64. Simultaneous Transport of Phosphatidylcholine and Proteins in the Frog Sciatic Nerve

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Previous studies on axonal transport in vertebrate species have shown the rapid flow of proteins associated with particulate fraction. In the present communication, evidence is presented for simultaneous transport of phosphatidylcholine and proteins, suggesting a flow associated with a membrane component.

Materials and methods. Bullfrogs, Rana catesbeiana, were kept at constant temperatures for at least 2 weeks before use. Under urethane anaesthesia, [4,5-3H] L-leucine (20–50 Ci/m mole), 4 μCi dissolved in 0.2 μl of frog Ringer solution, or [methyl-3H] choline chloride (15 Ci/m mole), 3 μCi in 0.2 μl, was injected into the 9th dorsal root ganglion through a capillary. In some animals indicated, a mixture of [U-14C] L-leucine (342 mCi/mmole; 0.8 μCi) and [3H] choline (3 μCi) in 0.4 μl of frog Ringer solution was similarly injected. Animals were kept at room temperature for 3 hours after the injection in order to ensure a sufficient incorporation of respective isotopes, and then returned to prescribed temperatures. At intervals specified, respective frogs were sacrificed by overdosage of urethane, the sciatic nerve together with the 9th ganglion removed, sectioned in 5 mm segments, each segment placed in a counting vial and solubilized in 0.5 ml of Soluene-100 (Packard), incubating the vial at 50°C for 2 hours with shaking. After cooling in the dark for hours following the addition of 10 ml of toluene-based scintillation fluid, radioactivities were counted in a Packard Model 3380 liquid scintillation spectrometer.

Subcellular fractionation of the sciatic nerve was carried out at 0–4°C as given below. Nerve portion of 45 mm long, 40 mm apart from the ganglion at its proximal end, was removed from the frog which had received simultaneous injection of [3H] choline and [14C] L-leucine and been kept at 25°C for 15 hours. Equivalent segments were collected from 5 animals, and ground in 9 volumes of 0.32 M sucrose using a glass homogenizer. The homogenate was centrifuged at 900 g for 10 minutes, at 10,000 g for 30 minutes and at 100,000 g for 90 minutes, with one wash each time, which respectively yielded
crude nuclear, mitochondrial, microsomal and supernatant fractions. The microsomal fraction was suspended in 1.5 ml of 0.32 M sucrose, gently homogenized, and a 1.2 ml portion layered on the top of a density gradient in 18 ml tube, consisted of continuous gradient ranging from 0.32 M to 0.9 M sucrose, with a cushion of 1 ml of 2.0 M sucrose at the bottom. This was centrifuged at 78,000 g for 11 hours in a Hitachi 65P preparative ultracentrifuge, using a RPS 25-3A rotor. Fractions of 65 drops were collected through a syringe pierced the bottom of the tube, precipitated in 5 percent trichloroacetic acid, together with 1 mg of bovine serum albumin as carrier, the sediment solubilized in Soluene-100 and radioactivities counted as described above.

For enzyme assays, nerves were collected from animals received no isotopes, and 10-fold as much the amount of tissue subjected to subcellular fractionation. Acetylcholinesterase activity was determined according to Ellman et al., NaK-ATPase according to Kurokawa et al. and 2',3'-cyclic nucleotide 3'-phosphohydrolase according to Kurihara et al.

**Fig. 1.** Typical flow patterns of labelled proteins in the frog sciatic nerve at 25°C. (a) Nine hours after injection of [3H] L-leucine (4 pCi) into the 9th dorsal root ganglion; (b) 18 hours after the injection. Value at 0 expresses the total CPM in the ganglion.

**Fig. 2.** Temperature dependence of the fast axonal transport of proteins. Note that the ordinate is given in logarithmic scale.
Results and discussion. More than 90 percent of $[^3]$H$[^3]$leucine was incorporated into the protein fraction within 4 hours at 25°C, and moved along the nerve. Autoradiographic examination detected no grains present outside the axon in nerve portions more than 20 mm distal to the ganglion. The flow was clearly demonstrated by a downward shift of a crest (Fig. 1), in accordance with observations made by Ochs and Ranish$^9$ in the cat sciatic nerve. Distance between the tip of the crest and the ganglion estimated at 6 different time intervals after injection was found proportional to time, which gave a flow rate of 152 mm/day at 25°C. The rate was temperature-dependent, $Q_{10}$ value being estimated to be 2.6 irrespective of temperatures examined (Fig. 2). An equivalent $Q_{10}$ value has recently been reported in the

Fig. 3. Typical flow patterns of radioactivities in the frog sciatic nerve after simultaneous injection of $[^3]$H choline (3 $\mu$Ci) and $[^4]$C L-leucine (0.8 $\mu$Ci) into the ganglion.

The frog was kept at 25°C for 15 hours after the injection.

Fig. 4. Effect of topical application of colchicine on the fast transport of labelled proteins.
(a) Colchicine-injected; (b) control (solvent-injected). Colchicine (0.56 M) dissolved in a mixture of 0.1 M Tris-HCl (pH 7.5): ethanol=4:1 (v/v), or the solvent only was injected under perineurium through a capillary at the site 75 mm apart from the ganglion 2 hours after the injection of $[^3]$H L-leucine into the ganglion. Frogs were kept at 25°C for further 36 hours, and then sacrificed.
goldfish optic nerve despite a difference in flow rate. Radioactivities constituting the crest were about 5 percent of the total incorporated radioactivity.

[^3H]Choline injected into the ganglion was incorporated into acid-precipitable fraction at a much slower rate than[^3H]leucine. Thus in the ganglion, radioactivity incorporated at 25°C comprised 60 and 70 percent of the total radioactivity 4 and 12 hours after injection, respectively. In nerve portions more than 20 mm distal to the ganglion, more than 90 percent of the radioactivity was recovered in acid-precipitable fraction. Phospholipids were extracted from the ganglion as well as from the nerve according to the method of Folch et al., evaporated to dryness under reduced pressure, dissolved in a small volume of chloroform, and subjected to thin layer chromatography, using silica gel plate and a solvent system of chloroform : methanol : acetic acid : water = 25 : 15 : 4 : 2 (v/v). Approximately 85 percent and 75 percent of the total acid-precipitable radioactivity were identified as phosphatidylcholine in the ganglion and nerve, respectively. Phosphatidylcholine moved along the nerve at a flow rate equivalent to that of proteins. On simultaneous injection of[^3H]choline and[^14C] leucine, both radioactive labels moved at the same rate (Fig. 3), although the flow of phosphatidylcholine was not attended by a crest formation. Flow of phosphatidylcholine proved to be temperature-dependent, with a $Q_{10}$ value equivalent to that in the protein flow. Both flows were completely blocked by colchicine introduced directly

![Fig. 5. Effect of colchicine on flows of phosphatidylcholine and of proteins. Values are expressed as percentage of the total CPM found throughout the dorsal root, ganglion and nerve. Animals which had received simultaneous injection of[^3H] choline (3μCi) and[^14C] L-leucine (0.8μCi), were kept at 25°C for various periods depicted in respective figures, and then sacrificed. Colchicine was injected at sites indicated by arrows, 2 hours after isotopes. --○--:[^3H] labels, −●−:[^14C] labels.](image-url)
into the nerve (Figs. 4 and 5). At proximal site adjacent to colchicine injection, accumulation of [\(^3\)H]-label was gradually increased during the period between 26 and 50 hours after [\(^3\)H]choline, while [\(^{14}\)C]-label, which amounted to approximately 3 percent of the total [\(^{14}\)C]-radioactivity, remained virtually unchanged (Fig. 5). The difference may well be explained by assuming that the pool size of rapidly transportable protein fraction is smaller than that of phosphatidylcholine. The finding in Fig. 5 also suggests the virtual absence of significant protein transport with intermediate flow rates between 152 mm/day and about 40 mm/day.

On subcellular fractionation, more than 80 percent of both [\(^{14}\)C] and [\(^3\)H]-labels were recovered in the particulate fraction, in accordance with results reported.\(^8\),\(^10\),\(^13\) Specific radioactivities of both [\(^3\)H]-phosphatidylcholine and [\(^{14}\)C]-proteins on a protein basis were found highest in microsomal fraction (Table I). In mitochondrial fraction, specific activities were lower than in microsomal fraction, in line with the finding that mitochondrial enzymes moved at a slower rate in the frog sciatic nerve in vitro.\(^11\)

### Table I. Subcellular distribution of radioactivities in the frog sciatic nerve after simultaneous injection of [\(^3\)H] choline and [\(^{14}\)C] L-leucine into the 9th dorsal root ganglion

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Protein (mg)</th>
<th>Total CPM</th>
<th>Specific activity (CPM/mg of protein)</th>
<th>Ratio [(^3)H]/[(^{14})C]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear</td>
<td>11.5</td>
<td>2284</td>
<td>1096</td>
<td>199</td>
</tr>
<tr>
<td>Mitochondrial</td>
<td>11.2</td>
<td>4314</td>
<td>1996</td>
<td>385</td>
</tr>
<tr>
<td>Microsomal</td>
<td>3.14</td>
<td>5400</td>
<td>2448</td>
<td>1720</td>
</tr>
<tr>
<td>Supernatant</td>
<td>4.97</td>
<td>2769</td>
<td>1324</td>
<td>557</td>
</tr>
</tbody>
</table>

Total amount of 440 mg of nerves, collected from 5 animals similarly treated, was homogenized and fractionated as described in the text. Protein was determined by the method of Lowry et al. (6), with bovine serum albumin as standard.

On continuous sucrose gradient centrifugation of the microsomal fraction, more than 50 percent of the protein was found located in a region of \(\rho\) around 1.08, and the remaining portion distributed in regions with \(\rho\) values ranging from 1.10 to 1.34. Specific radioactivities on a protein basis of both [\(^3\)H] and [\(^{14}\)C]-labels were exceedingly higher in high-density regions. Also, these regions were provided with very high activities of acetylcholinesterase and NaK-ATPase, two typical instances of the membrane enzyme. In contrast, a low activity of \(2',3'\)-cyclic nucleotide \(3'\)-phosphohydrolase, provisionally taken as a myelin marker, was found rather evenly distributed throughout the
range of the sucrose density gradient.

The present experiments demonstrate the presence of axoplasmic transport of phosphatidylcholine which occurs simultaneously with protein flow, and possibly associated with a membrane component. Rapid transport of a portion of acetylcholinesterase in the axon has been well documented by ligation experiments in various species of animals.\(^7,11,12\) Acetylcholinesterase activity found coincident with high specific radioactivities in microsomal subfraction may represent such a transportable portion of the enzyme.

In addition to the fast flow above described, a slow transport of proteins at a rate of 0.5–1 mm/day was also detected in the frog sciatic nerve.

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References