of the hypothesis that the salivary glands are essential or even necessary for the NGF
production. Against this assumption are a number of observations that are listed here only briefly. 1) Extirpation of the submaxillary salivary glands does not result in any adverse effect in sympathetic nerve cells, such as one would expect if the specific growth factor were produced by these glands as a hormone to be supplied to its target cells (74). 2) Sympathetic nerve cells of male and female mice are the same size, and yet the male salivary glands have a much higher NGF content than the female glands. 3) The NGF has not been detected in the salivary glands of other mammals even though it is present in trace amounts in the serum of all mammals tested.

The question has been raised, but still is not answered (70), whether this protein present in such a large quantity in male mouse salivary glands might not serve other purposes connected with digestion or perhaps with the gland's other function, namely the production and release of toxic substances in the saliva. The fact that this protein is present in snake venom makes this hypothesis plausible.

Whether the submaxillary glands synthesize the NGF protein in the gland's tubular portion or whether this protein is produced elsewhere, then exported and stored in the tubules, has been answered recently in favor of the first alternative. In vitro and in vivo experiments performed in three laboratories (19, 70, 103) gave decisive evidence for the in situ production of the NGF. In view of the considerable interest of these findings, we will briefly mention some of the experimental work that established this point.

The in vitro experiments consisted of the incubation of slices of salivary glands from adult male mice in Eagle medium supplemented with radioactive amino acids. Incubations were carried out for 60 min and 2 hr. The incorporation of labeled amino acids into protein was found to increase linearly with time. Likewise the NGF underwent a similar linear increase (70).

The in vivo experiments consisted of the injection with a microsyringe of radioactive amino acid in one of the two lobes of the salivary gland in adult male mice. The animals were sacrificed after 2 hr; the two lobes were separately homogenized, and the experimental and control lobes were pooled in two groups and then centrifuged. The results clearly showed a differential labeling of the NGF from the two lobes. In the lobe injected with the radioactive amino acids the labeled NGF was markedly higher than in the other lobe. These results give additional evidence for the in situ production of the NGF present in the salivary glands. In fact one would expect the NGF of the two lobes to have the same, or nearly the same, specific radioactivity if this protein were synthesized not in the salivary glands but elsewhere and then exported to the salivary glands and stored there (70).

Convincing indirect evidence for the in situ production of NGF was obtained by Burdman and Goldstein (19). These authors injected intravenously the specific antiserum to the NGF in mice. It was assumed that the antiserum present in the circulation of the treated animals would interfere with the accumulation of large quantities of the NGF in the salivary gland if this protein were produced elsewhere and then transferred through the circulation into the salivary gland. Experiments performed in many different conditions and in animals at different developmental stages consistently showed no effect by the antiserum on the content of the NGF in
the submaxillary salivary gland. Also it was shown by the same authors that intravenous injection of large quantities of NGF in mice could not be localized in the submandibular gland cells of the same animals as determined by fluorescent-antibody technique. They therefore came to the conclusion that the NGF is synthesized and stored in cells of the tubular portion of this gland.

GROWTH INHIBITION OF SYMPATHETIC NERVE CELLS BY ANTIBODIES TO NGF

The selective and permanent destruction of 90–95% of sympathetic nerve cells in newborn mice and other newborn mammals injected with a specific antiserum to the NGF was first reported in 1960 (72, 75). The antiserum was produced by Cohen by injecting the purified NGF protein into rabbits with Freund adjuvant, using techniques reported in the original articles (24, 65). Here we shall only briefly consider the in vivo and in vitro effects of the antiserum, since they bring once more into focus the key role of the NGF in the life of sympathetic nerve cells.

The antibody titer is first assayed in vitro. The assay technique consists of the addition of 0.2 ml of the antiserum at progressively higher dilutions to 0.2 μg of the purified NGF in 0.2 ml of isotonic sodium chloride (65). The mixture is incubated at 26°C for 1 hr and then added to the culture medium in the proportion of one-third of the total volume of the medium. Readings are made after 12 and 24 hr of incubation at 37°C. The titer of the antiserum is expressed as the reciprocal of the highest dilution that results in complete inactivation of the nerve growth factor. Under the best conditions the titer reaches the value of 2400 or even higher. Aliquots of this antiserum are then injected subcutaneously into newborn mice, rats, or other mammals in the amount of 0.05 ml/g of body weight. Daily injections of the antiserum in newborn mice for 3-5 days result in the permanent destruction of 95–98% of the sympathetic nerve cell population of the paravertebral sympathetic chain ganglia (72). A somewhat higher percentage of sympathetic nerve cells persists in the prevertebral ganglionic complex; this varies according to the potency of the antiserum. As a rule some 15–20% of these cells persist in the celiac ganglion, the most extensively investigated of all prevertebral ganglia. The persistence of adrenergic ganglionic complexes that innervate the male and female sex organs is the only instance of adrenergic sympathetic cells resistant to the antiserum (69). These ganglia also fail to respond to the nerve growth factor (62, 63).

Histological, physiological, and pharmacological studies on immonosympathectomized animals are reported in detail in a number of publications (53, 69, 104, 105). For the purpose of this review, it is of interest to consider the earliest effects elicited by the antiserum on its target cells, as revealed by examinations using both optic and electron microscopes. For reasons of convenience the superior cervical ganglia of newborn mice injected in vivo with the antiserum, or dissected out and incubated for short time periods in presence of the antiserum, were the object of choice in most of these investigations.

Regressive changes in sympathetic nerve cells are already visible by optic microscopy 12 hr after the first injection of the antiserum. Whereas in control
ganglia mitotic activity in the superior cervical and other sympathetic ganglia is very high at birth and continues to the 9th day, in ganglia of antiserum-treated mice mitotic figures are rarely observed and most of these appear atypical. A number of pyknotic cells are scattered throughout the ganglia; the number of dead and degenerating cells increases rapidly between the end of the 1st and the 2nd subsequent days. By the end of the 2nd and 3rd day of incubation, all mitotic activity has ceased. Neuroblasts are much smaller than controls and the cytoplasm is almost deprived of ribonucleic acid, as shown by a failure to stain with basic dyes and by the fact that nucleoli are barely visible (72). Dead cells and cell debris are scattered in large numbers among the neuroblasts and satellite cells that are present in larger proportion than in controls. In the following days satellite cells also undergo regression and the ganglia are reduced to diminutive sclerotic nodules barely detectable with the aid of a dissecting microscope.

Electron-microscopic studies of the same ganglia give evidence of the extraordinarily rapid effects elicited by the antiserum in vivo and in vitro. Two hours after the injection of the antiserum the sympathetic nerve cells show folding of the nuclear envelope, condensation of chromatin into large and small masses, dilation of the vacuolar system, and mitochondrial alterations. The in vitro effects of the antiserum are even more marked; extensive cytolytic lesions are already apparent after 4 hr of incubation (91). A comparison of these changes with those seen in electron-microscopic studies of tumor cells cultured in the presence of specific antisera shows that there are substantial differences between the latter and the effect mentioned above. Nuclear damage seems to be practically absent in the initial phase of the reaction of neoplastic cells to antisera (42, 47, 57). On the other hand, these data and other studies now in progress (unpublished observations from our laboratory) suggest that the nucleus of sympathetic nerve cells is the primary target of the antiserum to the NGF.

Further evidence for the precocious and lethal effects caused by the antiserum to the NGF comes from studies of the incorporation of uridine into RNA in sympathetic nerve cells. Four hours after the injection of the antiserum the incorporation of labeled uridine in the superior cervical ganglia of newborn mice is reduced to half of the control values (92).

CHEMICAL PROPERTIES OF NGF

NGF From Snake Venom

After the first discovery of NGF activity in snake venom, various samples of dried venoms from species of the three families of poisonous snakes were tested for their biological activity. All venoms examined showed similar growth-promoting properties. The crude venom of Elapidae and Viperidae were found to be 2-4 times more potent than Crotalidae (23). The venom of the moccasin was used by Cohen for purification of the NGF (22); approximately 25% of the activity present in 1 g of crude venom was recovered in 5.2 mg of protein, a purification of about 40-fold.
On a Spinco analytic ultracentrifuge, only a single component was detectable, with an $S_{20}$ of 2.2 S; the molecular weight was estimated to be about 20,000. The 280/260 absorption ratio was found to be 1.3; on acid hydrolysis and two-dimensional paper chromatography, the amino acid pattern was quantitatively identical to similar chromatograms prepared with bovine albumin. By the orcinol procedure, 1.6% of hexose was found to be present. Two additional lines of evidence support the view that the biological activity is associated with a protein: 1) the biological activity was completely destroyed on incubation with proteolytic enzymes (trypsin, pepsin, chymotrypsin); 2) the biological activity was lost on incubation with antiserum to snake venom. The possibility that the growth-promoting activity of the venom was due to one of the enzymes known to be present in the venom was carefully investigated by Cohen. Activity of a number of enzymes was assayed in snake venom and in the purified NGF but none was found to be associated with the nerve growth factor (22, 23).

In the original purification procedure devised by Cohen, which yields a pure and active NGF protein of about 20,000 mol. wt., a treatment with 6 M urea is used in the first step in order to avoid loss of biological activity. When milder fractionation procedures are used, the NGF activity is found associated with proteins in various molecular aggregates (2). Thus, when crude venom is fractionated on Sephadex G-100, the biological activity appears in a broad area corresponding to molecular weights around 40,000. Upon chromatography on a DEAE-cellulose column at pH 7.4, all the biological activity is recovered in the unabsorbed peak; when this component is analyzed by sucrose density-gradient centrifugation, the NGF activity appears to be associated with proteins of molecular weight in the range of 20,000. By chromatography of this fraction on a CM-cellulose column at pH 5, two active components are obtained. Both bind strongly to the column and are eluted with high salt concentration. On disc electrophoresis, on acrylamide gel at pH 6.6, the first component gives two closely spaced bands and the second component a single sharp band moving fast to the negative pole. In the ultracentrifuge these forms of NGF appear to have sedimentation coefficients where $S_{20}$ is 2.5 and 1.5, respectively. Molecular weights of about 22,000 and 12,000 were calculated by gel-filtration experiments (2). The specific activity of the larger NGF molecule is 2 times higher than that of the smaller NGF species. Complete amino acid analysis performed on these two forms of NGF from *Crotalus adamanteus* resulted in an almost identical amino acid composition for both of them. This finding suggests that they have the same primary structure and that the major differences between them must reside in their molecular size and/or conformation. Immunochemical analysis, using a microcomplement fixation, appears to confirm this assumption, since the $C'$-fixation curves of the various NGF samples are completely superimposable.

On the basis of their amino acid composition, a minimal molecular weight of 5000 has been calculated for the NGF from *Crotalus adamanteus*. This may actually represent the molecular weight of the NGF monomer, although a biologically active NGF of this size has not yet been isolated. It is suggested that the snake venom NGF is a protein that exists in multiple aggregate states of similar subunits. Definite correlation between the observed biological activity and a given form of the NGF...
must await further characterization of the basic monomer and of each pure aggregate.

**NGF From Mouse Submaxillary Salivary Glands**

As already reported the submaxillary glands of adult male mice are by far the richest source of NGF. Crude extracts are active in tissue culture at concentrations as low as 1–2 μg/ml. Cohen devised a procedure that gives a 100-fold purification of the active agent with an overall yield of about 20% (24). The purified NGF appears to be a protein with an $S_{20}$ of 4.33 $S$ and a molecular weight estimated at about 44,000. Biological activity is destroyed on incubation with proteolytic enzymes and is inhibited by specific antibodies. When tested in tissue culture with embryonic sensory ganglia, the most purified preparation evoked the characteristic fibrillar halo at the concentration of 0.015 μg/ml of medium. By immunological tests (in vitro inhibition of biological activity) a low degree of cross reaction was observed between the salivary and the venom NGF (24). Further studies are now in progress in our laboratory on the molecular properties of the NGF present in snake venom and in salivary glands and on the evolutionary changes that this protein exhibits from reptiles to mammals.

In crude extracts of submaxillary glands the NGF activity appears associated with a large molecular aggregate of over 120,000 mol. wt. This aggregate, on gel-filtration and ion-exchange chromatography, shows one single component when analyzed at neutral pH on starch-gel electrophoresis (79, 93); when analyzed instead by electrophoresis on acrylamide gel at pH 8.3, the same high-molecular-weight NGF preparation shows a number of components of different mobility. On elution of the gel sections, part of the applied activity can be recovered, and this is all present in a fine sharp band near the origin; the same active component migrates to the negative pole at pH 6.6. Dissociation of the large molecular aggregate also occurs on dialysis against acetate buffer (0.01 M, pH 5); chromatography of this material on a CM-Sephadex column at the same pH gives three main components: a pregradient peak comprising a group of rather acidic proteins, a second peak emerging between 0.1–0.2 M NaCl, and a third peak eluted at 0.5 M NaCl. The first and second peaks are completely inactive, whereas the applied NGF activity is recovered in the last peak. When analyzed by disc electrophoresis, this fraction shows a single basic component, which in the ultracentrifuge has a sedimentation coefficient of 2.3 $S_{20}$.

Our viewpoint is that the other protein components separated by CM-chromatography do not contribute to the biological activity of the NGF. This belief is supported by the following arguments.

1) The CM fraction obtained according to Cohen or with slight modification of the original procedure (24) when assayed in vitro shows a specific activity higher than each of the fractions obtained during the purification steps from the crude extract to this final NGF preparation. This result, first reported by Cohen in 1960 (24), has been consistently confirmed in all subsequent studies performed in our laboratory.
2) This same NGF form is fully active when injected in vivo. The impressive growth effects elicited in newborn mice were, in fact, obtained by injecting this NGF preparation.

3) The antiserum to the NGF was obtained by immunizing rabbits by injection of this NGF molecule. Thus, antibodies specific against this single protein call forth the destructive effects reported in this article. Since a full biological activity is present also in NGF preparations with higher molecular weight, the hypothesis is proposed that, as in the case of the snake venom NGF, the salivary NGF might also aggregate in multiples of the same NGF molecule. The two sedimentation coefficients of 2.3 and 4.4, alternatively obtained in various preparations of the CM fraction, suggest that the latter may represent a dimer of the former.

Other Models Proposed for NGF Molecule

Studies on the chemical structure of NGF isolated from the mouse submaxillary glands were performed by Schenkein and Bueker (94, 95), and more recently by Varon et al. (100, 101). Both models will be briefly considered below.

The purification procedure followed by Schenkein and Bueker consisted first of a precipitation of the gland homogenate with streptomycin sulfate, followed by alcohol precipitation of the supernatants; the ethanol precipitation was dissolved and salted out with ammonium sulfate at 40% and 90% saturation. This last precipitate, containing the biological activity, was then fractionated on a CM-column washed with alkali and then with distilled water until neutrality. Under these conditions the active material did not bind to the column and was eluted with the first protein peak. Further examination of this fraction by paper electrophoresis at pH 7.4 showed three bands that were labeled A, B, and C. In vitro bioassay of the individual bands showed no significant biological activity when tested separately, but when combined bands A and C gave a positive tissue culture test. According to the authors, the two components A and C were then obtained on a preparative scale by using electrophoresis on a starch column. Again, in vitro bioassay showed that fractions A and C singly possessed only a weak biological activity, whereas on recombination of the two fractions a restoration of the full biological activity followed. Dialysis after lyophilization of fractions A and C showed that C but not A was dialyzable. Quantitative amino acid analysis of fraction A showed the presence of all essential amino acids with the exception of methionine; a minimum molecular weight of 8600 was calculated. In the ultracentrifuge this fraction showed two peaks with $S_{20}$ of 2.41 and 4.24 respectively. Data on a quantitative amino acid analysis of fraction C allowed the calculation of a minimum molecular weight of about 3500.

In comparing these data with those originally reported by Cohen, Schenkein and Bueker suggested the possibility that the species with an $S_{20}$ of 4.24 might represent a dimerized form of A that in combination with C (mol. wt. 3500) would give the fully active NGF form of about 42,000 mol. wt. In summary, according to these results the active salivary NGF appears to be a protein, which under specific experimental conditions splits into two inactive components, one of which (C)...
seems to be a rather small polypeptide; recombination of the two parts restores the biological activity. Confirmation of these results from other laboratories is greatly to be desired.

A second model for the NGF molecule has been proposed by Varon et al. on the basis of association and dissociation experiments (100, 101). These authors used a three-step procedure to isolate the NGF from crude extracts of submaxillary glands; they used gel filtration on Sephadex G-100, chromatography at neutral pH on DEAE-cellulose, and a final gel filtration on Sephadex G-150. The final product accounted for 2% of the protein in the gland and, according to the authors, for 80% of the activity. The minimum amount in protein content required for giving a 3+ response in tissue culture (corresponding to our definition of 1 biological unit of the NGF) would be of the order of 13 ng; this specific activity is in the same range as that obtained in our laboratory according to Cohen’s procedure.

This fraction migrated on electrophoresis at neutral pH as a single component, which thus represented the NGF. Sedimentation analysis showed a major component with an $S_{20}$ of 7.1; the molecular weight of this NGF species was calculated to be approximately 140,000. This form of NGF appeared to be stable only within a limited range of pH (5–8); outside these limits the large aggregate dissociated into a number of subunits whose sedimentation coefficients were sufficiently close to each other as to sediment in a single boundary with an $S_{20}$ of 2.6, indicating a molecular weight in the range of 30,000. The dissociation of the NGF appeared to be reversible within given pH limits; thus over 80% of the high molecular weight was recovered if the completely dissociated system at pH 3.8 was dialyzed at neutral pH. The same dissociation was found on exposure to pH 9.7, but after exposure to pH 10.3 the reversibility was less complete; exposure to pH higher than 13.3 resulted in no recombination when the pH was adjusted to neutrality. According to Varon et al. the biological activity of the large aggregate decreased to about 25% of its value on dissociation of the large aggregate and was restored after dialysis at pH values under conditions where physical measurements showed recovery of the high-molecular-weight NGF form. On acrylamide gel electrophoresis at alkaline pH, after irreversible dissociation of NGF at high pH, three groups of proteins were observed: 1) a group of three acidic subunits, 2) a second group of three components of intermediate charge, and 3) a third group consisting apparently of a single basic subunit. Activity measurements on gel sections indicated that only the basic subunit had NGF activity. By chromatography on CM-cellulose at acid pH the three groups could be separated from each other, and again the most basic component was found to possess NGF activity, though considerably lower than that of the original NGF aggregate. From the various components separated on CM column, the original high-molecular-weight NGF species could be regenerated on dialysis at neutral pH, and apparently the activity level was increased more than twofold as a result of this recombination.

In summary, according to this model three types of different subunits account for the NGF molecule in its native state. Only one of these subunits, however, carries the specific biological activity; the others simply act as regulatory subunits “which permit control of biological activity through allosteric effects” (100, p.
1788). Varon et al. speculate about the possibility that interaction of the subunits may be of relevance in the extraganglionic environment or that they may serve to stabilize the activity of the active subunit or to maintain a constant concentration of this subunit through a dissociation equilibrium. The validity of this attractive model rests on the possibility of obtaining more convincing evidence in its favor. Perhaps the weakest point, as we see it, is that the NGF biological activity was judged only on the basis of in vitro experiments and never assayed in vivo. The same criticism applies to the model proposed by Schenkein and Bueker. As discussed previously, the in vitro response has only a limited value, and a twofold or even fivefold increase or decrease of biological activity may be very difficult to disclose for the reasons given on p. 542. Until more rigorous quantitative assays of the NGF become available using in vitro techniques, it seems highly desirable to grade this activity with parallel in vivo tests of each NGF preparation.

**METABOLIC EFFECTS OF NGF**

The growth effect elicited by NGF in the receptive nerve cells has been investigated biochemically; the main results of these studies are dealt with in the following sections.

**Effect on Glucose Metabolism**

Cohen first investigated the effect of NGF purified from snake venom on glucose oxidation by embryonic sensory ganglia cultured in vitro (23). He observed that the presence of glucose or mannose is required for the outgrowth of nerve fibers. If no energy source was added the outgrowth of fibers was initiated but ceased almost immediately. Glucose could not be replaced by D-fructose, L- or D-arabinose, D-ribose, D-galactose, glucuronic acid, gluconic acid, malic acid, L-ketoglutaric acid, succinic acid, or fumaric acid. Lactate and pyruvate could partially replace glucose. Using 14C-labeled glucose he observed that the NGF increased the oxidation of carbon 1 of glucose by 41–54%; less of the C-6 of glucose was oxidized by the ganglia and the stimulation brought by the NGF was 12–26%. The presence of fluoride or cyanide did not prevent the nerve fiber outgrowth nor the increase of glucose oxidation. These results were confirmed and extended with NGF purified from the mouse submaxillary gland. The relative yields of 14CO2 from glucose-1-14C were investigated in sensory and sympathetic ganglia incubated in Warburg flasks with and without NGF (7). In all these experiments, carried out for a 3 hr period, there was a marked increase in the oxidation of glucose-1-14C and only a slight increase in the yield of CO2 from 6-14C-glucose (6). Similar results were obtained when the NGF was injected in vivo in newborn animals and glucose oxidation was studied in the superior cervical ganglia. Control experiments with different nervous tissues known to be unresponsive to NGF did not show any change in glucose metabolism. The addition of fluoride to the medium at a concentration 10^-2 M did not prevent the stimulation of oxidation of 1-C-glucose (6, 25). These
results show that the NGF stimulates glucose utilization by the receptive nerve cells and that such stimulation appears to be mainly on a direct oxidative pathway. A number of enzyme activities representative of carbohydrate metabolism were also investigated in sensory and sympathetic ganglia incubated for different lengths of time with or without NGF. No evidence was obtained for some specific enzyme induction under the NGF stimulation (7); the common finding was instead that the NGF maintained all the enzyme activities tested at levels higher than controls. The effect was particularly evident in long-term experiments (6-24 hr) where most of the enzyme activities in the control ganglia underwent a progressive decline, whereas they were maintained at a steady-state level in the presence of NGF (4, 81, 84).

**Effect on Lipid Metabolism**

The growth response evoked by the NGF in vitro as well as in vivo is accompanied by an overall increase of synthetic processes in the receptive neurons. Experiments carried out with labeled acetate gave evidence that the rate of lipid synthesis is markedly stimulated in embryonic sensory and sympathetic ganglia incubated with NGF (5, 80). Within a 4-hr period of incubation the radioactivity incorporated into total lipids was, on the average, 60-90% greater in NGF-treated ganglia than in controls. Increased lipid synthesis was also observed in superior cervical ganglia from mice explanted in vitro and cultured for various periods of time (80). In experiments with embryonic sensory ganglia the lipid extracts were analyzed by thin-layer chromatography, followed by autoradiography. No significant differences in the relative intensity of labeling of the various components were detectable. The increase in the rate of lipid formation by the NGF-stimulated cells could be correlated with an increased availability of NADPH$_2$ (a necessary cofactor in the reductive synthesis of fatty acids) and/or with the induction of new enzyme molecules. The results of experiments with specifically labeled glucose indicate that the hexose monophosphate pathway is indeed activated in sensory or sympathetic ganglia under the influence of the nerve growth factor; the two major enzymes of the pentose pathway are specific for NADP, which is reduced to NADPH$_2$, and this may well serve as a reservoir of reducing potential for the synthesis of fatty acids. It is of interest to note that the stimulatory effect of NGF on acetate incorporation was completely abolished by actinomycin-D at a dosage that did not depress significantly the basal rate of incorporation in control ganglia (80). The significance of this finding is discussed below.

More recently the effect of NGF on lipid metabolism in embryonic sensory ganglia was investigated by using DL-2-$_{14}$C-mevalonate as a precursor (82, 83). Contrary to the results with $_{14}$C-acetate, the presence of NGF in the culture medium resulted in a 25% decrease of incorporation of labeled mevalonate after 4, 6, and 8 hr of incubation. Moreover, almost all the mevalonic acid incorporated was present in a sterol-like compound that was isolated and partially characterized. This steroid appears to be present in embryonic ganglia in fairly large amount (5% of dry weight) up to certain developmental stages (13-14 days), and it decreases
thereafter while cholesterol concentration gradually increases. Since NGF significantly decreased the synthesis of this steroid in embryonic ganglia explanted in vitro, the possibility that this metabolic effect might be related to some structural changes taking place in the stimulated nerve cells appears worth investigating.

Effect on Protein and RNA Synthesis

The growth effect elicited by the NGF on receptive nerve cells is accompanied by a rapid increase in net protein synthesis. Cohen first examined the effect of the growth factor on the incorporation of 14C-lysine into protein of embryonic sensory ganglia cultured in vitro (23). Under the influence of the venom NGF, the incorporation was increased 58–72% within a 20-hr period. The addition to the culture medium of the amino acid analog p-fluorophenylalanine (0.2 mM) almost completely inhibited the NGF effect.

More extensive studies of the NGF effect on protein synthesis were carried out with chick embryonic sensory and sympathetic ganglia and with superior cervical ganglia from newborn mice. The incorporation of a number of radioactive amino acid was consistently enhanced by the presence of the nerve growth factor in the medium even after only 3 hr of incubation. Other experiments indicated that the intracellular protein turnover is also stimulated by the growth factor (3).

A preliminary analysis of soluble proteins from embryonic sensory ganglia incubated for various periods of time in the presence of radioactive precursors revealed that the NGF selectively stimulates the rate of synthesis of some classes of proteins. When total soluble proteins were analyzed by sucrose density-gradient centrifugation, the radioactive profile of the NGF-treated samples consistently showed a relative increase in the labeling of heavier proteins (prealbumin zone) as compared with the controls. By column chromatography on DEAE-cellulose, soluble proteins from ganglia incubated 3–6 hr with and without NGF were separated in a discrete number of components; here the experimental pattern showed a marked increase in the labeling of the more acidic proteins as compared with controls. Selective stimulation of more negatively charged proteins was also observed when the protein extracts were fractionated by disc electrophoresis on acrylamide gel (39). These data indicate that the NGF modifies to variable degrees the rate of synthesis of different classes of proteins The addition of puromycin to the medium suppressed amino acid incorporation in both control and NGF-treated ganglia and inhibited the outgrowth of nerve fibers. Under these conditions, however, the stimulatory effect of NGF on RNA synthesis was not suppressed.

The stimulation of RNA synthesis, as indicated by incorporation of radioactive precursors, appears to be one of the earliest metabolic effects elicited by the NGF on explanted ganglia cultured in vitro. In early experiments carried out with snake venom NGF, the incorporation of 14C-adenine into RNA increased from 40 to 70% in a 20-hr period (23). The stimulatory effect of NGF on the incorporation rate of labeled precursors into RNA is even more striking in shorter periods of incubation. When embryonic sensory ganglia were incubated in a minimum essential medium containing uridine 3H, the incorporation rate into NGF-treated ganglia exceeded
that of control ganglia by more than 100% as early as 2 hr of incubation (3, 98). Time-sequence studies indicated that the NGF stimulation of RNA synthesis preceded that of protein synthesis (3, 62). Furthermore, ganglia preincubated with puromycin were still responsive to the NGF stimulation of RNA synthesis, thus suggesting that this effect is not mediated by de novo synthesis of some protein within the cells. Actinomycin D, at concentrations of 0.5 and 1 \( \mu \)g/ml, was found to cut down uridine incorporation to 15% in both control and experimental ganglia; at this concentration protein synthesis was only slightly inhibited for the first 3–4 hr of incubation but NGF stimulation of amino acid incorporation was completely abolished (3). The above findings seem to indicate that one of the primary effects elicited by the growth factor is at a step that involves new syntheses of DNA-primed RNA, which in turn is responsible for the enhancement of protein synthesis. Density-gradient profiles of \(^{3}H\)-uridine-labeled RNA extracted from ganglia incubated with and without NGF did not show any significant qualitative difference (98). The effect of NGF on the total RNA content and base ratios was also investigated in isolated neuroblasts from embryonic sensory ganglia cultured in vitro (17). A significant increase was found in the amount of RNA per cell after 17 hr of incubation only in the presence of NGF. No differences, however, were observed in base ratios between NGF-treated and control cell RNA's. In a more recent study Toschi et al. (99) analyzed the RNA extracted from ganglia incubated 5 hr with and without NGF in a medium containing \(^{32}P\)-orthophosphate. The RNA's were chromatographed on a methylated albumin column and the various components were analyzed for their nucleotidic composition. The main labeled component outlined by chromatography appeared to be a heavy, nonribosomal RNA, with a predominant nucleotidic composition of a DNA-like type. These results indicate that during the first 5 hr of incubation, there is only limited synthesis of R-RNA by sensory ganglia with or without NGF; during this time the cells produce high-molecular-weight RNA species with DNA-like base composition whose synthesis is greatly stimulated by the growth factor.

NGF AND OTHER SPECIFIC GROWTH FACTORS

The discovery of the epidermal growth factor (EGF) follows so closely that of the NGF, and its early history is so much interwoven with that of the NGF, that a brief report on its chemical properties, biological activity, and mechanism of production seems justified in a review devoted to the NGF. Moreover, both of these factors and also the erythropoietin-stimulating factor (ESF) show striking similarities in their biological activity as well as in other characteristics. Future studies on the NGF and on any of the other two agents should benefit therefore from a comparative analysis and a joint effort to elucidate the biological significance not only of one but all of them.

Epidermal Growth Factor

The epidermal growth factor (EGF) was discovered by Cohen in 1962 (25). Its action was first noted when the nerve growth factor was injected in newborn
mice, but at that time it was not recognized as a separate factor (71). The treated mice differed from untreated littermates not only in the marked volume increase of the sympathetic ganglia, but also in their stunted growth, failure of development of hair, precocious opening of the eyelids, precocious eruption of the upper and lower incisors, and calcification of these incisors before those of controls. Although some effects such as dwarfism or failure of hair growth could be supposed to be the result of some toxic effect of the injected, not highly purified preparation, the eyelid and incisor effects could hardly be explained that way. Using a biological assay based on the precocious opening of the eyelids, which occurred 6 days before controls in infant mice, Cohen was able to purify and identify this factor. He found the activity present in a heat-stable, nondialyzable protein whose more distinctive chemical characteristic was the absence of phenylalanine and lysine. Its molecular weight, estimated from the number of amino acid residues, is approximately 15,000. The factor is antigenic but no biological activity has been detected of injection of the antiserum into newborn animals (25). Additional evidence for the protein nature of this factor was obtained from studies of its chemical composition, electrophoretic and immunophoretic analysis, and evidence of its inactivation by proteolytic enzymes.

The purified EGF was injected daily into newborn mice and rats in the amount of 0.5–1 μg/g of body weight for periods of time ranging from 8 to 12 days. Histological evidence was obtained that the precocious eyelid opening is a reflection of enhanced epidermal keratinization and an increase in the overall thickness of the epidermis (26–29). Similar epithelial changes take place on the whole body surface, as well as in the epithelium lining the oral cavity, esophagus, and other sectors of the digestive tract. Newborn rabbits and dogs are likewise receptive to the EGF. In rodents 12–20 days of age, the injection of the EGF for a 3- to 4-week period results in a visible increase in the diameter of the tail and a thickening of the epithelium covering the plantar surface of the feet, while the fur-covered skin is not affected by the EGF. In vitro studies on epithelial tissues dissected out from different sources disclosed a very potent growth-stimulating effect that materializes in short-term experiments (9, 27). These studies gave evidence for a direct stimulatory action of the EGF on the target cells and at the same time offered an opportunity for analyzing the metabolic effects called forth by the EGF and for exploring its mechanism of action at the cellular and subcellular levels. Of these more recent investigations and those still in progress, one needs to mention only that the EGF markedly stimulates the net accumulation of protein and RNA in the target cells. Cell-free extracts from epidermis cultured in the presence of the EGF are more than twice as active as extracts from control cultures in incorporating labeled amino acids (49). This difference has been accounted for in the ribosomal fraction of the cells (50). A primary action of the EGF on nuclear RNA is considered unlikely on the basis of previous experiments with actinomycin D and puromycin: the latter but not the former prevents the EGF effect (49). In this respect the NGF and the EGF effects seem to be at variance (see p. 558).

Other features of the EGF are worth mentioning since they are similar, and in some respects are in fact identical, to those of the NGF. The EGF is present in large quantity in the submaxillary glands of the adult male mouse but it is absent in the same gland of animals before puberty and could not be detected in the salivary glands of female mice or of other mammals. Extirpation of the mouse
salivary glands performed in studies of the NGF (62) did not result in any appreciable "deprivation effects" in epidermal cell systems. No evidence was obtained from in vivo experiments as to whether the EGF is synthesized in the salivary glands or somewhere else and merely stored in these organs. Extrapolation from results obtained with the NGF (70) suggests the first alternative as the more likely. Tissue culture experiments indicate that serum from different sources stimulates epidermal growth (28). Argyris and Argyris (10, 11) and Patterson (88) observed an in vivo stimulation elicited by some tumors on adjacent epidermal tissue. It remains to be seen whether the EGF or some different growth-stimulating factor is responsible for these latter effects.

**Erythropoietin-Stimulating Factor**

An erythropoietin-stimulating activity was discovered at the beginning of the century by two French authors, Carnot and Défandré, and named after its action, hemopoietine (21). In the 6 decades that followed, this agent was referred to as hemopoietine, erythropoietin-stimulating factor (ESF), or simply as erythropoeitin. It has been the object of investigations that have become particularly intensive in this last decade, as exemplified by the review article of Gordon in 1959 (46) and by the symposium on erythropoiesis devoted to the analysis of this factor that appeared in 1962 (51). The chemical nature of ESF and the main features of this growth response are considered briefly.

The ESF has been assumed by most investigators to be a glycoprotein of fairly large molecular size. Although at present, as pointed out by Goldwasser, all data are still too inaccurate to yield more than a rough estimate (44) the molecular weight appears to be of the order of 60,000–70,000. The active principle is said by most authors to be a non-species-specific, heat-stable, nondialyzable substance, destroyed on incubation with proteolytic enzymes, and endowed with antigenic properties (45). This last property was studied particularly by Garcia and Schooley (41), who, on extraction and purification of the ESF from the urine of severely anemic patients, succeeded in preparing a rabbit antiserum and in proving its in vivo anti-ESF activity. The assumption of a specific anti-ESF activity is based on the observation of a marked reduction in radioiron incorporation in normal mice receiving injections of this antiserum. According to the authors, "These results support the concept that erythropoietin is involved in the normal homeostatic control of erythropoiesis" (40, p. 57). This important finding is quoted because of the similarity to the effects elicited by a specific antiserum to the NGF (see p. 549, 550).

The ESF is normally detectable in the plasma and urine of animals rendered anemic by cobalt treatment, exposure to lowered oxygen pressure, or treatment with phenylhydrazine (45, 85). Its presence in the urine of patients suffering from hypoplastic anemia (40, 85) and in human blood (55) has also been reported. Both the plasma and urine of animals made anemic with various treatments are routinely used for the extraction and further purification of the ESF. The site of formation of the ESF is far from settled (45, 51). At present the kidney is reputed to be the major site of production of this factor, in spite of the fact that the ESF is found only in
minimal quantity in this organ (38, 44, 45, 90). It is generally conceded that other tissues and also tumors (102) release ESF. Significant information about the mechanism of its action and the structural and biochemical events called forth by the ESF in the target cells was obtained from in vitro studies of the effect of this factor on bone marrow populations (1, 35, 54, 86, 89). Bone marrow stem cells respond to the ESF in vitro by increased stroma and RNA synthesis (35, 37). The stimulated RNA synthesis is abolished in the presence of actinomycin. These and other data reported by Goldwasser (44) suggest that the ESF exerts its action by regulating gene activity in erythroid cells. Tissue culture and perfusion techniques indicate that ESF increases the rate of red cell production and maturation as well as the release of erythrocytes into the circulation. A basic question remains unanswered (44): whether the ESF is a single substance with more than one site of action or whether the purest preparation yet available consists of more than one substance, each with its site of action. Goldwasser has recently proposed interesting models to account for the plural structural and biochemical events elicited by this factor (45).

Granulocytosis-Inducing Factor

A systematic screening of the submaxillary salivary gland extract for other potential growth-promoting activities resulted in the discovery of a protein fraction endowed with the property of greatly increasing the number of granulocytes in the circulation of newborn mice and of all other mammals so far investigated (8). Preliminary in vivo and in vitro experiments (70) suggest that a direct stimulatory action is elicited by this protein on bone marrow stem cells, which are thus channeled in the granulocytic differentiative pathways. Studies in progress are directed to the further purification of this protein fraction and to determine if it is another specific growth factor.

One may state that the growth response systems considered above share several common major features and differ from each other in only a few aspects. To summarize these features, the similarities and differences between the NGF, EGF, and ESF are listed.

1) All three factors were identified in protein molecules. 2) They are normally present at a very low concentration in the blood, organ extracts, and urine (NGF and ESF) of individuals belonging to different species, man included. 3) Two of these factors (NGF and EGF) are found in large quantities in the male mouse submaxillary salivary glands, which provide the source for the extraction of these factors. The kidney is reputed to play an important role in ESF production in spite of the fact that the ESF is present only in trace amounts in this organ. Considerations previously presented oppose the concept that the salivary glands produce the NGF and the ESF in an endocrine-gland fashion and supply these factors to their target cells. Likewise the kidney does not seem to fit in the role of an endocrine organ with respect to the ESF. The problem of the physiological source of these three factors remains unsolved. For each factor the possibility is suggested that more than one tissue or organ is engaged in its production and that the target cells receive it from the other cells that may be assembled in organs or disseminated.
everywhere in the organism. 4) All three factors are endowed with antigenic properties, though only in two systems (NGF and ESF) does the antigen-antibody reaction in the living organism result in destructive effects on the target cells. 5) The growth response is restricted to one cell type for the EGF and the ESF, whereas for the NGF two closely related nerve cell types respond to its action during embryonic life; only one of the two types of nerve cells, those in sympathetic ganglia, retains this capacity beyond the embryonic stages of life. 6) The growth factors exert their action directly on the target cells as proved by in vitro studies with the NGF, EGF, and ESF. 7) In all instances the effect materializes soon after the beginning of the incubation period and is characterized by clear-cut morphological and biochemical events in the target cells. 8) The same mechanism of action has been suggested for the NGF and ESF. In both instances studies with specific inhibitors of RNA and protein synthesis suggest that the primary site of action is at the transcription level, whereas similar experiments suggest that the EGF elicits a primary change in the ribosomes of its target cells. 9) A striking parallel is found in the extent of the growth response of the two cell types, epithelial and nerve cells, respectively, to the EGF and the NGF. In both instances the growth response is particularly impressive in cells in their early stages of differentiation and consists of an increase in the mitotic activity and acceleration of maturation processes. A size increase in fully differentiated sympathetic nerve cells finds no parallel in epithelial cells. This difference, however, is readily explained on the basis of intrinsic differences in the two cell types; nerve cells but not epithelial cells retain throughout life the capacity of increasing in size. The main feature of the growth response to the ESF is a numerical increase in immature red cells or reticulocytes. Since this factor exerts its action mainly on stem cells in bone marrow it has been referred to as the “primary inducer of erythroid differentiation” (44). This definition should be avoided, however, since the ESF differs in many respects from classical inducers (see following section), and also because this expression does not take into consideration other ESF effects, such as the early release of immature newly formed red cells into the circulation, the increase of several enzymatic activities in immature red cells (44), or the facilitation of the entry of iron into some cells of the marrow, which seems to indicate an influence on iron transport due to membrane changes (45). Specific growth factors and their possible significance are considered next.

BIOLOGICAL SIGNIFICANCE OF NGF

Before attempting an evaluation of the physiological significance of the NGF we shall briefly consider the biochemical properties of this factor and the main features of the growth response elicited in the target cells.

At the time of its discovery in 1954, the NGF was identified by Cohen et al. in a nucleoprotein molecule (31) and later in a protein molecule (30). Extensive analyses performed in several laboratories in subsequent years led to different models of the NGF molecule, but substantially confirmed the earlier findings on the protein nature of this factor. Though complete agreement has yet to be reached
on its ultimate size, and though its molecular structure is still the object of intensive investigation, it may be stated that the biological activity resides in certain parts of the polypeptide chain. Hence the NGF can be definitely set apart from the large and heterogeneous class of substances endowed with some growth-promoting activity such as vitamins, oligominerals, auxins, and nonprotein hormones. It belongs instead to another equally large and no less heterogeneous class of substances endowed with biological activity that share with the NGF the property of having a protein structure. We shall briefly consider three large groups of these substances, namely the enzymes, inducers, and protein hormones.

The possibility that the NGF owes its biological action to an enzymatic property was not readily discarded (24). It had in its favor the finding that the NGF is found in large quantity in snake venom and in mouse salivary glands, which are characterized by a wealth of hydrolytic enzymes. Furthermore, the observation that the NGF level in the mouse salivary glands under normal and experimental conditions closely follows the level of esterase and protease seemed to make this hypothesis even more plausible. However, no catalytic activity has been detected as yet in the most purified NGF preparation, in spite of extensive studies directed to uncovering this presumptive function. In the absence of experimental evidence, it seems at present unjustified to speculate further on the enzymatic nature of the NGF.

We shall now consider similarities and differences between the NGF, inducers, and hormones.

Inducers and the NGF share some properties in common. In both instances the activity is bound to protein molecules released by some cells and interacting with other cells. In induction these molecules play a key role in the differentiation of the reacting cells. Undifferentiated cells (if one deals with induction of primary embryonic systems), or cells in early differentiative stages (if secondary embryonic systems are concerned), are channeled into one of several alternative differentiative pathways on their reception of the protein molecules that transmit the inducer. The reaction is time-bound; it takes place only if the target cells are in the responsive stage (and this varies for each system) and ceases soon after the same cells have acquired biochemical and structural differentiative marks as a result of the induction. Induction, therefore, is regarded as the switching on of dormant genes in the target tissue, which triggers a series of processes leading to the synthesis of specific differentiative products. Inducer agents have been recently isolated in highly purified form [we refer the reader to the excellent recent review articles by Tiedemann (96, 97)] and are known to retain their activity when tested on in vivo and in vitro systems. Under normal conditions, however, there is evidence that induction occurs only through a cell-to-cell contact, or at best at very short distances of the order of a few microns.

None of the above conditions applies to the NGF. Although it is mainly effective on the embryonic target cells, these cells must already have gone through the first differentiative phases before they acquire the capacity of responding to the NGF. One of the two cell types retains throughout all its life cycle the capacity of responding to the NGF. The response is stimulus-bound but not time-bound. It
consists of a marked increment in the cells' growth potentialities as well as acceleration of early differentiative phases. In another way the NGF differs from inducers. The NGF molecule is present in the living organism in the body fluids and organ extracts. On injection, it circulates in the blood and reaches its target even if remote from the injection point. Although the differences listed above are contrary to the concept that the NGF belongs to the class of inducers, it should be stressed that in spite of these obvious differences they share an important aspect in common. In both instances a protein molecule released by some tissues plays a conspicuous role in the fate of other cell types. One could hardly conceive of the harmonious development of highly complex systems such as those of pluricellular organisms if interaction among tissues and dependent differentiation did not occur early in ontogenesis.

A thinner demarcation line can be drawn between the NGF and protein hormones (61). We are dealing in both instances with protein molecules released in the circulation and acting on target cells, whether assembled in organs or disseminated in the organism, or even dissociated in vitro. Both the NGF and protein hormones share the property of activating anabolic processes and, as a result, of enhancing growth in the target organs or cells. The NGF, as well as many protein hormones, exerts its influence on immature and mature receptive cells, and this influence is stimulus-bound. On withdrawal of the agent the effect ceases. The NGF, however, differs from "conventional hormones" in many other ways.

A basic tenet of classic endocrinology is the production of the hormone by organs or tissues easily identifiable on the basis of histological and biochemical data. A time-honored procedure is the extirpation of the organ or tissue that is the presumptive hormone producer and the subsequent finding that the hormone disappears from the circulation and that deprivation effects can be recognized in the target tissues. Extirpation of the mouse salivary glands in young and adult specimens fails to produce such effects. Though decisive evidence has been obtained for the in situ synthesis of the NGF, the exclusion of the mouse salivary glands does not result in any appreciable changes in its target cells, the sympathetic neurons. Further evidence contrary to the assumption that the NGF synthesized by the mouse salivary glands is utilized in hormone fashion by the sympathetic nerve cells has come from a number of well-ascertained facts reported earlier in this review.

In two other respects the NGF differs from "conventional" hormones: the magnitude of the effects and the strict specificity of the growth response. Both are unmatched by any of the known protein and nonprotein hormones.

The last and perhaps the most significant feature of the NGF system is represented by the dramatic effects elicited by the specific antiserum to this growth-promoting protein. The sudden disintegration and death of immature sympathetic nerve cells of newborn mice injected with this antiserum find no parallel in any antiserum effects to hormones. There is also another way to demonstrate the key role of the NGF in the life of the receptive cells. As reported above, embryonic sensory and sympathetic nerve cells survive and grow in a minimum liquid medium only if the medium is enriched with the NGF at a concentration as low as $10^{-7}$ g/ml.
Although the NGF differs in many significant ways from protein hormones it presents striking features in common with other specific growth factors. The two that come close to the NGF are, as reported, the EGF and the ESF.

The above considerations suggest that it is appropriate to list the NGF and other specific growth factors in a class distinct from those of inducers and hormones. Since the NGF and the other growth factors share many properties in common with these other substances, it is tempting to consider them as a class fitting between the inducers and the hormones. If this suggestion is valid it may be possible to discover other specific growth factors that will prove to play a key role on other cellular types in the same specific fashion as the NGF. Pending this evidence we shall, for the time being, consider the NGF as a protein endowed with a major function in the development and growth of its target cells.

SUMMARY AND CONCLUDING REMARKS

Although the primary site and the mechanism of action of the NGF on the target cells remain at present a matter of conjecture, and although it seems advisable to wait for more information before attempting to identify the former and explain the latter, one may with profit summarize the main features of the growth response elicited by the NGF on the receptive nerve cells and also outline a program for future work.

Among growth control mechanisms that operate in embryonic and post-embryonic life, the control exerted by the NGF on sensory and sympathetic nerve cells stands out by virtue of the magnitude of its effects, its target specificity, and the plurality of its actions.

The target cells become receptive to the NGF only after they have acquired unmistakable differentiative marks characteristic of one or the other of the two cell types. The NGF molecule is a normal constituent of the embryonic sensory and the embryonic and mature sympathetic nerve cells, and it is also present in many organs and tissue extracts; it is found in trace amounts in the serum of all mammals, man included. These findings lend support to the hypothesis that this protein is endowed with physiological significance. The same protein elicits striking growth effects on sensory and sympathetic nerve cells, but its range of action differs in magnitude and in time extension on the two cell types: only sympathetic nerve cells remain receptive to its action throughout their life cycle. The key role played by the NGF in the economy of these cells is further documented by the cytotoxic lethal effects elicited on immature sympathetic nerve cells by a specific antiserum to this molecule.

Since the magnitude of the growth response keeps pace with the amount of NGF supplied to the living organism, and the effect fades away soon after discontinuation of the treatment, a feedback mechanism must, under physiological conditions, control the production and release of this molecule. Neither its source of origin nor these hypothetical regulatory mechanisms have been identified. Equally unexplained at present is the role, if any, played by the salivary glands and the venom glands in supplying the receptive nerve cells with this factor.
Similar and equally challenging yet unsolved questions are raised by the discovery of two other specific growth factors, the epidermal growth factor and the erythropoietin-stimulating factor.

Progress in identifying the origin and in understanding the mechanism of action of any one of these three factors will advance our knowledge of the other two and possibly of still other unknown but similar factors. Future research should therefore aim not only at a more exhaustive and penetrating analysis of the NGF molecule and of its effects at a biochemical and ultrastructural level, but also at the investigation of the more general features of these growth regulatory factors that do not seem to fit into any of the well-known categories of agents that regulate growth and differentiative mechanisms in animal cells.

The investigations from the writers' laboratories have been supported in part by grants from the Public Health Service (NB-01602, NB-03777), the National Science Foundation (GIL Foundation (SD-333), and the Consiglio Nazionale delle Ricerche.

REFERENCES


