Transport of Axonal Enzymes in Surviving Segments of Frog Sciatic Nerve

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ABSTRACT Redistribution of axonal enzymes as a function of time in vitro was studied in an unbranched segment of frog sciatic nerve. Cholinesterase activity moved peripherally at a rate of 99 mm/day and centrally at 19 mm/day. One-quarter of the total nerve content of the enzyme was estimated to be in motion, one-eighth in each direction. Mitochondrial enzymes (hexokinase and glutamic dehydrogenase) moved peripherally at 20-31 mm/day, centrally at 11-20 mm/day. Only 10% of the total content of these mitochondrial enzymes was in motion. No movement of choline acetylase or 6-phosphogluconic dehydrogenase activity was seen even after 4 days in vitro. However, in a 12 day in vivo experiment choline acetylase moved toward the periphery at a rate of 0.34 mm/day. After a day or so in vitro the distal accumulations of cholinesterase and glutamic dehydrogenase decreased, with a concomitant and quantitatively equivalent increase in enzyme activities at the proximal end of the nerve. It is postulated that during incubation a mechanism for reversing the direction of flow develops in the peripheral stump of the nerve. Vinblastine inhibited central and peripheral flow of both cholinesterase and glutamic dehydrogenase. Movement of cholinesterase was not affected by ouabain, thalidomide, or phenobarbital, nor by K+ excess (110 mM) or absence.

INTRODUCTION

Rapid longitudinal movement of axoplasmic constituents continues in nerve segments isolated from the cells of origin and from the nerve terminals (Lubinska et al., 1964; Banks et al., 1969; Ochs and Ranish, 1970). An early paper by Lubinska (1956) may be interpreted as demonstrating axoplasmic flow1 in single nerve fibers of frog sciatic nerve in vitro. More recently, axoplasmic flow in vitro has been demonstrated in isolated segments of dog

1 Davison (1970) has pointed out that neither fast nor slow axoplasmic transport appears to have the characteristics of fluid flow. Since the word "flow" already has the authority of much usage and the convenience of brevity, we shall continue to use it, while disclaiming any implications with regard to the underlying mechanism.
nerve (Jankowska et al., 1969) and in cat (Ochs and Ranish, 1970; Ochs and Hollingsworth, 1971). In the present study, enzymes known (or thought) to be localized in specific subcellular structures were used as markers for these structures, and their redistribution in frog sciatic nerve was followed as a function of time in vitro. Hexokinase (EC 2.7.1.1) and glutamic dehydrogenase (EC 1.4.1.2) are mitochondrial enzymes; cholinesterase (EC 3.1.1.7) is known to be membrane bound; 6-phosphogluconic dehydrogenase (EC 1.1.1.44) is an example of a soluble enzyme. Choline acetylase (EC 2.3.1.6) was studied because of its neuronal localization. It is probably a soluble enzyme also, but there has been some dispute about this (Fonnum, 1970).

METHODS

Animals Frogs (R. pipiens) were obtained commercially. On arrival in the laboratory they were treated and maintained as described previously (Okada and McDougal, 1971).

Materials Vinblastine sulfate and vincristine sulfate were obtained from Eli Lilly and Company (Indianapolis, Ind.). The vincristine preparation contained 50 mg lactose for each 5 mg of active drug. The vinblastine preparation contained no carrier. Thalidomide (investigational lot No. 1045B) was kindly provided by Dr. David A. Blake, University of Maryland School of Pharmacy. Phenobarbital was obtained from Winthrop Laboratories (New York), tetraisopropylpyrophosphoramide (iso-OMPA) from Sigma Chemical Co. (St. Louis, Mo.), and m-aminobenzoic acid ethyl ester methane sulfonate salt (MS-222) from Calbiochem (Los Angeles, Calif.).

Enzyme substrates, coenzymes, and auxiliary enzymes were obtained from Boehringer Mannheim Corp. (New York), or Sigma Chemical Co.

Preparation of Nerve The brain and spinal cord were destroyed by pithing with a hard wire. The portion of the sciatic nerve which lies between hip and knee was used in all in vitro experiments. In this region the nerve divides into tibial and peroneal branches several millimeters above the knee, but is otherwise devoid of branches (Dunn, 1909). It was dissected free of surrounding tissues, care being taken to avoid damage to the perineurium, and ligated proximally and distally. The distal tie encircled both the peroneal and tibial branches. The ligated nerve was then removed to a bath of ice-cold frog Ringer solution: NaCl, 101 mm; KCl, 5.5 mm; CaCl₂, 2.1 mm; MgSO₄, 1.1 mm; NaHCO₃, 4 mm; and glucose, 4 mm. The solution was equilibrated with a gas mixture containing 95% O₂ and 5% CO₂. The pH was adjusted to 7.4 with HCl. Since the incubation vessels were not sealed, the solution gradually lost CO₂. The pH approached an equilibrium value near 8 during the first day in vitro.

Nerves were incubated at 22°C for periods up to 96 hr, then frozen in liquid N₂ and dried under vacuum at -35°C to -40°C. The entire dried nerve was cut into measured segments 0.5–15 mm long. Distal samples each contained two pieces, one tibial and one peroneal. After the perineurium was removed, each sample was weighed.
on a quartz fiber balance (Lowry, 1953). Samples 2 mm long or less were homoge-
nized in 45 μl of 0.1 M phosphate buffer, pH 7.8, containing bovine serum albumin
(0.05 %) using small quartz homogenizers with glass pestles. Longer segments were
homogenized in proportionately larger volumes. The homogenates were stored at
−80°C until assay.

In some experiments the composition of the incubation medium differed from that
of the Ringer solution described above, either by the addition of drugs or by changes
in ionic content. For these experiments, the nerves were preincubated in the altered
medium for 1 hr at 0°–4°C. Then the temperature was raised to 22°C and incubation
continued in the same medium as that used for the preincubation. Preliminary ex-
periments showed that no significant movement of cholinesterase occurs during incu-
bation for (at least) 8 hr in the cold. It was also shown that the movement of cho-
linesterase and glutamic dehydrogenase in control nerves during the next several
hours at 22°C was not affected by the 4°C preincubation.

A few experiments were done in vivo. Frogs were anesthetized with MS-222, and
one or two ties were placed on the sciatic nerve. The incisions were closed and the
animals kept for as long as 12 days. Then the frogs were killed, and the nerves were
removed, frozen, and dried as above.

**Enzyme Assays** The assays were performed according to published methods,
with occasional slight modifications. Each assay was linear with time and tissue con-
centration in the ranges used. The cholinesterase was measured according to the
method of Guth et al. (1964). Glutamic dehydrogenase was assayed according to
Garcia-Bunuel et al. (1962). The assay conditions for hexokinase and 6-phospho-
gluconic dehydrogenase are given in McDougal et al. (1961). The method of Mc-
caman and Hunt (1965), somewhat modified (e.g. by using triiodide precipitation,
Goldberg et al., 1969), was used to assay choline acetylase. Results of all assays are
expressed in terms of moles of product per kilogram dry weight of nerve per hour.

**Calculations** For each segment of every nerve the weight (w), the length, and
the enzyme activity (A) were determined. Thus, the average enzyme activity for the
whole nerve (Ā) and the mean weight per millimeter length (W) could be computed.
The amount of enzyme activity (C) which accumulated at a given end of the nerve
could then be calculated by using equation 1,

\[ C = \sum \left( \frac{(A - \bar{A}) \times w}{\bar{A} \times W} \right) \]

in which the indicated sum includes those segments adjacent to the ligature having
enzyme activities greater than \( \bar{A} \). Notice that C has the dimension of length and rep-
resents the increase in the amount of enzyme activity present in the terminal portion
of the nerve in terms of the average amount of activity in 1 mm of the same nerve
(“mm worth” of enzyme). Using C, nerves can be compared directly, despite differ-
ences in nerve enzyme activity (A) from frog to frog. Notice also that to calculate C
for any nerve, data from other nerves are not used.

To calculate the rate of movement of an enzyme in any nerve, one needs to know
how much of the total enzyme content of the nerve is in motion. It was assumed that
the average decrease in the level of enzyme activity in the central portion of nerves after prolonged incubation was a measure of the amount of moving enzyme. In addition, the data showed that initially some enzyme was in motion toward one end of the nerve segment, while some was moving toward the other. It was assumed that the ratio of the average maximal accumulation (plateau) of enzyme at one end of the nerve to the total amount of enzyme in motion was a measure of the amount of enzyme moving toward that end. With these two assumptions, the velocity \( V \) (in millimeters per day) was calculated from equations 1 and 2:

\[
V = \frac{C}{d \times f \times t} \tag{2}
\]

where
- \( d \) = average fractional decrease in enzyme activity in the central portion of the nerve after prolonged incubation. This is numerically equal to the fraction of enzyme activity in motion.
- \( f \) = fraction of movable enzyme in motion toward the (peripheral or central) end of the nerve.
- \( t \) = incubation time in days.

RESULTS

Characteristics of the Nerve Segment Used

Anatomically, the region of frog sciatic nerve between hip and knee has been shown to have 3000–4000 myelinated fibers, of which 25–30% are motor (Dunn, 1909).

In this portion of nerve, which was 2–2.5 cm long in the large frogs used in this study, there was no initial gradient of enzyme activity for any of the enzymes measured (e.g., Fig. 2, 0 h) (see Lubinska et al., 1963). Therefore, the weight-averaged activity \( \bar{A} \) of all the segments of a nerve served as an adequate control for the activity in any one segment.

Of the enzymes studied, only acetylcholinesterase and choline acetylase have a reasonable claim to a purely axonal localization in nerve. In experiments with frog brain, acetylthiocholine was hydrolyzed 15 times as rapidly as butyrylthiocholine (Fig. 1, legend). Also, the hydrolysis of butyrylthiocholine was almost completely inhibited by levels of iso-OMPA (10\(^{-4}\) M), which only inhibited the hydrolysis of acetylthiocholine 10\% (Fig. 1). Both observations suggest that almost all of the brain cholinesterase is acetylcholinesterase. Furthermore iso-OMPA at 10\(^{-4}\) M discriminated well between the two frog cholinesterases. Therefore the ability of cholinesterase from the sciatic nerve to hydrolyze acetylthiocholine was also examined in the presence and absence of 10\(^{-4}\) M iso-OMPA. As with brain, only 10\% of the cholinesterase activity was blocked by the inhibitor, suggesting that the nerve also contains largely acetylcholinesterase.

The activities of cholinesterase and choline acetylase in nerve were com-
pared with those in dorsal and ventral roots and in sensory ganglia (Table I). The expected relatively high levels of both enzymes were observed in ventral roots. Using the cholinesterase data for dorsal and ventral roots and sciatic nerve, it was estimated that around 18% of the axons in this region of

![Graph](image_url)

**Figure I.** Cholinesterase activity in frog brain using acetylthiocholine (AcSCh) or butyrylthiocholine (BuSCh) as substrate in the presence of varying concentrations of iso-OMPA. Brain with the indicated level of inhibitor was incubated for 30 min at 23°C. Then 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman's reagent) was added, and the reaction was started by adding the substrate. Control activity with acetylthiocholine was 0.32 mole/kg wet wt per hr; with butyrylthiocholine it was 0.021 mole/kg per hr.

| Table I | CHOLINE ACETYLASE AND CHOLINESTERASE ACTIVITIES IN DORSAL ROOTS, DORSAL ROOT GANGLIA, VENTRAL ROOTS, AND SCIATIC NERVE |
|--------|---------------------------------------------------------------------------------------------------|
|        | Cholinesterase                                                                                     |
|        | n | Choline acetylase | -iso-OMPA | +iso-OMPA |
|        | | | | | |
| Dorsal root | 4 | 0.05±0.02 | 59±4 | 57±2 |
| Dorsal root ganglion | 4 | 0.33±0.06 | 4500±220 | 1620±90 |
| Ventral root | 3 | 3.8±0.5 | 425±22 | 390±40 |
| Sciatic nerve | | 2.6±0.2 | 126±16 | 114±9 |

All roots and ganglia (numbers 8 and 9) came from the same frog. Where indicated iso-OMPA was included in the cholinesterase incubation medium at 10⁻⁴ M.

* In sciatic nerve, n = 13 for choline acetylase and n = 3 for cholinesterase, where n is number.

the nerve were of motor origin, in reasonable agreement with Dunn (1909). However, using the choline acetylase data in the same manner, it would seem that 68% of the axons should be of motor origin. We have no explanation for this difference.

Total cholinesterase activity was surprisingly high in dorsal root ganglia, and nearly two-thirds of the activity was inhibited by 10⁻⁴ M iso-OMPA.
The activity remaining after inhibition, presumed to be primarily acetylcholinesterase, was still four times that in ventral roots. Ganglionic choline acetylase activity, on the other hand, was only 10% of that in ventral roots. Since there is evidence (Pick, 1957) suggesting the presence of sympathetic neurons in spinal ganglia, and preganglionic axons in dorsal roots, much more work is needed before each anatomical element can be assigned its proper quota of enzyme activity.

Movement of Enzymes

CHOLINESTERASE When frog sciatic nerve segments were incubated at 22°C for several hours, the distribution of certain enzyme activities within the nerve was altered in a regular way. The activity increased at both ends of the segment, more rapidly at the peripheral than at the central end, and decreased in the center. A typical sequence for cholinesterase is shown in Fig. 2. It is clear that, under these experimental conditions, enzyme accumulation at both ends of the nerve was confined to the 1.5 mm adjacent to the ligatures.

The data suggest a rapid phase of cholinesterase accumulation at the peripheral end of the nerve for the first 3 hr, followed by a plateau lasting from the 8th to the 22nd hr. During the next 2 days, the enzyme level at this end dropped to half of the plateau level (Fig. 3). Centrally, cholinesterase accumulation was much slower, and continued for 48–72 hr. The fall in enzyme concentration at the peripheral end between 22 and 72 hr is approximately matched quantitatively by the rise at the central end during the same interval. Therefore, by 72 hr most of the movable enzyme had accumulated at the central end of the nerve.

During the first day in vitro, the average cholinesterase level of the central portion of the nerve, excluding 1.5 mm on each end, fell 25% (Fig. 3 B). No additional depletion occurred, and this reduction was taken as the value
of $d$, the amount of enzyme in motion, in equation 2. No change of total enzyme activity occurred during the prolonged incubation period (not shown).

The redistribution of acetylcholinesterase was compared to that of total cholinesterase by assaying segments from two 16-hr nerves in the presence and absence of $10^{-4}$ M iso-OMPA (Fig. 4). No evidence was found for movement of pseudocholinesterase.

![Figure 3](image)

**Figure 3.** A) Accumulation of cholinesterase activities in the peripheral and central 1.5 mm of nerve, and (B) the decrease in activity in the mid-portion of the nerve, as a function of incubation time in 30 nerves (control data from the experiment shown in Table IV are included here). The accumulated enzyme activities at the nerve ends are calculated in millimeters ("mm worth" of enzyme) according to equation 1 (Methods). Standard errors are indicated for three or more nerves, otherwise each point represents one nerve. The data for the mid-portion are given as the fractional change from the average activity per millimeter for the whole nerve.

**HEXOKINASE AND GLUTAMIC DEHYDROGENASE** Measurements of the movement of hexokinase and of glutamic dehydrogenase activities gave results similar to those for cholinesterase, but the accumulations were smaller and slower (Figs. 5 and 6). The accumulations of glutamate dehydrogenase at the ends of the nerve segment were somewhat greater than those of hexokinase, as was the decrease in activity in the central portion of the nerve. Therefore, the redistribution of glutamic dehydrogenase was studied in more detail than that of hexokinase.
FIGURE 4. Cholinesterase activity in two nerves incubated for 16 hr at 22°C and assayed in the absence and in the presence of $10^{-4}$ M iso-OMPA. The data are displayed as in Fig. 2.

FIGURE 5. Hexokinase (HK) activity in 13 nerves as a function of incubation time in vitro. In (A) the data for the central and peripheral ends, and for the mid-portion, are calculated as in Fig. 3. The data for the mid-pieces of the two nerves at 0, 2, 14, and 48 hr were nearly identical and are represented by one symbol at each time. In (B), average hexokinase activity in nerve is given as a function of incubation time in vitro.

FIGURE 6. Glutamic dehydrogenase (GDH) activity in 24 nerves as a function of incubation time in vitro (control data from the experiment shown in Table IV are included here). The data are represented as in Fig. 5. In (A), at 6, 48, and 96 hr the data for the mid-pieces of the two nerves are almost identical and are represented by one symbol at each time.
As in the case of cholinesterase, glutamic dehydrogenase activity in the peripheral end reached a plateau after a period of accumulation, but the plateau was reached after a longer time (22 hr) and was of shorter duration. Again the peripheral accumulation fell to \( \frac{1}{2} \) of the plateau level in the subsequent 2.5 days. At the central end a plateau was also reached at 22 hr but was only about \( \frac{1}{2} \) that at the distal end. Further central accumulation of the enzyme then occurred, matching quantitatively the decrease in enzyme activity at the other end.

The average activities for both glutamic dehydrogenase and hexokinase did not change during the prolonged incubation period (Figs. 5 B and 6 B). In both cases, the fractional depletion of the center was much less than that for cholinesterase (Figs. 5 A and 6 A). Notice that the central depletion of glutamic dehydrogenase was complete after 22 hr, as with cholinesterase. Whether the central depletion of hexokinase activity was complete by the end of the time studied cannot be said.

We have no explanation for the finding that in one nerve out of 20 no movement of glutamic dehydrogenase occurred (Fig. 6, 96 hr, symbols in parentheses). This is the only such occurrence so far, and an unsuspected analytical problem could be responsible for it. In this same nerve, movement of cholinesterase was not out of line with that found in the other two nerves at the same incubation time, and all three nerves were used to calculate the 96-hr points in Fig. 3.

It is apparent that cholinesterase moved more rapidly than hexokinase and glutamic dehydrogenase (Table II). Since the values of both the total movable enzyme \( (d) \) and the fraction moving in one direction \( (f) \) for hexokinase could only be guessed at, the calculated flow rates for this enzyme must not be taken too seriously. The way in which changes in \( f \) affect calculated flow rates may be seen for glutamic dehydrogenase (Table II).

**SOLUBLE ENZYMES** The distribution of the activity of 6-phosphogluconic dehydrogenase, an enzyme thought in other tissues to be soluble, did not change in the period studied (Table III).

Whether choline acetylase is soluble or particulate has been a matter of some controversy, and no direct studies appear to have been made in frog. In any event, the distribution of this enzyme, too, was unchanged during four days of incubation in vitro (not shown). In vivo experiments showed, however, that the enzyme does move in frog nerve, albeit slowly. Single ties, or double ties isolating a nerve segment of variable length, were placed on the sciatic nerves of five frogs, and the animals were kept for 12 days. In these nerves the choline acetylase activity was increased above the proximal tie, and decreased below the distal tie (Fig. 7). Assuming that all of the choline acetylase was contained within the axons and was available to move \( (d = 1) \) and that it was all going peripherally \( (f \) for peripheral flow equals 1,
TABLE II

RATES OF FLOW OF THREE PARTICULATE ENZYMES
IN FROG SCIATIC NERVE
IN VITRO

| Enzyme               | d   | Peripheral | Central | Flow rate
|----------------------|-----|------------|---------|------------|
|                      | mm  |            |         | mm per day |
| Cholinesterase       | 0.25| 0.5        | 0.5     | 99±9 (8)   |
| Hexokinase           | 0.12| 0.5        | 0.5     | 20±2 (6)   |
| Glutamic dehydrogenase* | 0.09| 0.65       | 0.35    | 28±3 (6)   |

The results were calculated from the data in Figs. 3, 6, and 7 using the points falling on the initial (linear) portions of the curves, the equations given under Methods, and the values for d (fraction of enzyme in motion) and f (fraction of moving enzyme in motion toward peripheral or central end) listed. The numbers of nerves used for each curve are given in parentheses.  
* Two conditions of f are assumed.

TABLE III

DISTRIBUTION OF 6-PHOSPHOGLOUCONIC DEHYDROGENASE ACTIVITY IN FROG SCIATIC NERVE AFTER 6-96 HR INCUBATION AT 22° C

| Time (hr) | Mean activity | Peripheral | Central |
|-----------|---------------|------------|---------|
|           | moles/kg per hr | mm        | mm      |
| 6         | 0.266         | -0.16      | -0.10   |
| 14        | 0.245         | -0.36      | -0.28   |
| 72        | 0.212         | -0.09      | -0.21   |
| 96        | 0.241         | -0.21      | -0.15   |

One nerve is represented at each time.

f for central flow equals 0), the rate of peripheral movement of choline acetylase was only 0.34 ± 0.01 mm/day. The enzyme activity between ties was quite low: 24, 60, and 10% of control on a weight basis (slightly higher on the basis of length) in segments 19, 16, and 4 mm long, respectively. There was no evidence of flow of choline acetylase within these segments (not shown). A few nerves were studied after shorter intervals (not shown), but the changes observed were quite small and in keeping with the rates calculated from the 12-day nerves.

Changes in Incubation Conditions

The rate of movement of both cholinesterase and glutamic dehydrogenase in both directions could be dramatically altered by preincubating the nerves in vinblastine (Table IV). Inhibition of movement of glutamic dehydrogenase
Figure 7. Choline acetylase (ChAc) activity in five nerves after ties had been in place for 12 days in vivo. As in Fig. 2, the width of each bar is proportional to the length of the segment analyzed. In (A), enzyme activity is expressed on the basis of dry weight (100% = 2.3 ± 0.5 mmoles/kg per hr), in (B) on the basis of length (100% = 0.24 ± 0.05 mmoles/km per hr). For three of the nerves, the data were normalized to the level of enzyme activity found in the unoperated nerve of the opposite side, and for the other two nerves to the level in their two most central segments. Three nerves had segments of 4–19 mm isolated between two ties. For this figure, data for samples taken above and below a single tie are combined with data for samples taken above the proximal tie and below the distal tie for the nerves with isolated segments. The SEM is indicated.

Table IV

|                      | Rate of transport |          |          |
|----------------------|-------------------|----------|----------|
|                      | Peripheral        | Central  |          |
|                      | mm per day        |          |          |
| Cholinesterase       |                   |          |          |
| Controls             | 99±3 (8)          | 19±1 (9) |          |
| +1 max vinblastine   | 16±3 (6)          | 8±2 (6)  |          |
| Inhibition           | 84%               | 57%      |          |
| Glutamic dehydrogenase|                  |          |          |
| Controls             | 28±3 (6)          | 20±1 (9) |          |
| +1 max vinblastine   | 2.6±0.7 (6)       | -6±4 (6) |          |
| Inhibition           | 91%               | 100%     |          |

Rates for cholinesterase in the presence of vinblastine were calculated using 3- and 8-hr nerves for the peripheral end and 8- and 16-hr nerves for the central end. Rates for glutamic dehydrogenase in the presence of vinblastine were calculated using 16- and 32-hr nerves. Control rates were taken from Table II, which includes data from control nerves incubated at the same time as the vinblastine-treated nerves.
was more complete than that of cholinesterase (Table IV). Preliminary experiments suggested that vincristine also blocked flow, but that the inhibition was less complete.

No influence upon the rate of peripheral accumulation of cholinesterase activity was seen in solutions containing only isotonic KCl, isotonic NaCl, or Ringer solution with 10 mM ouabain, 0.1 mM thalidomide, or 0.1 mM phenobarbital (Table V). Inhibition of accumulation at the central end could not have been observed in the 3 hr incubation period. However, no evidence of acceleration of central accumulation was seen.

The weight-averaged cholinesterase activity of frog nerve was inhibited about 30% by 10 mM ouabain, in good agreement with the findings of Logua and Kometiani (1970) using rat brain (Table VI). All other incubation conditions left the cholinesterase activity unchanged.

**TABLE V**

ACCUMULATION OF CHOLINESTERASE (C) IN THE PERIPHERAL AND CENTRAL ENDS OF FROG SCIATIC NERVE AFTER INCUBATION UNDER VARIOUS CONDITIONS

| Medium                           | n  | Distal mm | Proximal mm |
|----------------------------------|----|-----------|-------------|
| Frog Ringer                      | 8  | 1.7±0.2   | 0.3±0.2     |
| 110 mM KCl                       | 4  | 1.8±0.2   | 0.2±0.1     |
| 0 K⁺ (110 mM NaCl)               | 4  | 1.6±0.2   | 0.1±0.1     |
| Frog Ringer Plus 10 mM ouabain   | 4  | 1.5±0.2   | 0.07±0.06   |
| Plus 0.1 mM thalidomide          | 4  | 1.9±0.1   | 0.2±0.2     |
| Plus 0.1 mM phenobarbital        | 4  | 2.0±0.1   | -0.1±0.1    |

Nerves were first incubated for 1 hr at 2°-4°C and then for 3 hr at 22°C under the conditions indicated. Media with 0 K⁺ and 110 mM KCl were buffered with 10 mM tris(hydroxymethyl)-aminomethane (Tris) HCl, pH 7.55. All media contained 4 mM glucose.

**TABLE VI**

THE EFFECT OF VARIOUS INCUBATION CONDITIONS ON THE AVERAGE CHOLINESTERASE ACTIVITY IN FROG SCIATIC NERVE

| mmol/hr per kg | n  | Incubation time hr |
|----------------|----|--------------------|
| Normal         | 131±3 | 31                 |
| 1 mM vinblastine | 119±5 | 9                  |
| 1 mM vincristine | 125±16 | 6                  |
| 10 mM ouabain  | 81±7* | 4                  |
| 0 mM K⁺        | 128±2 | 4                  |
| 110 mM K⁺      | 139±5 | 4                  |
| 0.1 mM thalidomide | 123±12 | 4                  |
| 0.1 mM phenobarbital | 126±11 | 4                  |

Incubation conditions are given in Methods and in the legend of Table V.

* P ≤ 0.02 for a comparison between this value and any other in the table.
DISCUSSION

The present experiments clearly showed the presence of at least two components of axoplasmic transport in frog nerve in vitro. Cholinesterase moved as fast as the fastest components found in goldfish optic nerve in vivo by Elam and Agranoff (1971) and in frog sciatic nerve in vitro by Edström and Mattsson (1972). Mitochondria moved more slowly, but still nearly 100 times faster than slow flow, as demonstrated by choline acetylase (see above). This is in contrast to the hypothesis of Ochs (1972) who suggests that all rapid transport in mammals occurs at the same rate.

Furthermore, the flow of the membrane-bound cholinesterase and that of the mitochondrial enzymes is bidirectional, and in both cases central flow appears to be slower than peripheral flow. Many workers have demonstrated the existence of bidirectional flow (e.g. Dahlstrom, 1967; Lasek, 1967; and Zelená, 1968), and Kristensson and his colleagues have addressed themselves specifically to the problem of retrograde (centrally directed) flow (e.g. Kristensson et al., 1971).

The late drop in enzyme activity at the distal end, with a concomitant and equal rise at the proximal end of the nerve, occurring both with cholinesterase and with glutamic dehydrogenase, is a puzzling occurrence. A similar result has been obtained by Bray et al. (1971) using radioactive labeling in chicken sciatic nerve in vivo. We are tempted to speculate that these events are related to the reformation of the axonal tip preparatory to regeneration. In a normal neuron, a mechanism for reversing flow must be present to provide for the orderly return to the perikaryon of unused axonal constituents which arrive by fast flow at the axonal tip. Such a turnaround mechanism may also exist in the growing tip of the axon during morphogenesis. A turnaround seems unlikely to be present in mid-axon normally but must be formed at the cut end of the nerve during the early stages of regeneration. Without the capacity to redirect the movement of axoplasmic constituents, mitochondria and other organelles moving by fast flow would be expected to accumulate in the regenerating tip almost without limit, since the interval between nerve section and the reestablishment of functional peripheral connections by the regenerated axon may last many weeks, and since the rate of supply of materials by rapid transport seems to exceed the rate of utilization.

Several workers in this field have been hesitant to accept the existence of central flow as a feature of normal nerve (Weiss, 1970; Davison, 1970). Without going into the reasons for this reluctance, it seems fair to point out that the rate of rapid peripheral axonal transport appears to be quite resistant to environmental influences (Table V), with the exception of such things as vinblastine and cold sufficient to disrupt microtubules (Rodriguez Echandia...
and Piezzi, 1968). If the cell, too, had difficulty in modulating the transport rate, a satisfactory solution to the difficulty may have been to provide for a peripherally directed transport rate more than adequate to supply any physiological exigency, with a return of unused materials to the perikaryon. Back in the cell body, the concentrations of these materials might be expected to govern their rate of synthesis.

Lubinska and Niemierko (1971) found a distal flow rate for acetylcholinesterase in dog nerve of 260 mm/day, and a proximal rate of 134 mm/day, considerably higher than the rates we calculate for the same enzyme in frog, but the difference may be accountable in terms of the difference in temperatures for the two species. Lubinska and Niemierko (1971) calculate that $\frac{3}{4}$ of the enzyme in motion is going distally and $\frac{1}{4}$ proximally. Combined with the difference in rates, these calculations would mean that about four times as much enzyme is arriving at the terminals as is arriving at the perikaryon per unit time in dog. The situation in frog is almost the same, since the amounts of enzyme going proximally and distally are the same, but the distal rate is five times the proximal (Table II). The rate of mitochondrial flow in the hypogastric nerve of the cat (Banks et al., 1969) cannot be calculated because data enabling one to estimate $d$ are not given. If $d$ is assumed to be between 0.5 and 0.1, the rate of flow of mitochondria in this unmyelinated mammalian nerve would be between 90 and 450 mm/day.

Niemierko and Lubinska (1967) have shown that a relatively large fraction of the cholinesterase in dog nerve is apparently fixed, unavailable for movement along the axon. They suggest that the immovable fraction may be in the axolemma. The amount of fixed cholinesterase in frog nerve is similar. The amounts of mitochondrial enzymes which do not move in frog nerve are even greater. This is not surprising since mitochondria associated with Schwann cells would not be capable of translocation over long distances and the number of mitochondria in the paranodal apparatus of the Schwann cell is relatively very large (Williams and Landon, 1963). Furthermore, Zelená (1968) found the axoplasm in the center of isolated rat nerve segments to be cleared almost completely of mitochondria by axoplasmic flow. If this is true of frog nerve also, about 90% of the activity of these enzymes is attributable to Schwann cell mitochondria.

The blockade of axonal transport by vinblastine was quite complete (Table IV). While movement of mitochondria was inhibited more effectively than was that of cholinesterase, the incubation periods needed to demonstrate mitochondrial movement were longer. Therefore the difference in inhibition may merely reflect differences in vinblastine penetration. Edström and Mattsson (1972) have shown almost complete blockade of transport by vinblastine in another in vitro frog sciatic nerve preparation. Neither phenobarbital nor thalidomide (the latter in nearly saturated solution) had any measurable effect on the transport of cholinesterase (Table V).
So far we have been unable to demonstrate slow axoplasmic flow in vitro. Since the movement of choline acetylase in frog nerve in vivo was only 1% as fast as that of glutamic dehydrogenase, measurable changes would be difficult to detect, even after 4 days of incubation. The rate of movement of choline acetylase was almost the same as the rate of slow flow observed by Graffstein (1967) in goldfish optic nerve. The transport rate for mammalian choline acetylase which we calculate from the data of Ekström and Emmelin (1971) and Hebb and Silver (1961) was the same as that of slow flow in mammals (Weiss and Hiscoe, 1948) but 10 times the rate we observed in frog. (It seems unlikely that all of this difference between mammals and frog is attributable to the difference in temperatures.) Frizell et al. (1970), working with rabbit, obtained transport rates for choline acetylase which were about the same as those for cholinesterase. Their rates for choline acetylase are considerably faster, and for cholinesterase much slower, than the rates obtained by others working with mammals.

The lack of effects of altering the ionic composition of the incubation medium upon the rate of transport suggests two conclusions. In the first place, since in this preparation high K$^+$ depolarizes the nerve within an hour (Okada and McDougal, 1971; Osborn and McDougal, unpublished), it seems unlikely that the movement of intraaxonal constituents is the result of electrophoretic effects caused by injury currents (Weiss, 1970). Secondly, since both ouabain and 0 K$^+$ rapidly block the ion pumps in this preparation (McDougal, Partlow, and Osborn, in preparation), it may be concluded that rising Na$^+$ and falling K$^+$ concentrations within the axon do not change the rate of axonal flow. The results of Jankowska et al. (1969), showing the lack of effect of prolonged stimulation on the rate of flow of cholinesterase in dog nerve in vitro, are in keeping with this conclusion.

Difficulties do arise in the interpretation of studies of axonal transport using isolated nerve segments. For example, the nerves are surely damaged by isolation, and injury currents must flow for a time (see above and Table VI). Also the blood supply is interrupted, but phosphocreatine and lactate levels give no evidence of anoxia in such nerves in short-term experiments (unpublished). For experiments lasting days, care has been taken to provide glucose far in excess of calculated needs, but biochemical monitoring of the adequacy of the provisions has not yet been done.

Degeneration must, of course, occur in these nerve segments, as in all decentralized axons. However, Wallerian degeneration in frog nerve is much slower than in mammalian nerve (Honjin, et al., 1959). The first anatomical changes in frog do not appear until 36 hr after nerve section; axonal mitochondria swell in 2 or 3 days and become fragmented in 4. Furthermore, changes associated with decentralization of the axons seem more likely to reduce the rates of axonal transport than to enhance them.
Thus it seems significant that the fastest transport rate in isolated frog nerve is comparable to the rates found using tracer techniques in frog (in vitro; Edström and Mattsson, 1972) and goldfish (in vivo; Elam and Agranoff, 1971) with axons still attached to their cells of origin. In cat, Ochs and Ranish (1969) found little or no difference between the rates of transport in intact and decentralized axons.

A possible consequence of degenerative change is Schwann cell multiplication or "activation" and, in vivo, the infiltration of inflammatory cells. If the enzyme under study is not confined to the axon, and most enzymes in nerve are not, any one of these processes, occurring primarily in the region of the tie, would simulate transport. Several sorts of controls permit the experimenter to distinguish between injury-stimulated enzyme increase and axonal transport. One type of control is illustrated in Figs. 5 B and 6 B, and suggests no difficulty in these experiments on this score.

There are at least three advantages of the use of individual enzymes in the study of axoplasmic transport: (a) substances moving at different rates can be observed without confusion, even when the rates are such that the various substances are in measurable motion at the same time; (b) inhibitors or activators of flow having differential effects on different substances could be easily detected; and (c) central transport is as easily studied as peripheral movement, at least in segments of nerve devoid of major branches. When isotopically labeled compounds are used to study transport, many components with different rates of movement are marked, and substances with different velocities can be sorted out only with difficulty, if at all. Furthermore, probably because labeled substances moving with different velocities in both directions overlap, central flow is also difficult to study using isotopes, as the results of Edström and Mattson (1972) show. In chicken, a clear-cut demonstration of central flow has been achieved by Bray et al. (1971), however.

Finally, the use of in vitro preparations in the study of axoplasmic flow offers many obvious advantages. Among these is the possibility of studying incubation conditions which are incompatible with even short-term survival of the animal, such as some of those used in the experiments shown in Table V.

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