PHARMACOLOGICAL REVIEWS
Vol. 24, No. 2

Printed in U.S.A.

Axons of Sympathetic Neurons: Transport of Enzymes *in Vivo* and Properties of Axonal Sprouts *in Vitro*

IRWIN J. KOPIN[1] AND STEPHEN D. SILBERSTEIN[1,2]
National Institute of Mental Health, Bethesda, Maryland

THE function of adrenergic neurons is dependent upon the formation and release of norepinephrine (NE). Increased utilization of the transmitter leads to an immediate increase in the rate of its synthesis without alterations in the levels of the synthetic enzymes, presumably as a consequence of a feedback-control mechanism (37). With repeated or chronic increase in utilization of NE, however, changes in the levels of the enzymes involved in NE synthesis are found in the tissues. Thus after administration of reserpine, there is an increase in the levels of tyrosine hydroxylase (TH) (25) and dopamine-β-hydroxylase (DBH) (24) in the sympathetic ganglia and the heart. The changes in levels of TH in the ganglia precede the changes in the peripheral organs (36), suggesting that the increase in enzyme activity at the nerve terminals is a consequence of increased formation of the enzyme in the perikaryon. More enzyme in the peripheral tissue might be due to an increase in the density of innervation as well as to a greater concentration of enzyme in each terminal varicosity. Little is known, however, about the regulation of these enzymes in the ganglia, the control of the concentration of the enzymes in the nerve endings or the determinants of the density of terminal varicosities in the effector organs.

Axonal Transport of Enzymes

The axon of a neuron, which extends from the cell body to a distally located and specialized nerve ending, is required for communication between the cell parts and for maintenance of the nerve ending. The cell body contains the genetic information and protein synthetic mechanisms while the nerve ending is geared to function. Weiss (38) summarized the early evidence that, in addition to conducting nerve impulses, the axon was a route for continual flow of substances from the cell body to the nerve ending. The existence of such flow became increasingly clear after the introduction of isotopic techniques (7), and

[1] Laboratory of Clinical Science, National Institute of Mental Health, Bethesda, Maryland 20014.
[2] Research Associate in Pharmacology-Toxicology, National Institute of General Medical Sciences, National Institutes of Health, Bethesda, Maryland 20014.

it is now evident that there are at least two rates of axonal transport. The slower rate (1–3 mm/day) is presumably due to bulk flow, but there is also a much faster axonal transport (1–10 mm/hr) which is dependent on the integrity of the neurotubular protein in the axons (27, 39).

Although ligation of sympathetic axons results in accumulation of catecholamines proximal to the site of ligation (6, 9) and ^{14}C-NE injected into the coeliac ganglion of cats is transported through the axons of the splenic nerve (22), most NE released from sympathetic nerves is synthesized in the terminal varicosities (10, 22). The enzymes necessary for conversion of tyrosine to NE and the structures which store the catecholamine, however, are synthesized in the cell body and must be transported to the nerve endings *via* the axon. Kapeller and Mayor (15) demonstrated accumulation of granular vesicles above a constriction of splenic nerves. These vesicles are mainly larger and more completely granulated than those seen in the nerve terminals (8). DBH (18, 23) and chromagranin (23), the protein associated with catecholamine binding, also accumulate proximal to sympathetic axonal constrictions.

Dahlström (5) demonstrated that colchicine and vinblastine inhibit the proximo-distal migration of NE storage vesicles. Since colchicine and vinblastine are known to interact with and disrupt microtubules (27, 39), these structures have been implicated in the transport of the vesicles (2, 5, 13, 16). A close association of vesicular structures with microtubules has been found in the lamprey nervous system (33) and has been suggested in mammalian sympathetic axons (15), but the mechanism of vesicular transport remains unknown.

Local application of colchicine or vinblastine to the superior cervical ganglion of the rat results in a rapid increase in the levels of DBH (19) and decrease of levels of the enzyme in the salivary gland (fig. 1). When protein synthesis is inhibited by administration of cycloheximide, the levels of DBH in the ganglion decrease (fig. 2). If colchicine is applied to ganglia of animals treated with cycloheximide, there is no change in levels of the enzyme. These data indicate that accumulation of DBH in the ganglion is a result of new synthesis and that the decrease of enzyme levels in the ganglion seen when protein synthesis is inhibited is the result of transport of the enzyme out of the ganglion. The rate of increase of DBH in a colchicine- or vinblastine-treated ganglion may be used to estimate the rate of synthesis of the enzyme. The initial rate of increase of DBH in colchicine-treated superior cervical ganglia of rats is about 3.75 units/hr, which corresponds to approximately 5% of the content of the ganglion each hour.

The content of DBH in the submandibular salivary gland innervated by the ganglion treated with colchicine declines slowly during the first day (about 0.8 units/hr) and then more rapidly (almost 3 units/hr). If the initial rate of decline of DBH in the salivary gland (fig. 1) is attributed only to blockade of transport, then approximately one-fifth of the enzyme produced in the superior cervical ganglion is normally transported to the salivary gland.

The apparent rate of transport of DBH to the salivary gland (0.8 units/hr) is equivalent to 0.7% of the DBH content of the gland per hour. This turnover rate ($T_{1/2}$ = 4.1 days) is in close agreement with the rate of return to normal

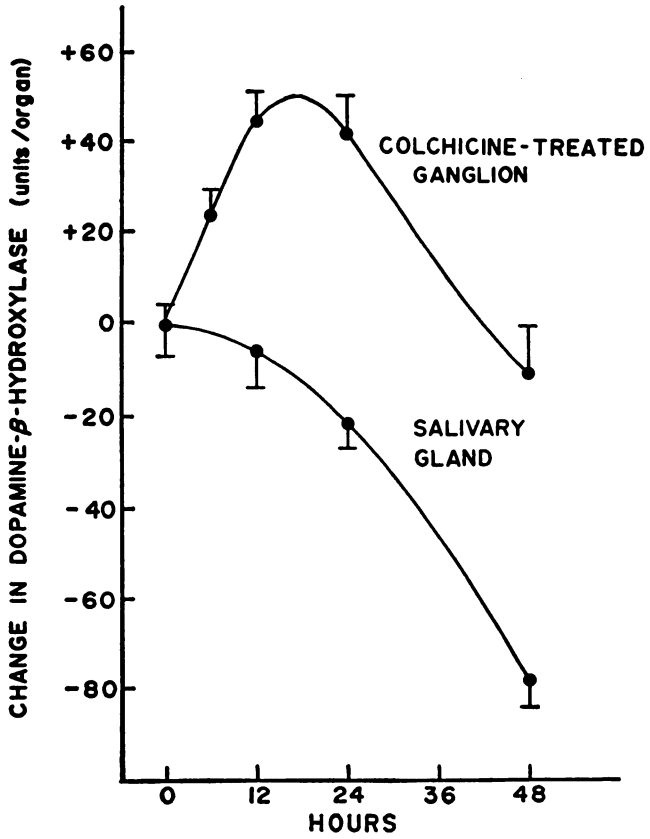

FIG. 1. Effect of local application of colchicine to the superior cervical ganglion on dopamine-β-hydroxylase (DBH) levels in the ganglion and salivary gland. Initial levels of DBH in ganglia and salivary gland were 80 ± 4 units/organ and 110 ± 8 units/organ, respectively.

of the NE content of heart ($T\frac{1}{2}$ = 4.6 days) which begins 2 days after depletion of the catecholamine by reserpine (13, 14), which presumably is an index of the rate of reappearance of vesicles at the nerve ending.

TH is a soluble enzyme and does not appear to be present in the storage vesicles. Changes in the levels of this enzyme are slower and are not as striking as those seen with DBH. Stopping axonal flow for 1 day produces no significant change in levels of TH in ganglia and only slight changes in ligated sciatic nerves (table 1). A slight increase in TH levels of colchicine-treated ganglia becomes evident by 36 hr (19). These observations confirm the findings of Laduron and Belpaire (17) in the cat splenic nerve and support the view that TH is not present in the granular vesicles and is transported by slow axonal flow. Recently, however, Wooton and Coyle (unpublished observations), in our laboratory, have found parallel increases in TH and DBH in ligated or colchicine-treated sciatic nerves.

After the initial increase in DBH levels in colchicine-treated ganglia, levels of the enzymes progressively diminish to about one-third the normal level.

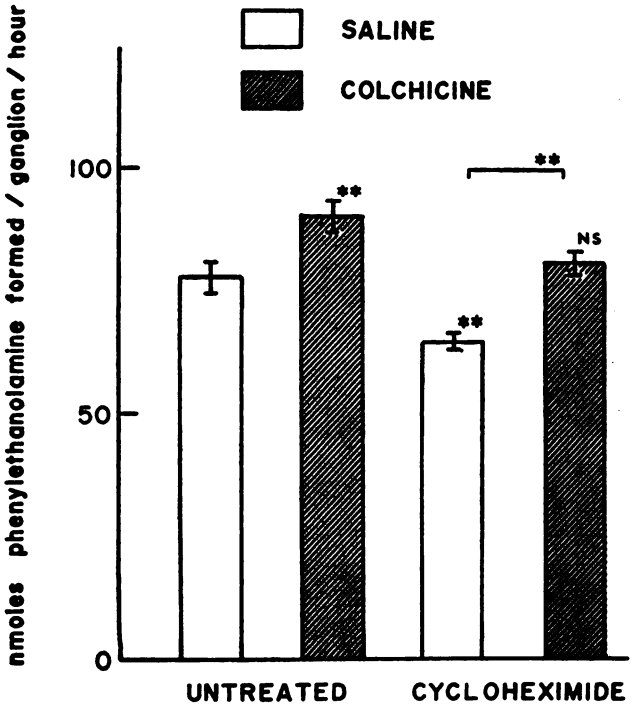

FIG. 2. Effect of cycloheximide on colchicine-induced elevation of dopamine-β-hydroxylase (DBH) in the superior cervical ganglion of the rat. A small volume (5–10 μl) of colchicine solution (0.05 M) was injected in and around the right superior cervical ganglion. Some rats received cycloheximide (2 mg/kg, subcutaneously) immediately before the application of colchicine to the ganglia. Rats were killed 4 hr later, and DBH levels were assayed.

TABLE 1

*Effect of stopping axonal flow for 1 day on tyrosine hydroxylase and dopamine-β-hydroxylase**

	Colchicine-treated Ganglia	Ligated Sciatic Nerve
Tyrosine hydroxylase	+10% (N.S.)	+45%
Dopamine-β-hydroxylase	+44%	+393%

* Data from Lamprecht *et al.* (19).

There is a similar decline in DBH levels in the ganglion after postganglionic section (fig. 3) or destruction of the sympathetic nerve endings by 6-hydroxydopamine (3). When an axon is severed, the distal portions die but the cell body reacts to provide the substance required for regeneration. Although histological evidence (chromatolysis) of a metabolic response to axonal injury by the cell body has long been recognized, the nature of the signal that ascends the axon to initiate these changes is unknown (4). Postganglionic section, local application of colchicine or vinblastine and destruction of sympathetic nerve

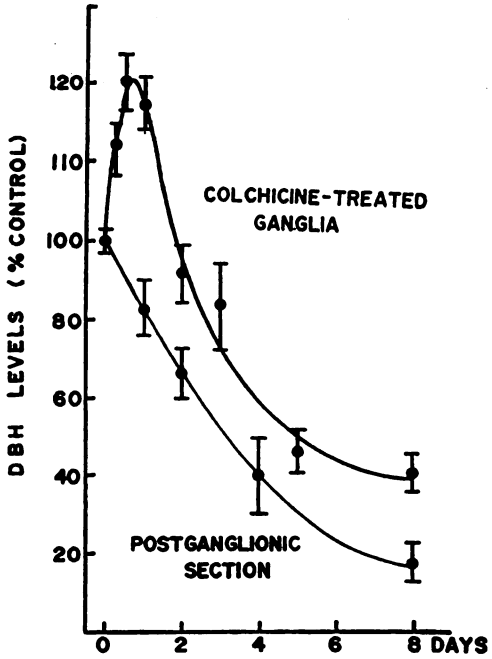

FIG. 3. Effect of postganglionic section or colchicine application on the dopamine-β-hydroxylase levels of the superior cervical ganglion of the rat.

endings by administration of 6-hydroxydopamine have similar effects on levels of catecholamines and DBH in the peripheral organs and sympathetic ganglia. The terminal axons cease functioning, and the reaction in the cell body results in an apparent switch in the metabolic priorities from the production of enzymes concerned with function to formation of structural components necessary for restoration of the nerve ending. Increased membrane surface formation during the period of reconstruction may account for the increase in ^{3}H-NE uptake by ganglia 3 to 4 days after local application of colchicine or vinblastine (11), postganglionic section or 6-hydroxydopamine administration (I. Hanbauer and I. J. Kopin, unpublished observations). The rapidity of the changes in the ganglia and nerve terminals is suggestive of a rate of signal transmission corresponding to the rate of transport by the microtubules. Involvement of the microtubules in conveying such information to the cell body from the axon is supported by the reduction in enzyme levels and increased ^{3}H-NE uptake seen after treatment of ganglia with drugs which disrupt the structure of the microtubules.

Sympathetic Ganglia in Organ Culture

Olson and Malmfors (26) showed that rat sympathetic ganglia transplanted to the anterior chamber of the eye could innervate the iris. This ganglion can be maintained *in vitro* in artificial media, and the neurons continue to respond to acetylcholine stimulation for over 1 week (20). Furthermore, when the ganglia

are cultured in contact with irises, the axons grow into the tissue and appear to reinnervate the muscle (30). These observations led to examination of the initial events which occur when the ganglia are removed and maintained *in vitro*.

Shortly (within 4 hr) after removal of the superior cervical ganglia of a rat to organ culture, there is a striking increase in the ability to take up NE which reaches a maximum of a 4- to 6-fold increase in 2 days (12, 29). There is also a more modest increase in the ability to take up metaraminol. Electronmicroscopic examination of ganglia maintained *in vitro* for 2 days revealed that multiple axonal sprouts had budded out of the perikaryon of the ganglioneurons which had survived explantation (about 50%). These structures, which were several micra in length, contained microtubules and small numbers of large granular vesicles usually associated with catecholamine binding. Electronmicroscopic radioautography of cultured ganglia incubated with ^{3}H-NE demonstrated that most of the radioactivity was associated with the axonal sprouts (29). Ganglia maintained in media containing colchicine or vinblastine did not develop enhanced uptake of NE, and there was a marked reduction in the number and extent of axonal sprouts.

Reserpine pretreatment completely blocked the ability of the ganglia to take up ^{3}H-NE but did not alter the modest increase in uptake of metaraminol. Colchicine and vinblastine, however, diminished uptake of metaraminol as well as NE. These observations suggested that intact neurotubular protein was required for development of membrane uptake and vesicular storage; reserpine interfered only with the latter.

The characteristics of the uptake of NE by the axonal sprouts are similar to those found in sympathetic nerve endings rather than those of ganglion cells (12). Uptake of ^{3}H-NE by the axonal sprouts was saturable and its K_m (1.9×10^{-6} M) was lower than that of the freshly removed ganglia ($K_m = 8.0 \times 10^{-6}$ M) and not significantly different from that of sympathetic nerve endings (0.6×10^{-6} M). Furthermore, ^{3}H-NE uptake by the axonal sprouts is inhibited by drugs such as cocaine, metaraminol and phenoxybenzamine to the same extent as the nerve endings; uptake of the catecholamine by ganglia is less sensitive to inhibition by these agents (table 2).

The changes in enzyme levels in ganglia maintained in organ culture in the

TABLE 2

*Characteristics of ^{3}H-norepinephrine uptake by superior cervical ganglia, sympathetic nerve endings and axonal sprouts**

	K_m	Percent Inhibition by		
		Cocaine	Metaraminol	Phenoxybenzamine
Ganglia	8.0×10^{-6}	30	22	47
Nerve endings	0.6×10^{-6}	59	42	62
Axonal sprouts	1.9×10^{-6}	52	41	63

*Data from Hanbauer *et al.* (12).

presence of nerve growth factor (21) are not striking. The levels of TH fall slightly, presumably due to the death of some of the neurons. DBH levels may increase slightly at first but decrease thereafter in the same manner as seen in ganglia from which the postganglionic fibers have been severed.

When incubated in media containing elevated levels of potassium, the levels of TH and DBH in ganglia (28) and adrenals (31) are elevated. Since this increase is blocked by protein synthesis inhibition, depolarization appears sufficient to initiate induction of the enzymes. In the adrenal gland, induction of TH by elevated levels of potassium is prevented by blocking release of the catecholamines (31).

The similarity of the properties of the mechanism for uptake of NE by axonal sprouts and sympathetic nerve endings and the presence of large granular vesicles in the sprouts prompted an examination of the possibility that the axonal sprouts might release NE. S. A. Vogel, S. D. Silberstein, K. R. Berv and I. J. Kopin (unpublished data) found that electrical field stimulation or potassium-induced depolarization produces a much greater release of ^{3}H-NE from cultured ganglion than from freshly removed ganglia. As at sympathetic nerve endings, bretylium diminishes and phenoxybenzamine enhances such stimulation-induced catecholamine release. Thus, almost as soon as they are formed, axonal sprouts of sympathetic ganglion cells resemble nerve endings in their ability to take up and store NE and to release the catecholamine in response to stimulation.

It is of particular interest that axonal sprouts, which contain only large granular vesicles, can release NE in response to stimulation. In the axons of sympathetic nerves, only large granular vesicles are found; however, at the nerve terminal varicosities, small vesicles predominate (8). After administration of a large dose of reserpine, there is a good correlation between the return of adrenergic function and uptake of labeled NE, but levels of endogenous catecholamine remain markedly depressed (1, 14, 34). The discrepancy between the reappearance of adrenergic function and restoration of NE stores after reserpine treatment may be the result of appearance of the large granular vesicles at the nerve endings. It has been suggested that the large granular vesicles are converted to small granular vesicles (8, 32). It is possible that the large granular vesicles, newly arrived from the cell body *via* rapid axonal transport on the neurotubules, are brought to the membrane terminals and are then preferentially used for discharge of their contents when the nerve ending is depolarized (fig. 4). Once their soluble protein contents are exhausted, they may be transformed into the smaller storage vesicles and be concerned primarily with synthesis and storage of the transmitter reserve. This hypothesis would also account for the blockade by colchicine or vinblastine of release of NE and DBH from sympathetic nerve endings (35) as well as the presence of two populations of vesicles and multiple stores of NE at sympathetic nerve endings.

Summary

Enzymes involved in catecholamine synthesis are formed in the cell body and transported to the nerve terminals. DBH, which is present in the vesicles, is

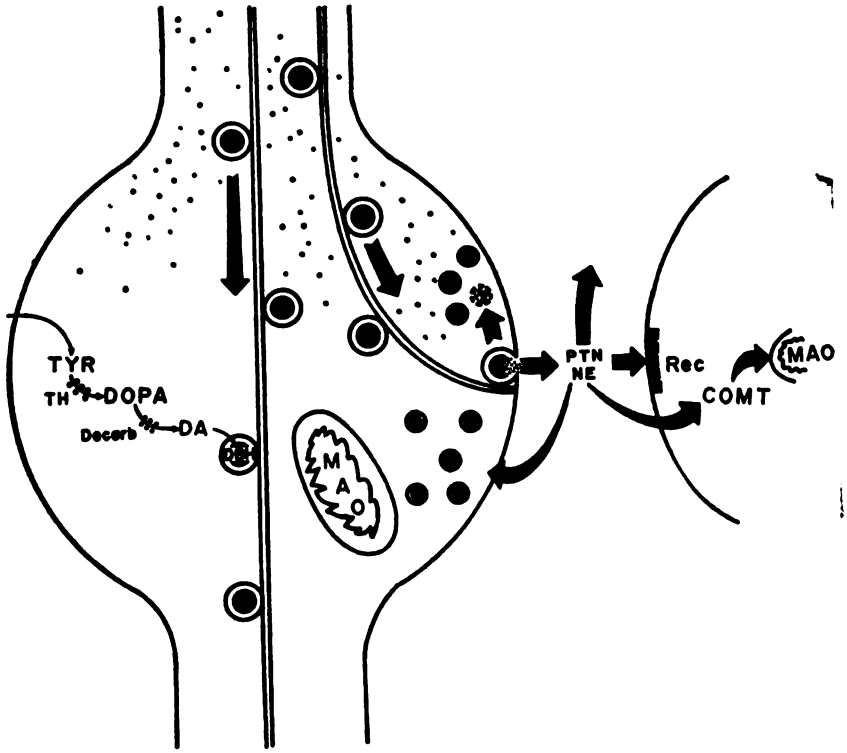

FIG. 4. Schematic representation of a sympathetic nerve varicosity. Large granular vesicles are transported to the axonal membrane, release their contents in response to a stimulus and form smaller granular vesicles. (See text for details.)

rapidly transported by a mechanism involving the neurotubules. The turnover of DBH is more rapid than that of TH.

Axonal damage or interference with integrity of the neurotubules results in diminished synthesis of catecholamine-forming enzymes with a switch to production of structural and membrane components. Levels of DBH and TH in the ganglia decline; however, uptake of NE increases, presumably as a consequence of increased membrane surface.

In vitro, axonal sprouts from cultured ganglia appear to have the properties of nerve endings almost as soon as they form. They avidly take up ^{3}H-NE, and although they contain only large granular vesicles, stimulation induces release of the labeled catecholamine. Thus large granules are sufficient to support adrenergic function.

The granules may be carried directly to the nerve terminal by the neurotubules. When the nerve impulse arrives and the contents of the large granular vesicle are extruded, the residual membranes with adherent proteins may form the smaller granular vesicles concerned with synthesis and storage of the reserve transmitter.

REFERENCES

1. Andén, N. E., Magnusson, T. and Waldeck, B.: Correlation between noradrenaline uptake and adrenergic function after reserpine treatment. Life Sci. 3: 19–25, 1964.
2. Banks, P., Mayor, D. and Tomlinson, D. R.: Further evidence for the involvement of microtubules in the intraaxonal movement of noradrenergic storage granules. J. Physiol. (London) 219: 755–761, 1971.
3. Berniljoin, W. S. and Molinoff, P. B.: Effects of 6-hydroxydopamine on the activity of tyrosine hydroxylase and dopamine-β-hydroxylase in sympathetic ganglia of the rat. J. Pharmacol. Exp. Ther. 178: 417–424, 1971.
4. Craig, B. G.: What is the signal for chromatolysis? Brain Res. 23: 1–21, 1970.
5. Dahlström, A.: Effects of drugs on axonal transport of amine storage granules. *In:* New Aspects of Storage and Release Mechanisms of Catecholamines, ed. by H. G. Schumann and G. Kroneberg, pp. 20–36, Springer-Verlag, Berlin, 1970.
6. Dahlström, A. and Haggendal, J.: Studies on the transport and life-span of amine storage granules in a peripheral adrenergic neuron system. Acta Physiol. Scand. 67: 278–288, 1966.
7. Droz, B. and Leblond, C. P.: Axonal migraion of proteins in the central nervous system and peripheral nerves as shown by radioautography. J. Comp. Neurol. 121: 325–346, 1963.
8. Geffen, L. B. and Ostberg, A.: Distribution of granular vesicles in normal and constricted sympathetic neurones. J. Physiol. (London) 204: 583–592, 1969.
9. Geffen, L. B. and Rush, R. A.: Transport of noradrenaline in sympathetic nerves and the effect of impulses on its contribution to transmitter stores. J. Neurochem. 15: 925–930, 1968.
10. Gewirtz, G. P. and Kopin, I. J. Effect of intermittent nerve stimulation on norepinephrine synthesis and mobilization in the perfused cat spleen. J. Pharmacol. Exp. Ther. 175: 514–520, 1970.
11. Hanbauer, I., Bloom, F. E. and Kopin, I. J.: Effect of local application of colchicine or vinblastine on the uptake of ^{3}H-norepinephrine by rat superior cervical ganglia. Fed. Proc. 31: 566, 1972.
12. Hanbauer, I., Johnson, D. G., Silberstein, S. D., Kopin, I. J. and Bloom, F. E.: Enhanced ^{3}H-norepinephrine uptake in superior cervical ganglia in organ culture. Pharmacologist 13: 203, 1971.
13. Hökfelt, T. and Dahlström, A.: Electron microscopic observations on the distribution and transport of noradrenaline storage particles after local treatment with mitosis inhibitors. Acta Physiol. Scand. 357: suppl., 10–11, 1971.
14. Iversen, L. L., Glowinski, J. and Axelrod, J.: The uptake and storage of ^{3}H-norepinephrine in the reserpine-pretreated rat heart. J. Pharmacol. Exp. Ther. 150: 173–183, 1965.
15. Kapeller, K. and Mayor, D.: The accumulation of noradrenaline in constricted sympathetic nerves as studied by fluorescence and electron microscopy. Proc. Roy. Soc. Ser. B Biol. Sci. 167: 282–292, 1967.
16. Kerr, P. and Livingstone, A.: Intraneuronal transport of noradrenaline in the rat. Mem. Soc. Endocrinol. 19: 671–682, 1969.
17. Laduron, P. and Belpaire, F.: Evidence for extragranular localisation of tyrosine hydroxylase. Nature (London) 217: 1155–1156, 1968a.
18. Laduron, P. and Belpaire, F.: Transport of noradrenaline and dopamine-β-hydroxylase in sympathetic nerves. Life Sci. 7: 1–7, 1968b.
19. Lamprecht, F., Weiss, V. K. and Kopin, I. J.: Effect of colchicine and vinblastine on the tyrosine hydroxylase and dopamine-β-hydroxylase content of the rat superior cervical ganglion and salivary gland. Fed. Proc. 31: 544, 1972.
20. Larrabee, M. G.: Metabolism of adult and embryonic sympathetic ganglia. Fed. Proc. 29: 1919–1928, 1970.
21. Levi-Montalcini, R. and Angeletti, P. V.: Nerve growth factor. Physiol. Rev. 48: 534–569, 1968.
22. Livett, B. G., Geffen, L. B. and Austin, L.: Proximo-distal transport of ^{14}C-noradrenaline and protein in sympathetic nerves. J. Neurochem. 15: 931–939, 1968.
23. Livett, B. G., Geffen, L. B. and Rush, R. A.: Immunohistochemical evidence for the transport of dopamine-β-hydroxylase and a catecholamine-binding protein in sympathetic nerves. Biochem. Pharmacol. 18: 923–924, 1969.
24. Molinoff, P. B., Berniljoin, W. S., Weinshilboum, R. M. and Axelrod, J.: Neurally mediated increase in dopamine-β-hydroxylase activity. Proc. Nat. Acad. U. S. A. 66: 453–458, 1970.
25. Mueller, R. A., Thoenen, H. and Axelrod, J.: Increase in tyrosine hydroxylase activity after reserpine administration. J. Pharmacol. Exp. Ther. 169: 74–79, 1969.
26. Olson, L. and Malmfors, T.: Growth characteristics of adrenergic nerves in the adult rat. Acta Physiol. Scand. 348: suppl., 1–11, 1970.
27. Schmitt, F. O.: The molecular biology of neural fibrous proteins. Neurosci. Res. Program Bull. 6: 119–144, 1968.
28. Silberstein, S. D., Berniljoin, W. S., Molinoff, P. B. and Lemberger, L.: Induction of dopamine-β-hydroxylase in rat superior cervical ganglia in organ culture. J. Neurochem. in press, 1973.
29. Silberstein, S. D., Johnson, D. G., Hanbauer, I., Bloom, F. E. and Kopin, I. J.: Axonal sprouts and ^{3}H-norepinephrine uptake by superior cervical ganglia in organ culture. Proc. Nat. Acad. Sci. U. S. A., in press.
30. Silberstein, S. D., Johnson, D. G., Jacobowitz, D. M. and Kopin, I. J.: Sympathetic reinnervation of the rat iris in organ culture. Proc. Nat. Acad. Sci. U. S. A. 68: 1121–1124, 1971.
31. Silberstein, S. D., Lemberger, L., Klein, D. C., Axelrod, J. and Kopin, I. J.: Induction of adrenal tyrosine hydroxylase in organ culture. Neuropharmacology, in press.
32. Smith, A. D.: Proteins of vesicles from sympathetic axons: chemistry, immunoreactivity and release upon stimulation. Neurosci. Res. Program Bull. 8: 337–382, 1970.
33. Smith, O. S., Jarlfors, U. and Beranek, R.: The organization of synaptic axoplasm in the lamprey (*Petromyzon marinus*) central nervous system. J. Cell Biol. 46: 199–219, 1970.
34. Stjärne, L.: Studies of catecholamine uptake, storage and release mechanisms. Acta Physiol. Scand. 62: suppl. 238, 1–97, 1964.

35. THOA, N. B., WOOTEN, F. G., AXELROD, J. AND KOPIN, I. J.: Inhibition of release of dopamine-β-hydroxylase and norepinephrine from sympathetic nerves by colchicine, vinblastine and cytochalasin-B. Proc. Nat. Acad. Sci. U. S. A. 69: 520–522, 1972.
36. THOENEN, H., MUELLER, R. A. AND AXELROD, J.: Phase difference in the induction of tyrosine hydroxylase in cell body and nerve terminals of sympathetic neurones. Proc. Nat. Acad. Sci. U. S. A. 65: 58–62, 1970.
37. WEINER, N.: Regulation of norepinephrine biosynthesis. Annu. Rev. Pharmacol. 10: 273–290, 1970.
38. WEISS, P.: The concept of perpetual neuronal growth and proximo-distal substance convection. *In:* Regional chemistry, ed. by S. S. Kety and J. Elkes, pp. 220–242, Pergamon Press, New York, 1961.
39. WEISS, P.: Neuronal dynamics and neuroplasmic flow. *In:* The Neurosciences: Second Study Program, ed. by F. O. Schmitt, pp. 840–850, Rockefeller University Press, New York, 1970.