STUDIES ON AXONAL FLOW OF DOPAMINE β -HYDROXYLASE AND TYROSINE HYDROXYLASE IN SCIATIC NERVES BY IMMUNO-FLUORESCENT AND BIOCHEMICAL METHODS*

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The transport of dopamine β -hydroxylase (DBH) and tyrosine hydroxylase (TH) in guinea pig sciatic nerves was studied by measuring the accumulation of these enzyme activities to a ligature. Axonal flow of DBH protein in rat sciatic nerves was also studied by immunofluorescence, and the anatomical results were compared with the biochemical ones.

The DBH immunofluorescence was increased in the proximal 3-mm segment of a ligature up to 48 hr after ligation. The DBH activity was also highly accumulated in the proximal 3-mm segment until 48 hr after ligation. Retrograde axonal flow of DBH or TH was observed in double ligation experiments at 5 hr after ligation, but at 24 hr the retrograde axonal flow apparently changed to the orthograde. The mean, or absolute axonal flow rate (mm/24 hr) of DBH in guinea pig sciatic nerves was 100, or 180, while that of TH was 20, or 32, respectively. The results suggest that DBH and TH may be localized in different intracellular compartments; DBH may be transported at a fast flow rate as a particulate enzyme, while TH at an intermediate flow rate mostly as a cytoplasmic enzyme.

It is generally agreed that the axoplasmic flow rate of an enzyme differs depending upon its subcellular localization in the axon; a particulate enzyme may flow down fast while a cytoplasmic enzyme slow. Many reports concerning the axonal transport of the enzymes involved in the biosynthesis of noradrenaline in adrenergic neurons have appeared (16, 18) in order to elucidate the intracellular localization of the enzymes. However, the results about the axonal flow rate of dopamine β -hydroxylase (DBH) [3,4-dihydroxyphenylethylamine, ascorbate: oxygen oxidoreductase (hydroxylating), EC 1.14.2.1] and tyrosine hydroxylase (TH) [L-tyrosine, tetrahydropteridine: oxygen oxidoreductase (3-hydroxylating), EC 1.14.3a] are somewhat controversial. Some reports (7, 18) indicated that TH, although predominantly localized in the non-particulate fraction of the peripheral nerves (15), is transported slightly slower than but almost as fast as DBH which is mainly localized in the particulate fraction, while other reports (16) showed that TH is transported much slower than DBH. The present work has been undertaken to clarify this controversy.

Dedicated to Professor Hideo Takamatsu in memory of his retirement.

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One of the authors (I. N.) had applied the immunofluorescent method for the elucidation of the anatomical characteristics of DBH accumulated in ligated rat sciatic nerve (13).

Based on these observation, axonal flow rate of DBH has been precisely examined and compared with that of TH in guinea pig sciatic nerves which have been found to have much higher enzyme activities than rat nerves. Monoamine oxidase (MAO) activity, a mitochondrial enzyme, was also measured as a control.

MATERIALS AND METHODS

Preparation of tissue samples:

Sprague-Dawley rats (200–250 g) and guinea pigs (200–300 g) were used for the experiments. Ligation of sciatic nerves were performed with 4–0 surgical silk (left *in situ*) under light ether anaesthesia, and segments of sciatic nerves were removed for assay at 5–72 hr after ligation. Contralateral sciatic nerves were used as control.

Biochemical assays:

Ten 3-mm nerve segments from guinea pigs were pooled and homogenized in 600 μ l of 0.1 M phosphate buffer (pH 7.4), containing 0.1% Cutscum (isooctyl-phenoxypolyethoxyethanol-containing detergent, Fisher Scientific Co.). The homogenate was centrifuged at $700\times g$ for 10 min, and the supernatant was used as the enzyme preparations.

TH activity was assayed by measuring the formation of ^{14}C -DOPA from L- ^{14}C -tyrosine (14). Incubation mixture (total volume, 250 μ l) contained: 200 mM Tris-acetate buffer (to give a final pH of 6.0), 1 mM FeSO_4 , 30 μ l of enzyme solution, 100 mM mercaptoethanol, 1 mM 6-methyltetrahydropterin, and 100 μ M L- ^{14}C -tyrosine (0.05 μCi). Incubation was carried out at 30°C for 15 min in air with constant shaking. TH reaction was linear only for 15 min, and not linear up to 60 min, but in a preliminary experiments, axonal flow rate of TH measured based on 60 min incubation gave identical values with that based on 15 min incubation.

DBH activity was assayed by measuring the formation of octopamine from tyramine by sensitive dual-wavelength spectrophotometry (9). By this method the maximum velocity can be measured with a saturated substrate concentration (20 mM) and under an optimum pH (5.0). Incubation mixture (total volume 1.0 ml) contained: 400 μ l of the diluted enzyme solution (50 μ l of the homogenate and 350 μ l of water), 0.2 M acetate buffer (pH 5.0), 10 mM N-ethylmaleimide, 1 μ M CuSO_4 , 10 mM sodium fumarate, 10 mM ascorbic acid, 1500 units (50 μg) of catalase, and 20 mM tyramine hydrochloride. Boiled diluted enzyme solution (95°C for 5 min) served for the blank. Incubation was carried out for 45 min at 37°C in air with constant shaking.

MAO activity was assayed fluorometrically by measuring the formation of 4-hydroxyquinoline from kynuramine (10). Incubation mixture (total volume 600 μ l) contained 400 μ l of the diluted enzyme solution (50 μ l of the homogenate and 350 μ l of water), 50 mM potassium phosphate buffer (pH 7.4), and 0.17 mM kynuramine dihydrobromide. Incubation was carried out at 37°C for 30 min in air with constant shaking.

Protein was measured by the method of Lowry *et al.* (11), as modified by Hartree (6).

Student's t-test was used to determine the significance of differences between means. The measure of variation used throughout was the standard error of the mean.

Immunofluorescent method:

Ten- μ frozen sections were cut and fixed in chloroform-methanol (2 : 1). Immunohistochemical staining procedure was performed using rabbit antiserum to DBH at first. Then antiserum to rabbit IgG, which had been prepared by using the affinity chromatography, was conjugated with FITC and used for staining (5, 13). Photographs were taken with a Leitz Orthoplan fluorescence microscope using an activation filter KP490 (12, 13). Kodak Tri-X panchromatic film was used.

RESULTS

Anatomical characteristics of DBH accumulation proximal and distal to a ligature in rat sciatic nerve:

Fig. 1 shows the distribution of the diameter of adrenergic axons at 24 and 48 hr after ligation of a rat sciatic nerve measured in DBH immunofluorescent pictures (13). At 24 hr, DBH appeared in "striated" areas as large as 20 μ in diameter. At 48 hr, the size of the area covered by DBH immunofluorescence was further increased.

The accumulation of DBH immunofluorescence distal to a ligature was marked at 5 or 24 hr after ligation. This suggests that the retrograde axonal flow of DBH is predominant at this early period.

Biochemical observation of accumulation of DBH in guinea pig sciatic nerve:

Rat sciatic nerve was used for the immunofluorescence studies for DBH since the previous works (3) based on noradrenaline fluorescence had been carried out with rat sciatic nerve. However, since TH and DBH activities in sciatic nerves of guinea pigs were much higher than those of rats (Table 1), guinea pigs are more appropriate animals and were used for the biochemical assays.

Accumulation of DBH, TH, MAO and total protein after double ligation of a guinea pig sciatic nerve is shown in Fig. 2. At 5 hr after the ligation DBH was markedly accumulated within 3 mm of a segment proximal to a ligature. A significant accumulation at the distal segment was also observed. The distal accumulation of DBH suggests that the retrograde axoplasmic flow is greater than the orthograde. The proximal accumulation was further increased within the 3-mm segment up to 48 hr. At 72 hr after ligation the accumulation of the activity decreased. The time course of accumulation of TH was similar to that of DBH, but the accumulation of TH was slower and broader than that of DBH. Any significant accumulation of MAO and total protein was not observed at 5 or 24 hr, and only slight accumulations of MAO and protein were observed at 48 hr.

Rates of accumulation of DBH and TH within the 3-mm and 9-mm nerve segments of a guinea pig sciatic nerve above ligation are shown in Fig. 3. Linear rate

FIG. 1. Distribution of the diameter of adrenergic axons in a rat sciatic nerve at 24 and 48 hr after ligation. The diameters of adrenergic axons with each 0.1-mm segment of the nerve were measured and was plotted against the distance. A single dot represents the diameter and the location of an axon.

of accumulation of TH was observed up to 48 hr either with 3-mm or 9-mm segments. In contrast, the rate of accumulation of DBH was linear only within 5 hr and fell down significantly after 5 hr. Both TH and DBH activities in a unit length (one mm) of the nerve segment are much higher in 3-mm segment than those in 9-mm segment, suggesting that most of the enzyme activities are concentrated in the 3-mm

TABLE 1. Comparison of catecholamine enzyme activities in sciatic nerves between guinea pigs and rats.

Species (n=5)	Enzyme activities			Protein (mg/cm nerve)
	Tyrosine hydroxylase	Dopamine β -hydroxylase (nmoles/hr/cm nerve)	Monoamine oxidase	
Guinea pigs	0.885 $\pm$ 0.055	12.43 $\pm$ 1.34	6.58 $\pm$ 0.49	0.55 $\pm$ 0.01
Rats	0.210 $\pm$ 0.006	1.44 $\pm$ 0.11	7.44 $\pm$ 1.43	0.61 $\pm$ 0.02

FIG. 2. Enzyme activities in 12-mm segments proximal (P) and 9-mm segments distal (D) to double ligations (L) of a guinea pig sciatic nerve. Nerves were ligated at 5, 24, 48, and 72 hr prior to killing animals. Tyrosine hydroxylase (TH), dopamine β -hydroxylase (DBH) and monoamine oxidase (MAO) activities were expressed as nmoles/hr/3 mm nerve, and protein concentration as mg/3 mm nerve. Each bar represents the mean $\pm$ S.E.M. for at least 3 experiments.

segment proximal to the ligature. The mean axonal flow rate (mm/24 hr) measured at 5 hr after ligation were: 100 for DBH and 20 for TH.

When an absolute axonal flow rate was measured, the following formula was used:

FIG. 3. Time course of accumulation of tyrosine hydroxylase (TH) and dopamine β -hydroxylase (DBH) above a ligature of a sciatic nerve. Enzyme activities were determined in 3-mm segments (●—●) or in 9-mm segments (○---○) proximal to a ligature. The enzyme activities were expressed as nmol/hr per each segment. The values are the means $\pm$ S.E.M. of at least 3 experiments.

$$\frac{24(T-C)L}{t(C-I)} \text{ mm/24 hr}$$

- T:** Enzyme activity in the segment proximal to a ligation,
C: Enzyme activity in the contralateral control segment,
I: Immobilized enzyme activity,
L: Length of the segment in mm,
t: Time between treatment and removal of the segment for assay in hr.

The absolute axonal flow rate (mm/24 hr) of TH or DBH was 32 or 180, respectively.

Changes in the rate of transport of DBH and TH within 3-, 6-, 9-, or 12-mm segments above a ligature of a guinea pig sciatic nerve measured at 5, 24, 48, and 72 hr were shown in Fig. 4. The rate of transport for DBH measured at 5 hr was high, and decreased gradually with time after ligation. In contrast, the rate of TH transport did not change so much when measured at 5, 24, or 48 hr, and slightly decreased at 72 hr.

DISCUSSION

The present work is intended to clarify the intracellular localization of DBH and TH based on the measurement of axoplasmic flow rate. Also, the observation on the axoplasmic flow of DBH by immunofluorescence was compared with the findings based on the biochemical activity measurements.

The flow rates of DBH and TH have been measured in different conditions, *i. e.*, by changing the length of the nerve segments assayed from 3 to 12 mm and with different periods of time from 5 to 72 hr after ligation.

Since essentially the entire accumulation is located within 3 mm on either side of the ligation by immunofluorescence, the appropriate length of the nerve segment measured may be 3 mm, which should be short enough so that rates of accumulation

FIG. 4. The mean axonal flow rates (mm/24 hr) of tyrosine hydroxylase (TH) and dopamine β -hydroxylase (DBH) measured at various periods of ligation of guinea pig sciatic nerve. The values are the means $\pm$ S.E.M. of at least 3 experiments at 5, 24, and 48 hr, and the means of 2 experiments at 72 hr.

can be measured with minimal dilution by existing levels of DBH, but not so short that accumulation extends above that ligation. The biochemical data on the accumulation of DBH activity coincided with the results of immunofluorescence. As shown in Fig. 2, DBH activity was accumulated within a 3-mm segment up to 48 hr after ligation. A similar pattern was also shown with TH activity. However, the pattern of accumulation of TH was somewhat different from that of DBH. The accumulation of TH was slower and broader than DBH.

The flow rate of DBH was not linear, whereas that of TH was linear, with time up to 48 hr (Fig. 3). Since most of activities are localized within a short segment, the activities in a unit length of the nerve segment are much higher in 3-mm segment than those in 9-mm segment. However, the rate of accumulation of TH and DBH is essentially similar to 3-mm or 9-mm nerve segments. The early drop of DBH flow rate can be due to: 1) reduced addition of the enzyme secondary to the inhibition of the transport of the enzyme from the cell body, and 2) increased rate of degradation and readsorption of the DBH containing-vesicles. A marked decrease

in the flow rate at 72 hr may be due to the increased degradation and clearance of the enzyme, since the immunofluorescence data of DBH indicated the disruption of the axonal membranes and extravasation of the vesicles into the extracellular space and possibly secondary inflammatory process.

The present result (Fig. 4) indicated the mean axonal flow rate of DBH in guinea pig sciatic nerve to be 100 mm/24 hr, and that of TH to be 20 mm/24 hr. Dahlström (3) firstly noticed a marked accumulation of noradrenaline above a ligature to the first few mm at 24 hr. Oesch *et al.* (16) reported the axonal flow rate of TH in rat sciatic nerve to be 18 mm/24 hr, which is very close to our mean value in guinea pig sciatic nerve. Brimijoin (2) used 3-mm segment to measure DBH activity in rat sciatic nerve, and found DBH accumulation in the 3-mm segment just above the ligature but not in the more proximal segment. His axonal flow rate for DBH was 110.5 mm/24 hr in rat sciatic nerve and is similar to our present data 100 mm/24 hr in guinea pig sciatic nerve.

These axonal flow rates do not take into consideration the immobilized parts of TH or DBH which may be localized outside the axons. Therefore, the flow rate should be called the mean flow rate. The percentages of the immobilized parts of TH and DBH in guinea pig sciatic nerve were 38% and 44%, respectively. The absolute axonal flow rates can be calculated by subtracting these immobilized parts of the enzyme activities, and 32 mm/24 hr for TH, and 180 mm/24 hr for DBH, respectively.

The differences in the patterns of accumulation of TH and DBH (Fig. 2) and in the rates of flow of both enzymes suggest that both enzymes may be localized differently in the neurons. Karlsson and Sjöstrand (8) tried to find some correlations between axonal flow rate and subcellular components; fast phase (40–150 mm/24 hr) for vesicles and endoplasmic reticulum, intermediate phase (6–12 mm/24 hr) for mitochondria, and slow phase (2 mm/24 hr) for soluble proteins. In the present study, the mean flow rate of DBH (100 mm/24 hr) indicates it to belong to the fast phase and thus its intragranular localization, whereas that of TH (20 mm/24 hr) indicates it to belong to the intermediate phase, suggesting that TH is not an entirely soluble enzyme, but still attaches to some intracellular organelles. Recent findings on immunoelectronmicroscopy indicate that TH is associated with neurotubules (17).

The immunofluorescent (Fig. 1) as well as the biochemical data (Fig. 2) clearly suggest that DBH and TH are also transported by retrograde axonal flow especially prior to 24 hr after ligation. The retrograde axonal transports of TH and DBH were clearly observed at 5 hr after ligation, but at 24 hr this retrograde axonal flow apparently changed to orthograde. The fact that retrograde accumulation of noradrenaline disappeared from 24 to 48 hr after ligation (3), agreed with our previous DBH immunofluorescent results (13) and also with our present biochemical results. An apparent distal accumulation is unlikely to have resulted from leak of DBH from the region above a ligature in view of the finding that noradrenaline accumulates primarily above the proximal but below the distal of two ligatures (4). If small amounts of vesicular components were transported centrally at a high rate, this process might have some messenger function such as feedback mechanism of information (1).

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