Biochem. J. (1973) **136**, 731–740
Printed in Great Britain

Rapid Transport of Phosphatidylcholine Occurring Simultaneously with Protein Transport in the Frog Sciatic Nerve

By TERUO ABE, TATSUYA HAGA and MASANORI KUROKAWA
Department of Biochemistry, Institute of Brain Research, Tokyo University Faculty of Medicine, Tokyo, Japan

(*Received* 6 *June* 1973)

1. Either L-[4,5-^{3}H]leucine or [*Me*-^{3}H]choline, or both L-[U-^{14}C]leucine and [*Me*-^{3}H]-choline, were injected into the ninth dorsal root ganglion of the frog, and peripheral transport of labelled proteins and/or phospholipids, mostly phosphatidylcholine, was studied by analysis of consecutive segments of the sciatic nerve. 2. At 25°C, approx. 5% of the ^{3}H-labelled protein was transported at the rate of 152mm/day. The rate was temperature-dependent with the Q_{10} value of 2.6. The flow was completely blocked by the local application of colchicine, but was unaffected by cytochalasin D. 3. [*Me*-^{3}H]-Choline was incorporated into phosphatidylcholine at a comparatively slow rate, but was transported in the nerve at a rate equivalent to that for ^{3}H-labelled proteins. 4. The simultaneous transport of phosphatidylcholine and the protein was further supported in the double-labelling experiments by an identical transport rate of ^{3}H-labelled phosphatidylcholine and ^{14}C-labelled proteins, by their identical temperature dependence, by simultaneous blockade with colchicine, and also by the parallel distribution of the two labels in subcellular fractions. Specific radioactivities on a protein basis of both ^{3}H and ^{14}C labels were highest in microsomal subfractions enriched with Na^+-plus-K^+-stimulated adenosine triphosphatase and acetylcholinesterase. It is suggested that ^{3}H-labelled phosphatidylcholine and ^{14}C-labelled proteins transported in the nerve reside in the same structural entity, most probably a membrane component.

Early work by Miani (1963) demonstrated the occurrence of axonal transport of phospholipids in rabbit nerves. The rate of transport, 72 and 39–41 mm/day in the vagus and hypoglossal nerves respectively, was rapid enough to discriminate the phospholipid flow from the proximodistal motion of axonal materials occurring at the rate of 1–2mm/day (Weiss & Hiscoe, 1948). In recent years, however, the presence of protein transport at much higher rates has been shown in a variety of nerves (for reviews, see Grafstein, 1969; Dahlström, 1971; Ochs, 1972*a*). Sjöstrand (1969) has demonstrated that in the rabbit vagus and hypoglossal nerves labelled proteins migrate at rates of 380–410 and 240–360mm/day respectively. Evidence has also accumulated that a large proportion of rapidly transported proteins is associated with the small-particulate fraction (McEwen & Grafstein, 1968; Ochs *et al.*, 1969; Sjöstrand & Karlsson, 1969; Elam & Agranoff, 1971).

Although the structural counterpart in axons of the small particulate fraction and also molecular species of transported proteins are largely unknown at present, it seems unusual that phospholipid, a major membrane constituent, moves down in the axon at a rate entirely different from that of proteins. This indicated the need for comparative studies on the rate of phospholipid and protein transport in the same axon. In the present study, attention is focused on phosphatidylcholine, in view of its predominant occurrence in the small-particulate fraction of the brain (Eichberg *et al.*, 1964). Our results show that phosphatidylcholine rapidly migrates in the axon* simultaneously with proteins. A preliminary account of this work has been published (Abe *et al.*, 1973).

Materials and Methods

Chemicals

The following chemicals were obtained from the sources indicated: L-[4,5-^{3}H]leucine (20–50Ci/mmol), [*N*-*Me*-^{3}H]choline chloride (15Ci/mmol) and L-[U-^{14}C]leucine (342mCi/mmol) were from The Radiochemical Centre, Amersham, Bucks., U.K. Colchicine was from E. Merck A.-G., Darmstadt, Germany. Adenosine 2′:3′-cyclic monophosphate, Tris–ATP and bovine serum albumin (crystallized and freeze-dried) were from Sigma Chemical Co., St. Louis, Mo.,

* In the present paper, the term 'axon' is used for the peripheral portion of sensory fibre contained in the sciatic nerve, in view of the proposal made by Bodian (1962).

U.S.A. Di-isopropylphosphorofluoridate was from British Drug Houses Ltd., Poole, Dorset, U.K. Soluene-100 and [^{3}H]toluene (2.48×10^6 d.p.m./g) were from Packard Instrument Co., Downers Grove, Ill., U.S.A. Cytochalasin D (Zygosporin A) was a gift from Shionogi Co., Osaka, Japan. Phosphatidylcholine (from egg yolk) and sphingomyelin (from human erythrocytes) were preparations by Dr. S. Handa, Department of Biochemistry of this Faculty.

Treatment of animals

Bullfrogs (*Rana catesbeiana*) weighing 200–400g were fed in the dark at respective constant temperatures for at least 2 weeks before use. Under urethane anaesthesia, the ninth dorsal root ganglion was exposed under a binocular microscope and radioisotopes were injected into it through a capillary with a tip width of 15μm. Commercial preparations of L-[4,5-^{3}H]leucine, [*Me*-^{3}H]choline and L-[U-^{14}C]-leucine were freeze-dried and dissolved in appropriate volumes of frog Ringer solution so as to give 20, 15 and 4 μCi/μl solutions respectively. Frog Ringer solution contained, in final concns.: NaCl, 110mM;KCl, 1.9mM; $CaCl_2$, 1.1mM; $NaHCO_3$, 2.4mM; NaH_2PO_4, 0.083mM; glucose, 11mM. Portions (0.2μl) of radioisotope solutions were stored in oil-wells filled with liquid paraffin (cf. Kato & Lowry, 1973). For use, one drop of radioisotope solution was sucked into the tip of a capillary, which was filled with liquid paraffin and connected to polyfluoroethylene tubing. The tip was inserted in the ganglion by the aid of a micro-manipulator, and radioisotope solution (0.2μl) was carefully injected during the period of 2min by pressing a syringe connected to the tubing. Quantitative injection of the radioisotope solution was ensured by watching the movement of the boundary between liquid paraffin and frog Ringer solution. In some animals as indicated, a mixture of L-[U-^{14}C]leucine (0.8μCi) and [*Me*-^{3}H]choline (3μCi) in 0.4μl of the Ringer solution was similarly injected. After suture, the animal was kept at 25°C for 3h to ensure sufficient incorporation of the radioisotopes, and then at a prescribed constant temperature in the dark in a specially designed box. In experiments in which temperature-dependence of the axonal transport was examined, at least four frogs were used for each temperature. Fall in rectal temperature from 25°C to 2–3°C was attained within 2h. Tetracycline was given orally to animals when appropriate.

In some indicated experiments, 5μl of 0.56M-colchicine dissolved in 0.1M-Tris–HCl (pH7.5)–ethanol (4:1, v/v), or of 10mM-cytochalasin D in dimethyl sulphoxide, or of 100mM-EGTA [ethanedioxybis(ethylamine)tetra-acetate], adjusted to pH7.4 with 1M-Tris, was injected at 25°C 2h after the injection of radioisotope, into the nerve site 70–80mm distal to the ganglion by the use of a capillary, 80μm at the tip. In controls solvents only were injected.

Treatment of the nerve

Analysis of the consecutive nerve segments. The sciatic nerve, together with the ninth dorsal root ganglion and the dorsal root, was removed bilaterally, washed in frog Ringer solution and sectioned in 5mm segments. Nerve contralateral to that injected with radioisotope served as control. Each of the segments was placed in a vial and solubilized in 0.5ml of Soluene-100 by shaking the vial at 50°C for 2h. After cooling, 10ml of scintillation fluid containing, in 1 litre of toluene, 4g of 2,5-diphenyloxazole and 0.25g of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene, was added, and vials were kept in a cooled dark place for several hours before measurement of radioactivity in a Packard model 3380 liquid-scintillation spectrometer. The quenching showed negligible variation, obviating the need for correction. In double-label experiments, the spillover of ^{14}C in the ^{3}H channel was approx. 15%, which was appropriately corrected.

Incorporation of radioactivity into the acid-precipitable fraction. At prescribed time-intervals after injection of radioisotope the ganglion and 5mm segments of the nerve near the ganglion were removed. The ganglion and each nerve segment were homogenized in 2ml and 4ml of ice-cold 5% (w/v) trichloroacetic acid respectively, centrifuged, and the sediment was washed two more times, each with 1ml of 5% (w/v) trichloroacetic acid. For incorporation of L-[4,5-^{3}H]leucine, but not of [*Me*-^{3}H]choline, the sediment was heated at 80°C in 1ml of 5% (w/v) trichloroacetic acid for 20min, centrifuged, and extracted three times each with 2ml of diethyl ether–ethanol (4:1, v/v) at room temperature. The final sediment was solubilized and radioactivity measured as described above. Radioactivity in the combined trichloroacetic acid supernatant was measured in a 1.0ml sample with 10ml of scintillation fluid containing, in 1 litre of toluene–Triton X-100 (2:1, v/v), 5.5g of 2,5-diphenyloxazole and 0.1g of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene. Appropriate correction for quenching was made with [^{3}H]toluene as an internal standard.

Phospholipid analysis. At 15h at 25°C after the injection of [*Me*-^{3}H]choline, the ganglion and the 35mm-long nerve segment, 40mm away from the ganglion at its proximal end, were removed. These were homogenized and washed in 5% (w/v) trichloroacetic acid as described above. Phospholipids were extracted from the acid-insoluble precipitate essentially as described by Folch *et al.* (1957). The precipitate was homogenized at 0–4°C in 3ml of chloroform–methanol (2:1, v/v). The insoluble material was collected by centrifugation and washed

twice each with 1ml of chloroform–methanol (2:1, v/v). The combined extracts were shaken with 0.2vol. of 0.9% NaCl solution. The two phases were separated by centrifugation and the upper phase was removed. The lower phase was washed three times with fresh upper-phase solvent containing chloroform–methanol–aq. 0.9% NaCl (3:48:47, by vol.). The final lower phase was evaporated to dryness under reduced pressure, dissolved in 1ml of chloroform–methanol (2:1, v/v), and any insoluble material was removed by passage of the extract through a Pasteur pipette plugged with glass wool. This was again dried and dissolved in a small volume of chloroform. Samples were subjected to t.l.c. on silica gel plates (E. Merck A.-G.; 20cm×20cm; layer thickness 0.25mm), developed by chloroform–methanol–acetic acid–water (25:15:4:2, by vol.) or chloroform–methanol–water (65:25:4, by vol.) for 3h at room temperature. Spots of respective phospholipids were detected by using I_2 vapour, or with the reagent described by Dittmer & Lester (1964). Phosphatidylcholine and sphingomyelin were identified by comparison with authentic samples, a mixture of these being developed with each plate. Areas detected by I_2 vapour were scraped off, oxidized in a Packard model 305 automatic sample oxidizer, and then radioactivity was measured with 15ml of scintillation fluid consisting of 720ml of dioxan, 45ml of methanol, 135ml of toluene, 100g of naphthalene, 5.0g of 2,5-diphenyloxazole and 0.3g of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene. In some experiments, lipid samples were subjected to mild alkaline hydrolysis (Dawson, 1960) to examine the possible presence of radioactivity in the alkali-stable phospholipid fraction.

Subcellular fractionation. All operations were carried out at 0–4°C. The 45mm-long nerve segment, 40mm away from the ganglion at its proximal end, was removed from a frog that had received simultaneous injections of [*Me*-^{3}H]choline (9μCi) and L-[U-^{14}C]leucine (2.4μCi), and been kept at 25°C for 15h. Perineural connective tissue was peeled and discarded. Pooled samples from five animals (440mg wet wt.) were homogenized in 9vol. of 0.32M-sucrose, by using a glass homogenizer, with 30 up-and-down strokes of the pestle rotating at approx. 400rev./min. The homogenate was centrifuged at 900*g* for 10min, at 10000*g* for 30min and at 100000*g* for 90min, with one wash each time, which respectively yielded pellets P_1, P_2, P_3 and supernatant fractions. The P_3 fraction was suspended in 1.5ml of 0.32M-sucrose, gently homogenized, and a 1.2ml portion layered on top of a density gradient in an 18ml tube, consisting of a continuous gradient ranging from 0.32M- to 0.9M-sucrose (15ml), with a 'cushion' of 1ml of 2.0M-sucrose at the bottom. This was centrifuged at 78000*g* for 11h in a Hitachi 65P preparative ultracentrifuge, in a RPS 25-3A rotor. Fractions (65 drops) were collected through a syringe pierced through the bottom of the tube, and samples of each (0.5ml) precipitated with 0.5ml of 10% (w/v) trichloroacetic acid, together with 1mg of bovine serum albumin as carrier, and the sediment was washed three times each with 1ml of 5% (w/v) trichloroacetic acid. The final precipitate was solubilized in Soluene-100 and radioactivity was measured as described above. Radioactivities of other subcellular fractions were similarly assayed after trichloroacetic acid precipitation, but without bovine serum albumin, except for the supernatant fraction. The quenching in the various fractions showed negligible variation, obviating the need for correction.

Assays

For enzyme assays, approx. 4g of the sciatic nerve collected from frogs not injected with label was subjected to subcellular fractionation by the procedure described above. Acetylcholinesterase and cholinesterase activities were determined by the method of Ellman *et al.* (1961), with minor modifications as described by Kato & Kurokawa (1967). Since no significant cholinesterase activity (butyrylthiocholine-splitting activity minus butyrylthiocholine-splitting activity in the presence of 1μM-diisopropylphosphorofluoridate) was detected in the P_3 fraction in the present experiment, acetylthiocholine-splitting activity minus acetylthiocholine-splitting activity in the presence of 0.1mM-diisopropylphosphorofluoridate was taken to represent acetylcholinesterase activity. Na^+-plus-K^+-stimulated ATPase (adenosine triphosphatase) activity was determined as described by Kurokawa *et al.* (1965). Activity of 2′:3′-cyclic nucleotide 3′-phosphohydrolase was determined by the method of Kurihara & Takahashi (1973), but without the pretreatment of samples with sodium deoxycholate, because of the difficulty of obtaining concentrated samples of the P_3 subfractions. Protein was determined by the method of Lowry *et al.* (1951), with dry bovine serum albumin as standard.

Results and Discussion

Incorporation of radioactivity

L-[4,5-^{3}H]Leucine injected into the ninth dorsal root ganglion was rapidly incorporated into the protein at 25°C (Fig. 1). Half-maximal incorporation was attained within approx. 30min of injection, and 90% of the total radioactive label became acid-insoluble in about 4h. In the nerve portion no more than 20mm away from the ganglion, 10–15% of the total radioactivity remained acid-soluble even 12h at 25°C after injection of label, but in portions beyond 20mm the corresponding value was less than 5%.

[*Me*-^{3}H]Choline was incorporated into the acid-

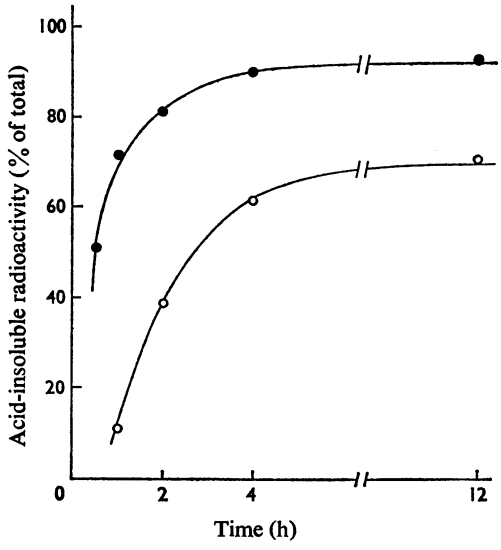

Fig. 1. *Incorporation of* L-[4,5-^3H]*leucine and of* [*Me*-^3H]*choline at 25°C into the acid-insoluble fraction in the ninth dorsal root ganglion of the frog*

●, L-[4,5-^{3}H]Leucine; ○, [*Me*-^{3}H]choline. Each point represents the average of duplicate experiments.

insoluble fraction at a slower rate than with L-[4,5-^{3}H]leucine (Fig. 1). Although the incorporation reached a plateau around 4h after the injection, approx. 30% of the total radioactivity in the ganglion remained acid-soluble even 12h after the injection.

Incorporation of [*Me*-^3H]*choline into phosphatidylcholine*

At 15h at 25°C after the injection of [*Me*-^{3}H]-choline, approx. 30 and 8% respectively of the total radioactivities were acid-soluble in the ganglion and in the nerve, and 99 and 90% respectively of the total radioactivity in acid-insoluble precipitates were recovered in the phospholipid fraction in the ganglion and nerve. On t.l.c. separation, 85–90% of the radioactivity in the phospholipid fraction was recovered as phosphatidylcholine in both the ganglion and the nerve (Fig. 2). Approx. 95% of the radioactivity in the phospholipid fraction from both the ganglion and the nerve was alkali-labile under hydrolysis conditions described by Dawson (1960). Most of the alkali-stable radioactivity seemed to have derived from sphingomyelin, as suggested by t.l.c. (Fig. 2). Contribution by choline plasmalogen to the radioactivity in the phosphatidylcholine fraction after t.l.c. would thus be negligible.

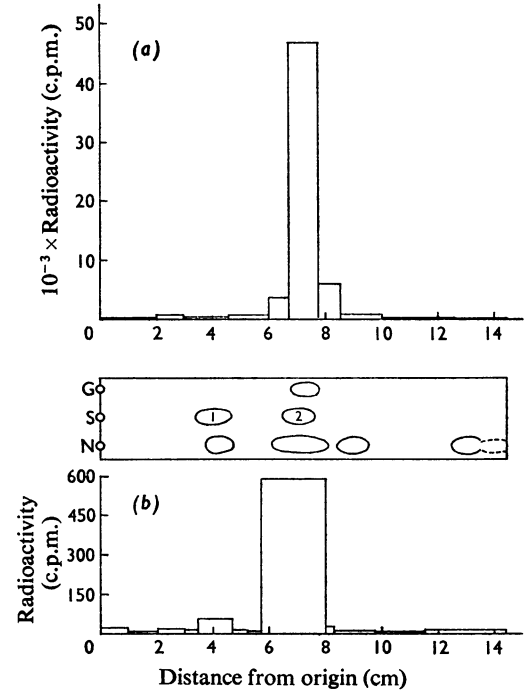

Fig. 2. *Distribution of radioactivities on t.l.c. of the phospholipid fraction extracted from the ninth dorsal root ganglion (a) and the sciatic nerve (b) of the frog*

Tissues were removed from a frog that had been kept at 25°C for 15h after the injection of [*Me*-^{3}H]choline (3μCi), and phospholipids were extracted and chromatographed as detailed in the Materials and Methods section. T.l.c., schematized in the centre of the figure, was developed with chloroform–methanol–acetic acid–water (25:15:4:2, by vol.). Extract from the ganglion was applied at G, that from the nerve at N, and a mixture of authentic sphingomyelin (1) and phosphatidylcholine (2) at S. Solvent front is shown by the vertical line at the right end.

Flow patterns and flow rate

For labelled proteins the flow was clearly demonstrated by a downward shift of a peak of radioactivity, the distal side of which fell precipitously to the baseline value (Fig. 3*a*). Low radioactivities, invariably found in the region of the baseline, appeared to be of extra-axonal origin, probably via the general circulation, since the nerve contralateral to that injected with label was also labelled to a similar extent. The flow pattern shown in Fig. 3(*a*) bears a close resemblance to that reported in the sciatic nerve of the cat (Ochs & Ranish, 1969) and of other mammals

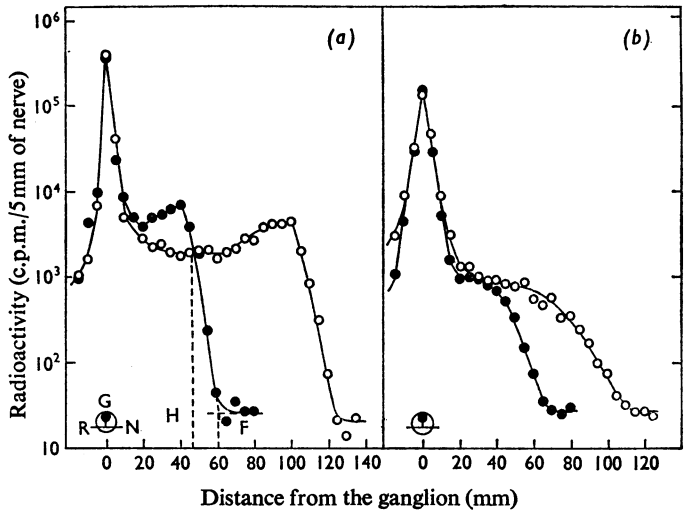

Fig. 3. *Flow patterns at 25°C of labelled proteins and phosphatidylcholine in the frog sciatic nerve and the dorsal root*

(*a*) L-[4,5-^{3}H]Leucine (4μCi) was injected into the ninth dorsal root ganglion. Dorsal root-ganglion–sciatic nerve preparation was removed 9h (●) and 18h (○) after the injection, sectioned in 5mm segments and radioactivity in each segment was measured. (*b*) [*Me*-^{3}H]Choline (3μCi) was injected into the ganglion and the radioactivity was measured 12h (●) and 18h (○) after the injection. R, dorsal root; G, the ninth dorsal root ganglion; N, sciatic nerve. F, distance from the ganglion to the intersection of the forward face of the crest with the baseline; H, distance from the ganglion to the half-height of the forward face.

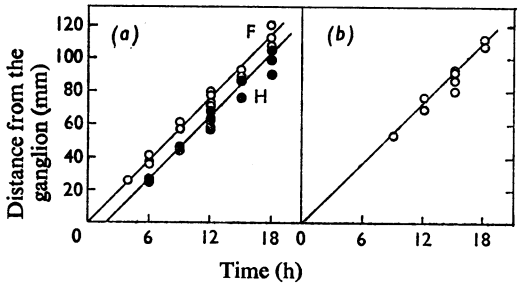

Fig. 4. *Downward shift of labelled proteins and phosphatidylcholine in the frog sciatic nerve at 25°C as a function of the time elapsed after the injection of respective precursors into the ninth dorsal root ganglion*

(*a*) L-[4,5-^{3}H]Leucine (4μCi) was injected into the ganglion and at the times indicated on the abscissa, the nerve was removed and measured for radioactivity. On the ordinate is shown the distance from the ganglion to the intersection of the forward front of radioactivity with the baseline (○; designated as F; see Fig. 3*a*), or the distance from the ganglion to the half-height of the forward face (●; designated as H; see Fig. 3*a*). (*b*) [*Me*-^{3}H]Choline (3μCi) was injected into the ganglion, and at intervals indicated on the abscissa, the nerve was removed and measured for radioactivity. Distance from the ganglion to the intersection of the radioactivity front with the baseline is shown on the ordinate.

(Ochs, 1972*b*). The peak comprised approx. 5% of the total incorporated radioactivity.

The distance from the ganglion to the intersection of the forward face of the peak with the baseline (F in Fig. 3*a*), or the distance from the ganglion to the half-height of the forward face (H in Fig. 3*a*) bears a linear relationship to the time elapsed after the injection of label (Fig. 4*a*). On this basis, flow rate is calculated to be 152mm/day at 25°C. There seems to be little variation in flow rate among axons, in view of the fact that the line F in Fig. 4*a* runs parallel with the line H. That the line F passes through the zero point indicates the fact that the newly synthesized protein leaves the perikaryon and begins to move down the axon without any appreciable delay.

In contrast with protein transport, the transport of phosphatidylcholine was not attended by a peak formation (Fig. 3*b*). However, in a sciatic nerve, which had been ligated by a thin cotton thread at its initial site 6h after the injection of [*Me*-^{3}H]choline into the ganglion and kept for a further 10h at 25°C, a peak of radioactivity was clearly demonstrated (results not shown). This implies that the pool size of transported phosphatidylcholine is larger as compared with that of proteins. The slope of radioactivity of phosphatidylcholine tends to become less steep with the lapse of time (Fig. 3*b*), which would indicate the occurrence of the base-exchange reaction (for review, see McMurray & Magee, 1972) during down-flow in axons. The flow rate of phosphatidylcholine was

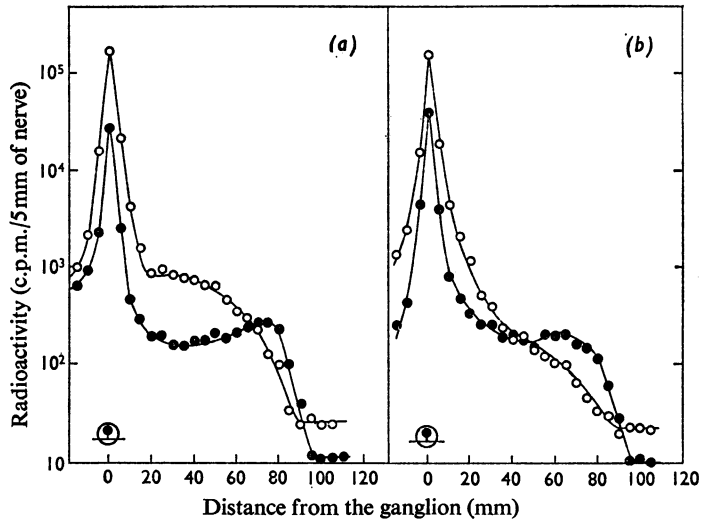

Fig. 5. *Distribution of radioactivities in the dorsal root, dorsal root ganglion and the sciatic nerve of the frog after simultaneous injection of* [*Me*-^{3}*H*]*choline and* L-[*U*-^{14}*C*]*leucine into the ganglion*

(*a*) At 15h after the injection at 25°C; (*b*) at 3h at 25°C, and then 45h at 10°C after the injection. A mixture of [*Me*-^{3}H]choline (3μCi) and L-[U-^{14}C]leucine (0.8μCi) was injected into the ganglion in both (*a*) and (*b*). ○, ^{3}H, representing phosphatidylcholine; ●, ^{14}C, representing proteins.

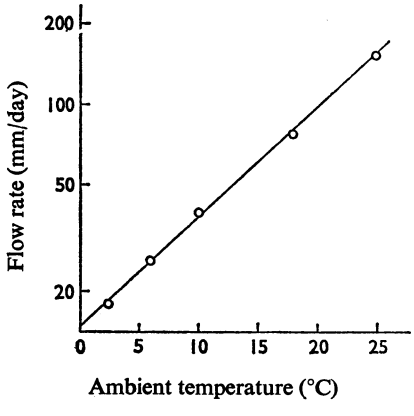

Fig. 6. *Temperature dependence of the protein transport in the frog sciatic nerve*

Transport rates were measured in frogs kept at different ambient temperatures shown on the abscissa, in a similar way to that outlined in Fig. 4. Rates are plotted on the ordinate on a logarithmic scale. At least four animals were used at each temperature.

calculated to be 150mm/day at 25°C (Fig. 4*b*), being equivalent to that of protein transport.

Further evidence for identical flow rate of proteins and phosphatidylcholine was obtained in double-labelling experiments, in which the forward front of ^{14}C-labelled proteins and of ^{3}H-labelled phosphatidylcholine was found to move simultaneously along the nerve at two different temperatures examined (Figs. 5*a* and 5*b*).

Temperature dependence of the flow rate

The rate of flow was determined in frogs which had been kept at five different ambient temperatures after the injection of L-[4,5-^{3}H]leucine. The linearity between the distance from the ganglion to the front of the radioactivity and the time after injection of label observed at 25°C (Fig. 4*a*) was also valid at all temperatures examined, which provided the basis for the calculation of flow rates at various temperatures. The rate was temperature-dependent, with a Q_{10} value of 2.6, irrespective of temperatures examined (Fig. 6). An equivalent Q_{10} value was obtained also for phosphatidylcholine transport (cf. Figs. 5*a* and 5*b*).

A comparative Q_{10} value has been reported for protein transport in the optic nerve of goldfish, although the rate of transport was different (Grafstein *et al.*, 1972). The Q_{10} value of 2.6 is somewhat higher than that generally estimated for enzyme reactions (1–2; Dixon & Webb, 1964), and implies the need of higher activation energy in the rate-limiting process of the rapid axonal transport.

In the frog sciatic nerve *in vitro*, Edström &

Mattsson (1972) found a rapid protein transport occurring at a rate of 60–90 mm/day at 18°C. If the Q_{10} of 2.6 applies to the nerve *in vitro*, the rate at 25°C is calculated to be 120–180mm/day, in reasonable agreement with the rate *in vivo*. Partlow *et al.* (1972) have reported the rate of downward transport of acetylcholinesterase in the frog sciatic nerve *in vitro* to be 99mm/day at 22°C. Based on a Q_{10} of 2.6, this indicates a rate of 132mm/day at 25°C. Identity in the rate of axonal transport of proteins and acetylcholinesterase as shown in the cat sciatic nerve (Ranish & Ochs, 1972) may thus hold also in the frog sciatic nerve.

Blocked axonal transport

Local injection of colchicine completely blocked protein transport (Fig. 7*a*). The amount of radioactivity accumulating at the site of the blockage comprised 5% of the total incorporated radioactivity, which was equivalent to that constituting the peak. The transport of phosphatidylcholine was similarly blocked by colchicine (Fig. 7*b*). In double-labelling experiments, it was noted that the accumulation of ^{3}H-labelled phosphatidylcholine at the site of blockage gradually increased from 0.7 to 1.3% of the total incorporated radioactivity during the period of 24 to 48h after the colchicine injection, whereas the accumulation of ^{14}C-labelled proteins remained virtually unchanged. This suggests that the pool size of rapidly transported protein fraction is smaller than that of phosphatidylcholine. The fact that protein labelling did not increase during the above-mentioned period suggests the virtual absence of significant protein transport with intermediate flow rates between 152mm/day and approx. 40mm/day at 25°C.

Unsuccessful attempts were made to find an agent other than colchicine that effectively blocks the transport. Possible implications of microtubules in axonal transport have been proposed for some time (for review, see Davison, 1970). The demonstration by Weisenberg (1972) that Ca^{2+} affects the depolymerization–polymerization equilibrium of microtubules *in vitro* would thus imply the involvement of Ca^{2+} in the mechanism of axonal transport. Ochs (1972*a*) has reported that oxalate blocks axonal transport, presumably by decreasing the amount of free Ca^{2+} in the axon. In the present experiment, however, direct injection of EGTA solution into the nerve failed to affect the rapid protein transport.

Also, no indication of blockage of the protein transport by cytochalasin D was obtained in our present system. Cytochalasin B was reported to produce a dose-related decrease in the amount of transported proteins in the rat sciatic nerve *in vitro*, but no reproducible inhibition was observed in the rat optic system *in vivo* (Crooks & McClure, 1972). McGregor *et al.* (1973) observed a slight suppression by cytochalasins A and B of protein migration in the

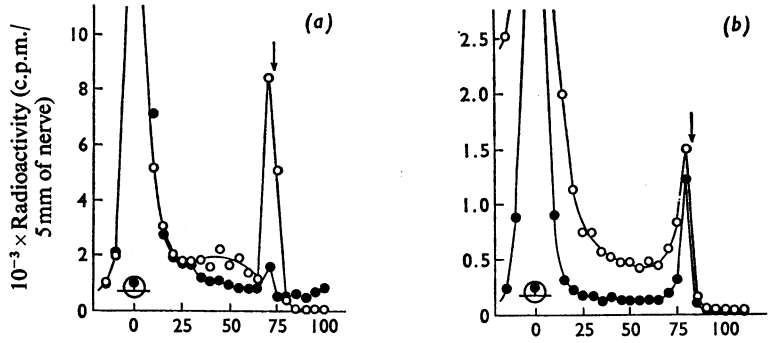

Fig. 7. *Blockade of the axonal transport of proteins and phosphatidylcholine in the frog sciatic nerve by local application of colchicine*

(*a*) Blockade of the protein transport. L-[4,5-^{3}H]Leucine (4μCi) was injected into the dorsal root ganglion and, after 2h at 25°C, 5μl of 0.56M-colchicine was injected into the nerve site indicated by the arrow (○). The animal was kept at 25°C for a further 36h and then killed. The control animal (●) received only the solvent, but otherwise was treated in an identical way. (*b*) The nerve was double-labelled as described in Fig. 5. ○, ^{3}H-labelled phosphatidylcholine; ●, ^{14}C-labelled proteins. Colchicine was injected into the site shown by the arrow 2h at 25°C after the injection of radioactive precursors into the ganglion. The nerve was removed from the frog kept at 25°C for 36h after colchicine application.

chicken sciatic nerve *in vivo*. However, they ascribed the suppression to a decreased uptake of the precursor, rather than suggesting the involvement of the microfilament system, which is postulated to be the primary site of cytochalasin action (Wessells *et al.*, 1971). Also, the rapid axonal transport of glycoproteins in the frog sciatic nerve *in vitro* was reported to be unaffected by cytochalasin B, although the uptake of [^{3}H]glucosamine into ganglionic cell bodies was markedly inhibited by this drug (Anderson *et al.*, 1972).

Subcellular distribution of the radioactivity

On subcellular fractionation, more than 80% of both ^{3}H and ^{14}C labels were recovered in the particulate fraction, and specific radioactivities on a protein basis of both [^{3}H]phosphatidylcholine and [^{14}C]proteins were highest in the P_3 (microsomal) fraction (Table 1).

After centrifugation of the P_3 fraction on a continuous sucrose gradient, distribution of [^{3}H] phosphatidylcholine and [^{14}C]proteins was surprisingly in parallel in various density regions (Fig. 8).

Table 1. *Subcellular distribution of radioactivities in the frog sciatic nerve after the simultaneous injection of [Me-^{3}H]choline and* L-*[U-^{14}C]leucine into the ninth dorsal root ganglion*

A 45mm-long segment of the sciatic nerve was removed from the frog kept at 25°C for 15h after the injection of [*Me*-^{3}H]choline (9μCi) and L-[U-^{14}C]leucine (2.4μCi) into the ninth dorsal root ganglion. In each run, nerves obtained from five animals similarly treated were analysed. The P_1, P_2 and P_3 fractions were sedimented by successive centrifugation of the homogenate at 900*g* (10min), 10000*g* (30min) and at 100000*g* (90min) respectively. Values are means from three experiments.

Fractions	Protein (mg)	Total radioactivity (c.p.m.) ^{3}H	Total radioactivity (c.p.m.) ^{14}C	Specific radioactivity (c.p.m./mg of protein) ^{3}H	Specific radioactivity (c.p.m./mg of protein) ^{14}C	Ratio ^{3}H/^{14}C
P_1	11.5	2284	1096	199	95	2.1
P_2	11.2	4314	1996	385	178	2.2
P_3	3.14	5400	2448	1720	780	2.2
Supernatant	4.97	2769	1324	557	266	2.1

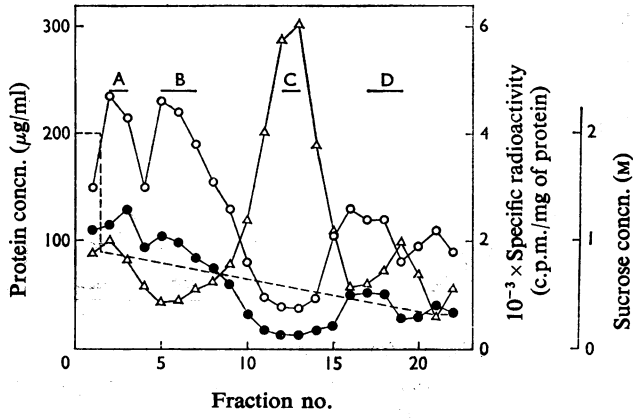

Fig. 8. *Distribution of radioactivities in the microsomal subfractions of the frog sciatic nerve after the simultaneous injection of [Me-^{3}H]choline and* L-*[U-^{14}C]leucine into the dorsal root ganglion*

The microsomal (P_3) fraction was obtained as in Table 1. The fraction was further subjected to centrifugation on a continuous density gradient of sucrose ranging from 0.32 to 0.9M. △, Protein concn.; ○, specific radioactivity of ^{3}H-labelled phosphatidylcholine on a protein basis; ●, specific radioactivity of ^{14}C-labelled proteins on a protein basis. The broken line indicates sucrose density gradient, the concentration of which is shown on the ordinate at the right-hand side. For details of subfractions A–D see Table 2 and the text.

Table 2. *Distribution of acetylcholinesterase, Na^+-plus-K^+-stimulated ATPase and 2′:3′-cyclic nucleotide 3′-phosphohydrolase activities in the microsomal subfractions*

For the P_3 (microsomal) fraction see the legend of Table 1, and for subfractions A, B, C and D, Fig. 8. Acetylcholinesterase activity is expressed in terms of nmol of acetylthiocholine hydrolysed minus acetylthiocholine hydrolysed in the presence of 0.1 mM-di-isopropylphosphorofluoridate/min per mg of protein (23°C), Na^+-plus-K^+-stimulated ATPase in nmol of P_i released/min per mg of protein (37°C), and 2′:3′-cyclic nucleotide 3′-phosphohydrolase in μmol of P_i released/min per mg of protein (37°C). Values are means of two determinations.

	Specific activities in					
Enzymes	Homogenate	P_3	A	B	C	D
Acetylcholinesterase	4.1	18	64	31	2.0	0
Na^+-plus-K^+-stimulated ATPase	0.97	3.2	9.3	9.9	1.5	0.61
Cyclic nucleotide phosphohydrolase	0.14	0.35	0.48	0.61	0.19	0.26

It thus seems most probable that both labels reside in the same structural entity. Specific activities on a protein basis of Na^+-plus-K^+-stimulated ATPase and acetylcholinesterase were extremely high in regions A and B (Table 2), where specific radioactivities of both ^3H and ^{14}C labels were highest (Fig. 8). However, the parallelism between acetylcholinesterase and specific radioactivities is of a limited significance, because the great bulk of the enzyme activity should have derived from the motor fibres (cf. Partlow *et al.*, 1972), which are not labelled under the present experimental conditions. It is nevertheless relevant to note that the rapid protein transport of the same rate occurs in both sensory and motor fibres in the cat sciatic nerve (Ochs & Ranish, 1969). In region D, which possessed moderately high specific radioactivities, specific activities of the above two enzymes were low (Table 2), but it remains to be determined whether or not the apparent difference between regions A and B and region D implies different axonal structures. In contrast to the above two enzymes, the specific activity of 2′:3′-cyclic nucleotide 3′-phosphohydrolase, provisionally taken as a myelin marker (Kurihara & Takahashi, 1973), was rather evenly distributed throughout the range of the sucrose gradient, indicating a lower contribution of the myelin component in characterizing the distribution of radioactivities in the P_3 subfractions.

General conclusions

The transport rate of the phospholipids reported by Miani (1963) in the rabbit vagus and hypoglossal nerves was exceedingly low compared with that of protein transport as reported by Sjöstrand (1969) in the same nerve. This is in apparent contrast with our present finding that phosphatidylcholine moves down the axon in association with proteins. It is noted, however, that Miani (1963) used [^{32}P]P_i for labelling phospholipids. The possible inadequacy of using ^{32}P labels for demonstrating rapid axonal transport is suggested for the following reasons. First, the rapid exchange reaction, which is apparent under conditions used by Miani (1963) may cause an underestimation of the flow rate. Also, Ochs & Ranish (1969) have demonstrated that in the cat sciatic nerve, ^{32}P-labelled materials move down more slowly as compared with ^3H-labelled proteins. It remains to be shown, though it is very likely, whether multiple rates as demonstrated in the protein transport (Karlsson & Sjöstrand, 1971) also hold for phospholipid transport in the axon.

In our present experiments, available evidence indicates that the transported phosphatidylcholine and proteins reside in the same structural entity, most probably a membrane component. It is tempting to speculate that a sort of precursor material of the axonal and synaptic membranes is continuously supplied by the transport system such as that demonstrated in the present experiment.

We thank Dr. T. Kato for his advice in microtechniques. Thanks are also due to Dr. S. Handa for his generous gift of authentic samples of phosphatidylcholine and sphingomyelin and for his helpful discussions.

References

Abe, T., Haga, T. & Kurokawa, M. (1973) *Proc. Jap. Acad.* **49**, 297–302

Anderson, K.-E., Edström, A. & Mattsson, H. (1972) *Brain Res.* **48**, 343–353

Bodian, D. (1962) *Science* **137**, 323–326

Crooks, R. F. & McClure, W. O. (1972) *Brain Res.* **45**, 643–646

Dahlström, A. (1971) *Phil. Trans. Roy. Soc. London Ser. B* **261**, 325–358

Davison, P. F. (1970) *Advan. Biochem. Psychopharmacol.* **2**, 289–302

Dawson, R. M. C. (1960) *Biochem. J.* **75**, 45–53

Dittmer, J. C. & Lester, R. L. (1964) *J. Lipid Res.* **5**, 126–127

Dixon, M. & Webb, E. C. (1964) *Enzymes*, 2nd edn., pp. 145–166, Longmans Green, London

Edström, A. & Mattsson, H. (1972) *J. Neurochem.* **19**, 205–221
Eichberg, J., Whittaker, V. P. & Dawson, R. M. C. (1964) *Biochem. J.* **92**, 91–100
Elam, J. S. & Agranoff, B. W. (1971) *J. Neurochem.* **18**, 375–387
Ellman, G. L., Courtney, K. D., Andres, V. & Featherstone, R. M. (1961) *Biochem. Pharmacol.* **7**, 88–95
Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957) *J. Biol. Chem.* **226**, 497–509
Grafstein, B. (1969) *Advan. Biochem. Psychopharmacol.* **1**, 11–25
Grafstein, B., Forman, D. S. & McEwen, B. S. (1972) *Exp. Neurol.* **34**, 158–170
Karlsson, J.-O. & Sjöstrand, J. (1971) *J. Neurochem.* **18**, 749–767
Kato, T. & Kurokawa, M. (1967) *J. Cell Biol.* **32**, 649–662
Kato, T. & Lowry, O. H. (1973) *J. Neurochem.* **20**, 151–163
Kurihara, T. & Takahashi, Y. (1973) *J. Neurochem.* **20**, 719–727
Kurokawa, M., Sakamoto, T. & Kato, M. (1965) *Biochem. J.* **97**, 833–844
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
McEwen, B. S. & Grafstein, B. (1968) *J. Cell Biol.* **38**, 494–508
McGregor, A. M., Komiya, Y., Kidman, A. D. & Austin, L. (1973) *J. Neurochem.* in the press
McMurray, W. C. & Magee, W. L. (1972) *Annu. Rev. Biochem.* **41**, 129–160
Miani, N. (1963) *J. Neurochem.* **10**, 859–874
Ochs, S. (1972*a*) *Science* **176**, 252–260
Ochs, S. (1972*b*) *J. Physiol. (London)* **227**, 627–645
Ochs, S. & Ranish, N. (1969) *J. Neurobiol.* **2**, 247–261
Ochs, S., Sabri, M. I. & Johnson, J. (1969) *Science* **163**, 686–687
Partlow, L. M., Ross, C. D., Motwani, R. & McDougal, D. B. (1972) *J. Gen. Physiol.* **60**, 388–405
Ranish, N. & Ochs, S. (1972) *J. Neurochem.* **19**, 2641–2649
Sjöstrand, J. (1969) *Exp. Brain Res.* **8**, 105–112
Sjöstrand, J. & Karlsson, J.-O. (1969) *J. Neurochem.* **16**, 833–844
Weisenberg, R. C. (1972) *Science* **177**, 1104–1105
Weiss, P. & Hiscoe, H. B. (1948) *J. Exp. Zool.* **107**, 315–395
Wessells, N. K., Spooner, B. S., Ash, J. F., Bradley, M. O., Luduena, M. A., Taylor, E. L., Wrenn, J. T. & Yamada, K. M. (1971) *Science* **171**, 135–143