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Rapid Axoplasmic Transport of Tyrosine Hydroxylase in Relation to Other Cytoplasmic Constituents

(constricted chicken sciatic nerves/dopamine β-hydroxylase/norepinephrine/acetylcholinesterase)

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ABSTRACT **The transport of norepinephrine and two key enzymes involved in its synthesis, tyrosine hydroxylase (EC 1.14.3a) and dopamine β-hydroxylase (EC 1.14.2.1), has been studied in relation to other axonal constituents in ligated chicken sciatic nerves. Norepinephrine, tyrosine hydroxylase, and dopamine β-hydroxylase activity all increased proximal to the constriction over a 20-hr period. The rate of transport of norepinephrine, tyrosine hydroxylase, and dopamine β-hydroxylase were calculated as 2.6, 1.9, and 3.4 mm/hr, respectively. The only enzyme marker to show a similar rate of accumulation was acetylcholinesterase (EC 3.1.1.7), a putative marker for endoplasmic reticulum. The rapid axoplasmic transport of tyrosine hydroxylase from the cell bodies to the terminals of sympathetic neurons may be adequate to account for the elevated amounts of this enzyme in chronically active axon terminals, without the necessity to invoke peripheral axonal synthesis of the enzyme to explain such elevated amounts.**

Both the enzymatic composition of adrenergic synaptic vesicles and their origin within the neurone are controversial. A unitary particle containing all the transmitter-synthesizing enzymes has been proposed on kinetic grounds by Udenfriend (1), but while it is generally agreed that dopamine β-hydroxylase (EC 1.14.2.1) is located within norepinephrine storage vesicles, the degree to which tyrosine hydroxylase (EC 1.14.3a) and DOPA decarboxylase (EC 4.1.1.26) are isolated as particulate enzymes in nerves depends on homogenization conditions—thus, they are usually considered to be soluble enzymes (2, 3).

Recently, a life cycle for synaptic vesicles in adrenergic neurones has been proposed (4–6) whereby precursor vesicles containing enzymes and other proteins involved in norepinephrine synthesis, storage, and release are produced in the neurone cell bodies and translated by rapid axoplasmic transport to the axon terminals, where they are transformed into synaptic vesicles. An alternative view is that synaptic vesicles are assembled locally in axon terminals (7).

Ligated sympathetic nerves have been used to study the axoplasmic transport of norepinephrine storage vesicles. A rapid accumulation of norepinephrine, dopamine-β-hydroxylase, and other radioactive proteins have been found (4, 5). However, it has been reported that tyrosine hydroxylase (8) and DOPA decarboxylase (9) failed to accumulate in the first 24 hr. The insensitivity of the assays used in these studies compared with the radiochemical methods now available led us to restudy the transport of these enzymes.

In the present study, the distribution of tyrosine hydroxylase in ligated sciatic nerves of chickens has been studied in relation to other enzymes, both those involved in catecholamine metabolism and those that constitute markers for various known particulate and soluble components of axons.

A similar study of DOPA decarboxylase is in progress (Dairman, Geffen, Nagatsu, and Udenfriend, to be published).

METHODS AND MATERIALS

Ligation of Sciatic Nerve. Leghorn cockerels, 10–12 weeks of age, were anesthetized with a mixture of halothane–nitrous oxide–oxygen (1:33:66). The left sciatic nerve was constricted about 5 cm from the spinal cord with a silk thread (10). The wound was sutured and, at intervals of 5, 10, and 20 hr after ligation, the chickens were killed by cervical dislocation. Both sciatic nerves were desheathed and 1-cm segments were taken proximal to the constriction and from the unligated nerved, frozen in liquid N_2, weighed, and stored overnight at −24°. In one experiment, a ligature was placed on the left sciatic nerve 5 cm from the spinal cord; 10 hr later, a second ligature was placed 2-cm distal to the first and another ligature was placed on the right sciatic nerve. After a further 10 hr, the chick was killed and 1-cm segments proximal to the ligations were taken for assay.

Biochemical Assays. Each nerve segment was homogenized in 0.5 ml of ice-cold 150 mM KCl containing 1 mM Tris·HCl (pH 7.0) in a Kontes Duall glass homogenizer (size 20) with a Teflon pestle. Aliquots were taken immediately for estimation of tyrosine hydroxylase, dopamine-β-hydroxylase, and norepinephrine, and the remainder was frozen in small aliquots and stored at −24° for 1–2 days for the remaining analyses.

Tyrosine Hydroxylase activity in 50 μl of homogenate was estimated by a modification of the method of Mueller, Thoenen, and Axelrod (11), whereby 0.1 mM brocrescine (4-bromo-3-hydroxy-benzyloxyamine, Smith & Nephew, Welwyn Gardens, U.K.) was added to the incubation mixture to inhibit aromatic-aminoacid decarboxylase activity. Substrate concentrations were 25 μM L-[3,5-^{3}H]tyrosine (1 Ci/mmol) (The Radiochemical Centre) and 1 mM 6,7-dimethyl-5,6,7,8-tetrahydropterine (Calbiochem). Reagent blanks and zero-time tissue blanks contained about 2000–3000 dpm; tissue samples contained 9000 dpm or more.

Dopamine β-Hydroxylase activity in 50 μl of homogenate, diluted 1 to 4 with cold Tris·HCl (5 mM, pH 7.4) containing

0.1% Triton X-100, was measured by the method of Molinoff, Weinshilboum, and Axelrod (12); 1 mM phenylethylamine was used as substrate and 10 μM $CuSO_4$ was present to neutralize the endogenous enzyme inhibitor.

Norepinephrine in 10 μl of homogenate was estimated by the method of Iversen and Jarrott (13).

Phosphoglucoisomerase (EC 5.3.1.9) (ref. 14), *acetylcholinesterase* (EC 3.1.1.7) (ref. 15), *β-glucuronidase* (EC 3.2.1.31) (ref. 16), *monoamine oxidase* (EC 1.4.3.4) (ref. 17), and *5′-nucleotidase* (EC 3.1.3.5) (ref. 18) activities were determined as enzymic markers for soluble cytoplasm, endoplasmic reticulum, lysosomes, mitochondria, and plasma membranes, respectively.

Protein was determined by the method of Lowry *et al.* (19).

The validity of the methods for enzyme assay were established in control experiments that showed that the measured activities were proportional to both homogenate concentration and incubation time. Appropriate blanks were determined and subtracted from the observed values.

RESULTS

The accumulation of norepinephrine, tyrosine hydroxylase, and dopamine-β-hydroxylase activity was measured in homogenates of the 10-mm segment of nerve proximal to the ligature, and compared after different intervals with a corresponding portion of unligated nerve (Fig. 1). All increased about linearly over 20 hr. At 10 hr, all were significantly increased compared with control values ($P < 0.001$ by analysis of a t-test). Although the percent accumulation of tyrosine hydroxylase was not significantly different from that of norepinephrine ($P = 0.3$), the accumulation of dopamine-β-hydroxylase was significantly different from that of tyrosine hydroxylase ($P < 0.05$).

The rapid accumulation of tyrosine hydroxylase has not been previously detected; as a check that the accumulation resulted from axoplasmic transport and not from a local

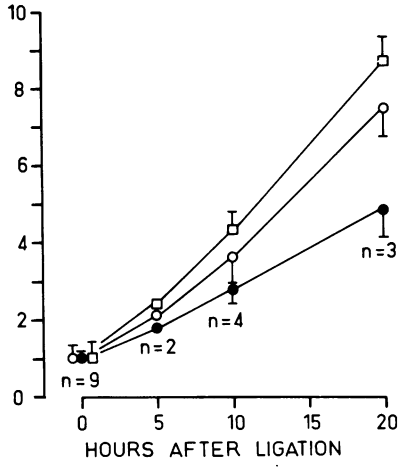

FIG. 1. Rate of accumulation of norepinephrine, O——O; tyrosine hydroxylase, ●——●; and dopamine-β-hydroxylase, □——□; activity in a 1-cm segment of ligated sciatic nerve. Control values are given at 0 hr, and each *point* represents the mean ± SE (with the number of observations given as *n*). The *ordinates* are in arbitrary units; the value of 1 unit is shown in parentheses: norepinephrine (92 ng/cm), tyrosine hydroxylase (26.6 pmol/hr per cm) and dopamine-β-hydroxylase (0.45 nmol/hr per cm).

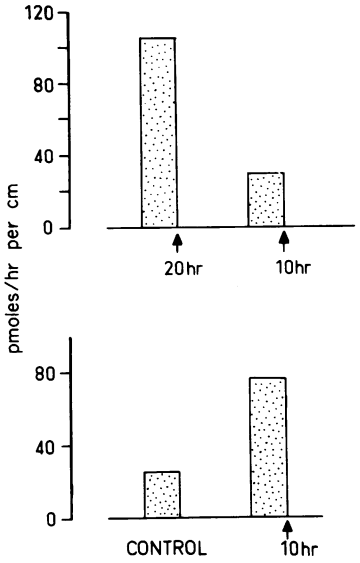

FIG. 2. Accumulation of tyrosine hydroxylase activity above a double ligation of the left sciatic nerve (*top*) and above a single ligation of the right sciatic nerve (*bottom*).

reaction to injury, a double ligation experiment was performed. This showed that there was no accumulation of tyrosine hydroxylase activity during 10 hr above a second ligature placed distal to the first (Fig. 2).

Flow rates of 2.6 mm/hr (norepinephrine), 3.4 mm/hr (dopamine-β-hydroxylase), and 1.9 mm/hr (tyrosine hydroxylase) were calculated by the method of Dahlstrom and Haggendahl (20), which assumes that the accumulation of norepinephrine, dopamine-β-hydroxylase, and tyrosine hydroxylase activity is solely derived from axoplasmic transport.

Homogenates were also assayed for enzyme markers of soluble cytoplasm (phosphoglucoisomerase), endoplasmic reticulum (acetylcholinesterase), lysosomes (β-glucuronidase), mitochondria (monoamine oxidase), and plasma membranes (5′-nucleotidase). Only acetylcholinesterase showed an increase in activity (about 3-fold) over 20 hr (Table 1).

DISCUSSION

Tyrosine hydroxylase activity accumulated rapidly in ligated sciatic nerves of chickens at about the same rate as nor-

TABLE 1. *Accumulation of several enzymes in ligated sciatic nerves*

Enzyme	Hours after ligation 0*	5†	10†	20†
Acetylcholinesterase (μmol/hr per cm)	19.2 ± 1.2	33.0	44.4	54.6
5′-Nucleotidase (μmol/hr per cm)	1.42 ± 0.03	1.43	1.90	1.55
Monoamine oxidase (nmol/hr per cm)	21.9 ± 3.0	23.0	26.8	32.2
β-Glucuronidase (nmol/hr per cm)	49.3 ± 7.1	51.3	42.0	59.2
Phosphoglucoisomerase (μmol/hr per cm)	79.3 ± 8.6	90.1	76.2	89.3

* Mean ± SE; $n = 3$.
† Single determinations.

epinephrine and dopamine-β-hydroxylase, both constituents of the large granular vesicles that are rapidly transported somatofugally in sympathetic neurones (see ref. 5). This finding suggests that *in vivo* tyrosine hydroxylase may be associated with norepinephrine storage vesicles, at least during axoplasmic transport. The only other enzyme to show a rate of accumulation comparable to tyrosine hydroxylase and dopamine-β-hydroxylase was acetylcholinesterase, which is marker for endoplasmic reticulum in cholinergic neurones (21–23).

The evidence for rapid axoplasmic transport of vesicles containing norepinephrine has been reviewed recently (4, 5). Norepinephrine and dopamine-β-hydroxylase have been estimated to be transported at rates of 1.4–10 mm/hr in different nerves; the transport rates of norepinephrine, dopamine-β-hydroxylase, and tyrosine hydroxylase found in the present study to be 2.6, 3.4, and 1.9 mm/hr, respectively, fall within this range.

However, tyrosine hydroxylase has been reported to be slowly transported in unligated rat sciatic nerve (24); in ligated dog splenic nerves, tyrosine hydroxylase activity in the proximal segment was reported to decrease (8). Both these studies measured tyrosine hydroxylase in a 30,000 × *g* supernatant, whereas we used a whole homogenate. If the tyrosine hydroxylase was attached to a rapidly transported particle, then these disparate findings could be reconciled without recourse to species difference and assay methods. A rapid accumulation of DOPA decarboxylase has recently been found in constricted rat sciatic nerves (Dairman *et al.*, to be published).

The method of calculating axoplasmic transport of a substance from the rate of accumulation above a ligature assumes that there is either negligible synthesis and degradation at the constriction, or that they are in balance. It also assumes that the ligature itself has no effect on the subsequent production and transport of the substance and, in the case of enzymes, that factors inhibiting or enhancing their activity do not also accumulate. The linearity in accumulated enzyme activity over 20 hr obviates some of these assumptions. The parallel rapid increase in calculated transport rates of norepinephrine, dopamine-β-hydroxylase, and tyrosine hydroxylase in the first 20 hr after ligation is sufficient to revive an earlier suggestion (1) that all of the enzymes involved in norepinephrine synthesis may be contained in subcellular elements.

Prolonged increased sympathetic activity leads to a rise in tyrosine hydroxylase activity in sympathetic neurones (11). Thoenen, Muller, and Axelrod (24) studied the rate of induction of tyrosine hydroxylase in stellate ganglia and heart of rats as a consequence of reserpine administration. They observed a phase lag of 2 days between induction of the enzyme in the sympathetic cell bodies and in nerve terminals, and concluded either that a rapid transport of tyrosine hydroxylase from the ganglion or that local axonal synthesis of tyrosine hydroxylase initiated by some messenger from the cell bodies was responsible. Thoenen *et al.* (24) favored the latter view, because the slow flow of tyrosine hydroxylase calculated in their experiments could contribute only 12% to the increase in the enzyme activity observed in the heart. Our results provide an alternative explanation based on the rapid axoplasmic flow of tyrosine hydroxylase, possibly associated with the norepinephrine- and dopamine-β-hydroxylase-containing particles that are postulated to be transported somatofugally from cell bodies to terminals in sympathetic neurones (4–6).

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