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Comparison between the Effect of Neuronal Activity and Nerve Growth Factor on the Enzymes Involved in the Synthesis of Norepinephrine

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Introduction

THE response of effector cells to the activity of neurons which control their function is not confined to actual effects such as changes in ionic permeability of the cell membrane or changes in their intermediary metabolism. The response also involves changes in the macromolecular composition of the effector cells. Recent studies have shown that a prolonged increase in the activity of cholinergic nerves supplying the terminal peripheral adrenergic neurons, or the adrenal medullary cells, produces a marked rise in the *in vitro* activity of tyrosine hydroxylase (TH) (9, 18, 28, 29, 45, 46) the enzyme which catalyzes the usually rate-limiting step in the synthesis of norepinephrine (24). This rise in the *in vitro* activity of TH is neurally mediated (19, 42, 43), results from an increased synthesis of new enzyme protein (30, 34), and represents a long-term adaptation to increased transmitter utilization. However, this neurally-mediated regulation of the synthesis of a well defined macromolecule is of more general interest in providing concrete evidence for the so far vaguely defined and poorly understood "trophic action of neuronal activity on effector cells." These neurally-mediated changes in the macromolecular composition of effector cells could also be of importance for the long-term storage of information in the central nervous system, the development of drug tolerance not related to the induction of drug-metabolizing enzymes, and the regulation of ontogenetic processes.

Very recent studies revealed a striking similarity between the effect of increased activity of preganglionic cholinergic nerves in adult rats and the administration of nerve growth factor (NGF) to newborn animals on the enzyme pattern in the terminal adrenergic neurons (39, 40). Therefore, questions arise as to whether neurally and NGF-mediated enzyme induction involves similar mechanisms, whether they are mutually related to each other, what their relative importance is to the normal development of the terminal adrenergic neuron, and also whether there is a retrograde effect of the terminal adrenergic neuron on the macromolecular composition of the preganglionic cholinergic nerves.

Relationship between Neurally-Mediated Induction of Tyrosine Hydroxylase and Other Enzymes Involved in the Synthesis of Norepinephrine

After previous studies had shown that a prolonged increase in the activity of peripheral (29, 38, 42) and central (33, 38) adrenergic neurons resulted in an augmented synthesis of TH, the question arose as to whether this effect was restricted to TH or whether other enzymes responsible for the synthesis of norepinephrine were also involved. This aspect is of particular interest, since in bacterial systems the genetic information for enzymes involved in a given metabolic pathway is often located in adjacent chromosomal areas, which form an operational unit governed as a whole by substances acting as inducers or repressors (1). If the genetic information for norepinephrine-synthesizing enzymes were arranged in a similar way, *i.e.*, in a single operon, one would not only expect an increased activity of TH but also of the other enzymes involved in the synthesis of norepinephrine. To study this question, cold exposure of animals seemed to be a very suitable procedure, as it avoids possible drug effects not related to an increased activity of preganglionic cholinergic nerves, and as it has already been shown that cold stress produces an increased norepinephrine turnover and an increased *in vitro* activity of TH in both peripheral (13, 38) and central adrenergic neurons (36, 38). Cold exposure (4°C) of rats for 1 to 4 days leads to a gradual increase in the *in vitro* activity of TH and dopamine-β-hydroxylase (DBH) both in superior cervical ganglia and adrenals (fig. 1). However, the activity of the third enzyme engaged in the synthesis of norepinephrine, dopa decarboxylase, does not change in either of the two organs. In the adrenals TH and DBH increases run virtually parallel and reach a level of more than twice that of the controls after 4 days. In contrast, in the superior cervical ganglion the increase in DBH is much smaller than that of TH. However, since the amount of enzyme protein present in a given neuron represents the resultant of both synthesis and degradation, the difference between the increase in TH and DBH in superior cervical ganglia could result from differences in the rate of degradation rather than synthesis. Indeed, the turnover of TH in superior cervical ganglia is much slower than that of DBH, as far as can be judged from the rate of decay of enzyme activity after inhibition of protein synthesis by cycloheximide (40). For TH no consistent decrease in enzyme activity could be detected within 6 to 9 hr when doses of cycloheximide were administered which reduced the incorporation of ^{3}H-leucine into protein to 5 to 7%. However, DBH activity decayed gradually, having a half-life of 13 hr, which in cold-exposed animals was not significantly different from that of controls (40). It has to be mentioned that the virtually parallel increase in TH and DBH activity produced by cold exposure in the adrenal medulla (fig. 1) is not fully representative of all other experimental conditions producing a transsynaptic induction of these enzymes in the adrenal medullary cells. For instance administration of reserpine or gradually increasing doses of morphine produce a considerably larger increase in TH than DBH.

The lack of increase in dopa decarboxylase activity which was observed not only after cold stress but also after reserpine administration (5) cannot be ex-

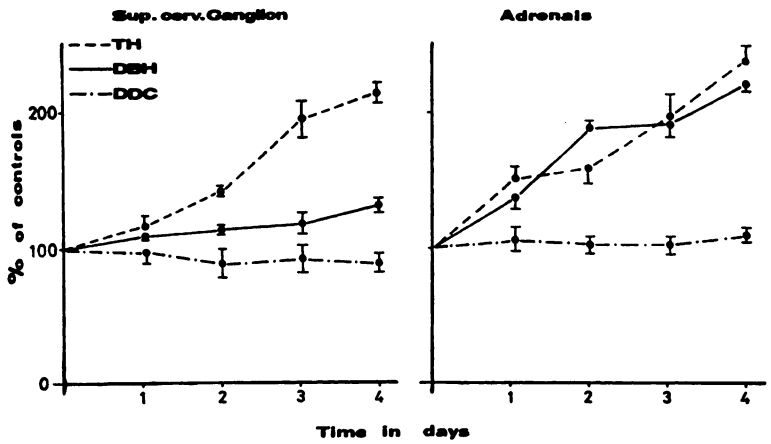

FIG. 1. Effect of cold exposure on tyrosine hydroxylase (TH), dopamine-β-hydroxylase (DBH) and dopa decarboxylase (DDC) activity in superior cervical ganglia and adrenals of the rat. The animals were exposed to 4°C for 1 to 4 days and the *in vitro* activity of TH, DBH and DDC was compared with that of controls kept at 24°C. The activity of TH was determined according to Mueller *et al.* (29), DDC according to a modification (40) of the method of Håkanson and Owman (14) and DBH according to Duch *et al.* (10). The enzyme activities are expressed in percent (mean ± S.E.) of controls (100%) n = 6–8.

plained by turnover differences, as its half-life was found to be 12 hr in the superior cervical ganglion, which is very similar to that of DBH (40).

The turnover rate of an enzyme determined either by inhibition of protein synthesis or after pulse labeling with immunological methods does not only depend on the proteolytic degradation of the enzyme, but also on additional factors such as loss of enzyme by neurosecretion or transport of enzyme protein from the cell body to distal parts of the neuron, which for technical reasons cannot be included in the determination (40).

From the information available so far it can be concluded that not all the enzymes involved in the synthesis of transmitter substances of adrenergic neurons and adrenal medullary cells are regulated as an operational unit. Whether TH and DBH are regulated by the same mechanisms cannot be decided with certainty. In the adrenal medulla, the synthesis of these two enzymes is regulated both by the activity of the splanchnic fibers supplying the adrenal medulla (20, 25, 26, 34, 40, 43) and the hypophyso-adrenocortical system (19, 29, 31, 47). In contrast the synthesis of phenylethanolamine-N-methyl transferase (PNMT), the enzyme which catalyzes the conversion of norepinephrine to epinephrine is mainly regulated by adrenocortical hormones (19, 48), with the regulation by nerve impulses being of minor importance (19, 44).

Specificity of the Effect of Nerve Growth Factor (NGF) on Enzymes Involved in the Synthesis of Norepinephrine

Ever since the discovery of the growth promoting effect of NGF on sympathetic ganglia (22, 23) the question has been raised as to whether the response to this

factor represents only a general growth confined to sympathetic neurons, accompanied by a general increase in the synthesis of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), proteins and lipids (21, 23), or whether there is also a selective stimulation of metabolic pathways which are characteristic of this particular nerve cell type. The injection of newborn rats with NGF results not only in an enhanced growth but also in an accelerated differentiation of the neuroblast in the superior cervical ganglion (22, 39). The morphological changes are accompanied by a selective induction of TH and DBH (fig. 2), enzymes which are selectively located in adrenergic neurons or adrenal chromaffin cells (25, 28, 35, 37). In contrast, the activity of dopa decarboxylase and monoamine oxidase, enzymes with a much more general distribution (2, 7, 12, 15, 16, 17), increase only in proportion to the general growth (39). The slight increase in specific activity may represent a relative increase in the volume of neuronal *versus* satellite cells. Therefore, it can be concluded that there is not only morphological but also biochemical evidence that NGF does not only promote the general growth of sympathetic ganglia but also their differentiation, manifested by the selective induction of enzymes which are characteristic of this particular type of nerve cell.

Nerve Growth Factor (NGF) and Preganglionic Cholinergic Nerves; Their Relative Importance to the Development of the Terminal Adrenergic Neuron

The striking similarity between the effect of increased activity of preganglionic cholinergic nerves in adult rats and the effect of NGF in newborn animals on the

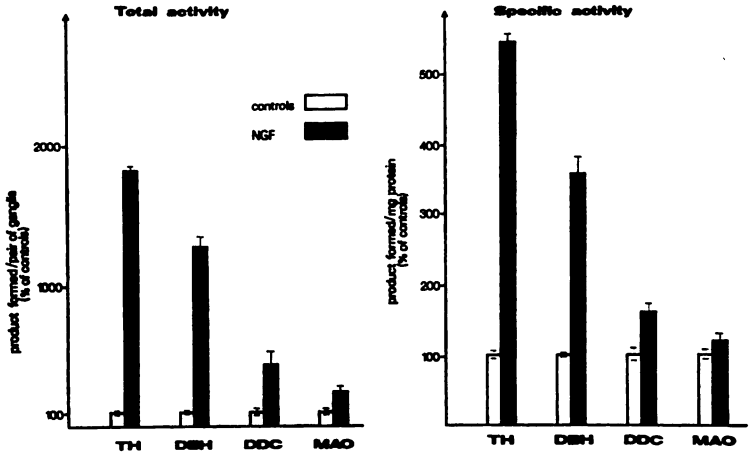

FIG. 2. Effect of nerve growth factor (NGF) on enzymes involved in the synthesis and metabolic degradation of norepinephrine in the superior cervical ganglion of the rat. Newborn rats were treated with 10 μg/g of NGF for 10 days. The activities of all enzymes studied in the superior cervical ganglion are expressed in percent of saline-injected controls (100%) both for total and specific activity. TH, tyrosine hydroxylase; DBH, dopamine-β-hydroxylase; DDC, dopa decarboxylase; MAO monoamine oxidase. [According to Thoenen *et al.* (39).]

enzyme pattern of the superior cervical ganglion (39) poses the question as to what extent these two factors are responsible for the normal development of the peripheral sympathetic nervous system.

In recent experiments Black *et al.* (3, 4) have shown that in mice the development of choline acetyltransferase (CAT), an enzyme selectively located in cholinergic neurons (12, 41), is a reliable biochemical measure for the formation of synapses in the superior cervical ganglion and that the formation of synapses precedes the rise in TH in the cell body of the terminal adrenergic neuron. However, in rats there is no apparent relationship between the rise in CAT and the development of enzymes involved in the synthesis of the adrenergic transmitter (fig. 3). The activity of CAT is very low at birth and reaches its maximal level after 30 days, amounting to a 50-fold increase in total activity and a 15-fold increase in specific activity (41). In contrast, the increase in the activity of enzymes characteristic of adrenergic neurons is much smaller. The maximal increase in the specific activity of TH amounts to only 40%, which is reached after 10 to 14 days (41). The activity of dopa decarboxylase increases virtually in parallel with the general growth of the ganglia and the initial, slight increase in specific activity never reaches statistically significant levels (41). Of all the enzymes engaged in the synthesis of the adrenergic transmitter, DBH is the only one which shows to some extent a parallel development with that of CAT (fig. 3). However, the increase in the former from birth to adult life is much smaller than that of the latter, amounting to a 2.5-fold increase in specific and an 8-fold increase in total activity (41).

From these findings it can be concluded that—at least in rats—there is no clear-cut evidence for a relationship between the formation of synapses in the superior cervical ganglion and the development of the terminal adrenergic neurons. In view of these results and the extensive destruction of the peripheral sympathetic nervous system after administration of nerve growth factor antiserum (NGF-AS) to newborn animals (22, 23), it is conceivable that NGF is the predominant factor responsible for the development of the peripheral adrenergic neurons. However, this does not exclude a regulatory function of other humoral factors yet unknown and that the formation of a small number of synapses could represent a prerequisite condition for the action of NGF. To answer this question the superior cervical ganglia of 3-day-old rats were decentralized on one side and the effect on general growth of the ganglia was followed by measuring their protein content, and that of differentiation by determining their changes in TH and DBH activity. Completeness of decentralization was confirmed by measuring CAT in the superior cervical ganglia 14 days after transection of the preganglionic trunk. The activity of this enzyme was reduced to less than 3% as compared to that of the intact (100%) side.

Decentralization delayed but did not block both general growth and differentiation of the terminal adrenergic neuron. At the time of decentralization (3 days after birth) the protein content/ganglion amounted to 26 $\pm$ 2 μg. Fourteen days after surgery the protein content had risen to 63 $\pm$ 3 μg/ganglion on the intact side and to 43 $\pm$ 3 μg/ganglion on the decentralized side. Figure 4 shows that the increase in both specific and total activity was impaired by decentralization

but the latter to a larger extent than the former. This becomes apparent above all in the case of dopamine-β-hydroxylase which exhibits the largest increase in total and specific activity of all the enzymes involved in the synthesis of the adrenergic transmitter.

The effect of NGF on both general growth and selective induction of TH and

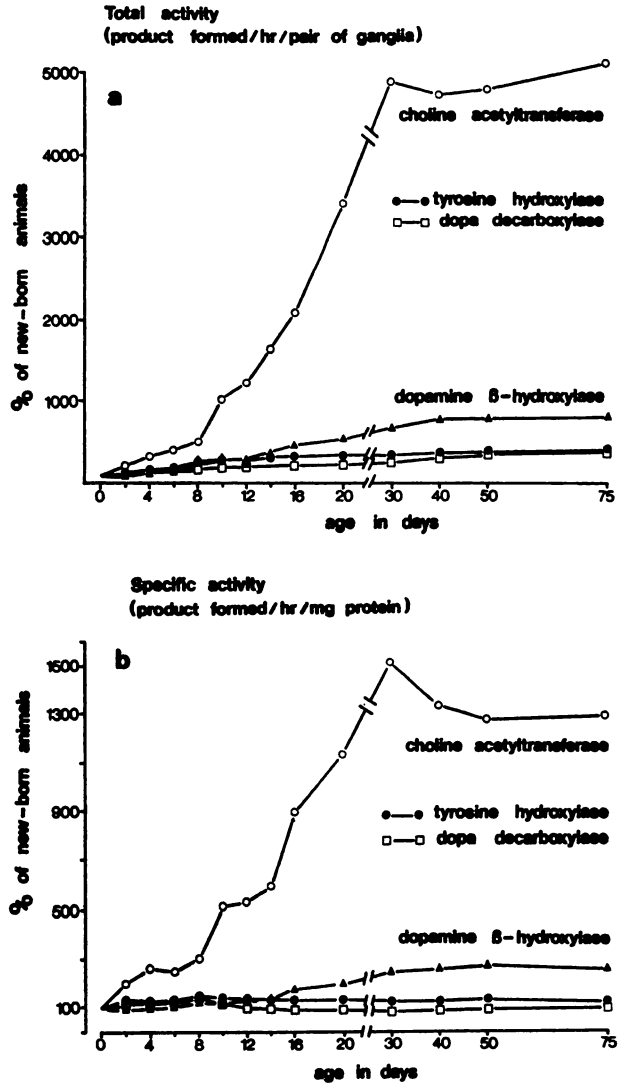

FIG. 3. Time-course of the development of choline acetyltransferase and enzymes involved in the synthesis of norepinephrine in the superior cervical ganglion of the rat from birth to adult life. The changes in total (fig. 3a) and specific activity (fig. 3b) are expressed in percent (mean ± S.E.) of the values of newborn (100%) animals (n = 7–9). The activity of choline acetyltransferase was determined according to Fonnum (11), tyrosine hydroxylase according to Mueller *et al.* (29), dopamine-β-hydroxylase according to Molinoff *et al.* (27), dopa decarboxylase according to Håkanson and Owman (14).

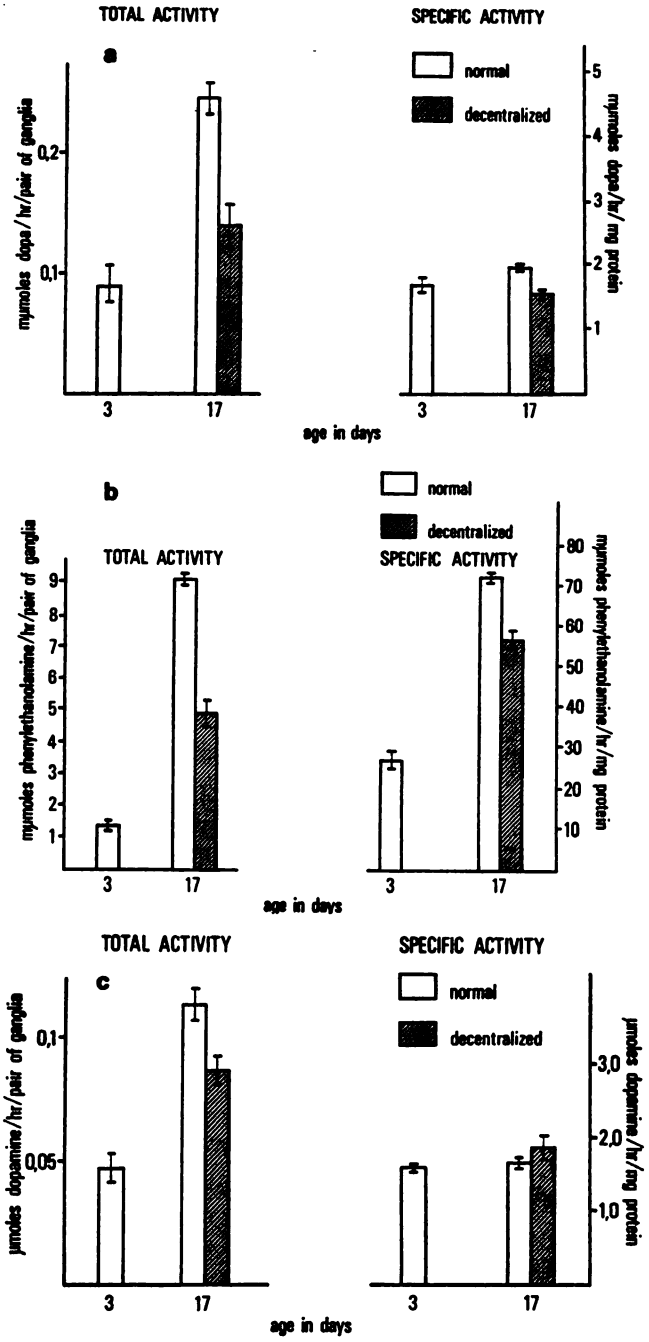

FIG. 4. Effect of decentralization on enzymes involved in the synthesis of norepinephrine in the superior cervical ganglion of the rat. The left preganglionic trunk was transected under ether anesthesia in 3-day-old rats. Fourteen days after surgery the animals were killed and the total and specific activity of tyrosine hydroxylase (fig. 4a), dopamine-β-hydroxylase (fig. 4b) and dopa decarboxylase (fig. 4c) of the intact and decentralised side was compared with that of 3-day-old animals, *i.e.*, the activity at the time of decentralization. The values given represent the mean ± S.E. n = 7–8.

DBH was not impaired by decentralization, *i.e.*, the proportional rise on the experimental and intact side was the same (fig. 5). Therefore, it can be concluded that the effect of NGF does not depend on an intact preganglionic innervation and that the activity of the preganglionic cholinergic nerves seems to represent an additive factor only, contributing mainly to the general growth. Moreover the main factor responsible for the development of the peripheral sympathetic nervous system seems to be NGF, if one accepts that the selective destruction of adrenergic neurons by NGF-AS results from the removal of NGF. However,

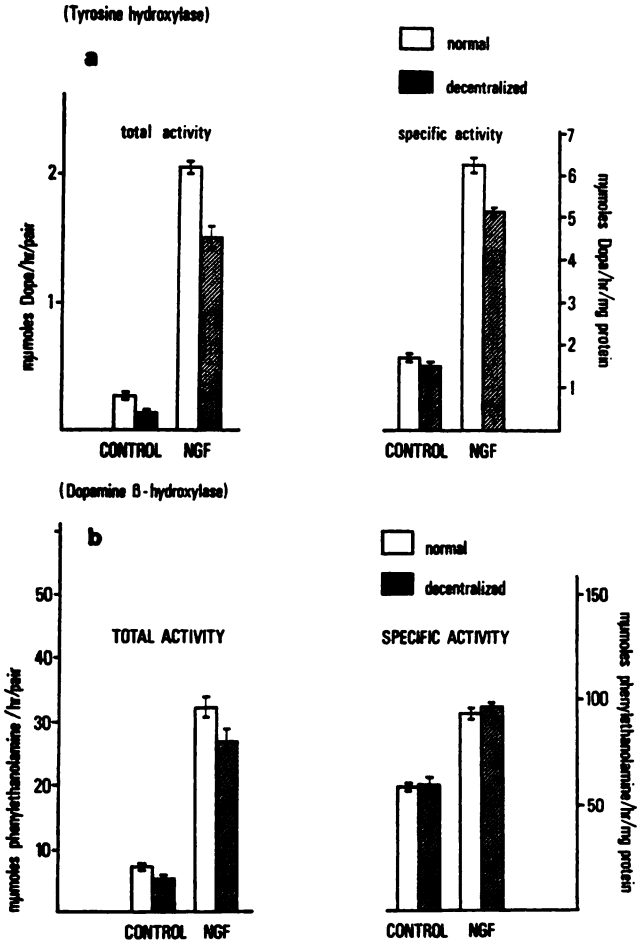

FIG. 5. Effect of decentralization on the response to nerve growth factor (NGF) (6). After decentralization of the left superior cervical ganglion 3 days after birth, half of the animals were treated for 10 days with 10 μg/g of NGF. Twenty-four hours after the last dose the NGF-treated animals were killed together with the saline-injected littermates. The activity of tyrosine hydroxylase (fig. 5a) was determined according to Mueller *et al.* (29), that of dopamine-β-hydroxylase (fig. 5b) according to Molinoff *et al.* (27). The values given represent the mean ± S.E. n = 7–8.

very recent experiments of Hendry and Iversen (personal communication) have shown that the efficiency of NGF-AS does not depend so much on its potency to precipitate NGF as to the degree to which NGF-AS has complement-binding properties, suggesting that not only the removal of NGF but also the formation of a lytic antiserum-complement complex on the cell surface of the adrenergic neurons could be of importance.

Retrograde Transsynaptic Effects in the Superior Cervical Ganglion of the Rat

The results presented so far have shown that the activity of the preganglionic cholinergic nerves produces characteristic changes in the macromolecular composition of the terminal adrenergic neuron of adult rats, and that the preganglionic fibers also contribute to the normal development of the adrenergic neurons from birth to adult life. A further question now arises as to whether this "trophic" action is unidirectional only, *i.e.*, confined to the effect of preganglionic nerves on postganglionic neurons or whether there is also a retrograde effect. A retrograde transsynaptic effect has been shown in the brain, where, after lesioning of particular areas such as the visual and cingulate cortex and the optic nerve tract there occurs not only orthograde but also retrograde transsynaptic degenerations (8). Black *et al.* (4) have shown that treatment of newborn mice with 6-hydroxydopamine (6-OHDA) results not only in a marked decrease of TH (representing a measure for the degree of the destruction of the adrenergic cell bodies) in the superior cervical ganglion, but also in a marked reduction of CAT, which is selectively located in cholinergic nerve terminals (12, 41). It is possible that the severe damage to the adrenergic neurons produced by 6-OHDA could lead to preganglionic changes by embodying the cholinergic nerve terminals into the proteolytic and inflammatory processes occurring as a consequence of the destruction of the adrenergic cell bodies. In an attempt to get more accurate information on this question we studied whether there are differences between the time-course of the reduction of choline acetyltransferase and the enzymes selectively located in adrenergic neurons, *i.e.*, TH and DBH. Newborn animals were treated with 6-OHDA (150 mg/kg given subcutaneously for 10 days) and the activities of pre- and postganglionic enzymes were compared at 20 days with those of untreated littermates. In 6-OHDA treated animals the total activity of CAT was reduced to 15 %, TH to 7 % and DBH to 10 %. From this it might appear that preganglionic cholinergic nerve terminals and adrenergic cell bodies are damaged to a very similar extent. However, it must be born in mind that the rise in choline acetyltransferase activity is much steeper than that of TH and DBH. If the enzyme level at the second day after birth (when the 6-OHDA treatment began) is taken as a point of reference (100 %), it becomes apparent that TH and DBH gradually decay (fig. 6), and reach a level of 20 and 35 % respectively at the age of 20 days. In contrast, the activity of CAT shows a slight but steady increase, and reaches a level of 210 % as compared to that at the beginning of the 6-OHDA treatment. Although direct damage of the preganglionic cholinergic nerves by proteolytic and inflammatory processes as a consequence of

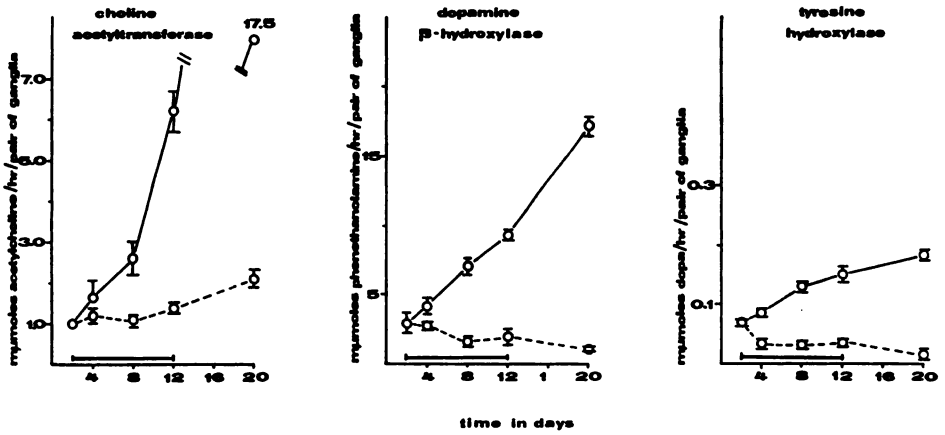

FIG. 6. Effect of 6-hydroxydopamine on the time-course of the development of choline acetyltransferase (CAT), tyrosine hydroxylase (TH) and dopamine-β-hydroxylase (DBH) in the superior cervical ganglion of the rat. From the 2nd to the 12th day after birth the animals were given daily injections with 150 mg/kg of 6-hydroxydopamine. In intervals of 2 and 4 days groups of 6–7 treated rats (O-----O) and saline-injected (O——O) controls were killed and the enzyme activities determined in the superior cervical ganglia. The values given represent the mean ± S.E. CAT activity was determined according to Fonnum (11), TH according to Mueller *et al.* (29) and DBH according to Molinoff *et al.* (25).

the destruction of the adrenergic cell bodies by 6-OHDA cannot be excluded, the difference in the time-course of the enzymes characteristic for cholinergic and adrenergic neurons points toward a true "trophic" response of the preganglionic cholinergic nerves to the destruction of the adrenergic cell bodies.

To decide whether there are not only negative (destruction) but also positive retrograde transsynaptic effects we treated 2-day-old rats for 10 days with 10 μg/g NGF daily. This treatment led not only to a marked increase in growth and differentiation of the sympathetic ganglia, accompanied by a selective induction of TH and DBH, but also to an increase in CAT (fig. 7). In the superior cervical ganglion the increase amounted to 70% as compared to saline-injected littermates. In contrast, in the heart and adrenal medulla there were no statistically significant ($P < .05$) changes. Thus, the effect is confined to ganglia with cholinergic-adrenergic but not cholinergic-cholinergic synapses as is the case in the heart. At cholinergic-adrenergic synapses an increase in CAT occurs only if the adrenergic cell is responsive to NGF. This is the case for the main part of pre- and paravertebral ganglia but not for the chromaffin cells of the adrenal medulla (22, 23).

It can be concluded that not only orthograde but also retrograde trophic effects are present in superior cervical ganglia. In the case of the orthograde effect it seems to be most likely that acetylcholine is the first messenger of the "trophic" action, as far as can be judged from the transsynaptic induction of enzymes which can be abolished by ganglionic blocking agents (32). The mechanism of the retrograde transsynaptic effect is as yet unclear and the elucidation of the factors involved remains to be established by future investigations.

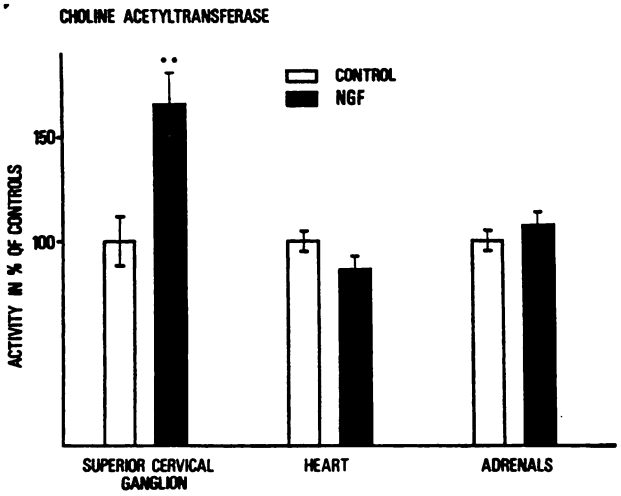

FIG. 7. Effect of nerve growth factor (NGF) on choline acetyltransferase (CAT) activity in superior cervical ganglion, heart and adrenals of the rat. Newborn rats were treated for 10 days with 10 μg/g of NGF. Twenty-four hours after the last dose the animals were killed and the CAT activity was determined according to Fonnum (11). The values given represent the mean $\pm$ S.E. ** $P < .01$ (n = 7).

Conclusion

Both a prolonged increase in the activity of the preganglionic cholinergic nerves in adult rats and the administration of NGF to newborn animals result in an induction of TH and DBH in the terminal adrenergic neuron, whereas the third enzyme involved in the synthesis of norepinephrine, dopa decarboxylase, remains unchanged. These findings indicate that the three enzymes responsible for the synthesis of the adrenergic transmitter are not located in a single operon and are not regulated as an operational unit, as has been shown in bacterial systems for enzymes involved in a given metabolic pathway.

The striking similarity between the effect of NGF and increased preganglionic activity raises the question as to whether NGF and transsynaptic enzyme induction involve the same mechanism, or at least possess some common regulatory factor(s), and to what extent NGF and preganglionic nerve activity are responsible for the normal development of the terminal adrenergic neuron. Decentralization of superior cervical ganglia 3 days after birth delayed but did not block both general growth and differentiation of the adrenergic neuron. Moreover, decentralization did not impair the effect of NGF on general growth of the adrenergic neuron and the selective induction of TH and DBH. These findings indicate that the effect of NGF does not depend on an intact preganglionic cholinergic innervation, that NGF is most probably the main factor responsible for the development of the peripheral terminal adrenergic neurons in rats and that the formation of synapses and the activity of the preganglionic cholinergic nerves represent an additive factor only, involving general growth to a larger extent than differentiation.

The fact that destruction of adrenergic neurons decreases, whereas administra-

tion of NGF increases the activity of choline acetyltransferase (a reliable biochemical correlate for the development of preganglionic cholinergic nerve terminals) in ganglia with cholinergic-adrenergic synapses but not with cholinergic-cholinergic synapses, provides evidence that the trophic effect of neuronal activity is not only orthograde but also retrograde; the mechanism of the latter is as yet unclear.

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