DEMONSTRATION OF A RAPIDLY TRANSPORTED NEURONAL PROTEIN ASSOCIATED WITH THE MYELIN FRACTION OF NERVE

A DISSERTATION

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BY

JOY ANNETTE EDWARDS, B.A.

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ABSTRACT

An *in vitro* preparation was utilized to determine if a rapidly transported protein which appears in the myelin fraction of nerve is similar to rapidly transported proteins which have previously been observed released from nerve axons. Three sciatic nerves, and their accompanying eighth and ninth dorsal root ganglia were isolated from frogs (*Rana catesbieana*), and placed in a modified Warburg flask. A center well of the flask contained the ganglia, bathed in 0.2 ml Ringer solution containing 100 μCi ^{3}H-leucine. The nerves passed from the center well through a hole to an outer well containing 3.0 ml Ringer solution with cycloheximide, but without radiolabeled leucine. After 24 hours, the nerves were removed, and the ganglia excised and discarded. Myelin proteins were isolated from the nerves and separated by sodium dodecyl sulfate gel electrophoresis, and the radiolabeled profile of the gel determined. Radiolabeled transported proteins released by the nerve into the outer well were also electrophoresed, and their radiolabeled profile determined. To compare the myelin proteins synthesized by the Schwann cells with the myelin proteins labeled by the neuron and with the proteins released from the axons, the nerves, without the ganglia, were incubated in 3.0 ml Ringer containing 300 μCi ^{3}H-leucine for 24 hours. The myelin proteins were examined as above. The results show that the

neuronally labeled and transported protein isolated with myelin is of high molecular weight, about 100k daltons, whereas the proteins synthesized by Schwann cells correspond to the major myelin proteins of the peripheral nervous system, the P_0, P_1, and P_2 proteins, with molecular weights of 40k, 28k, and 19k daltons, respectively. The protein released by the nerve axons is similar to the neuronally labeled and transported protein isolated with myelin, in that it also has a molecular weight of about 100k daltons. This conclusion was strengthened by the observation that the two proteins co-electrophorese when placed on the same gel. The results show that the neuronally labeled and axonally transported protein from isolated myelin can be released from isolated myelin by treatment with 10 mM EDTA, thus indicating that the bonding to myelin is dissociable.

INTRODUCTION

An accepted hypothesis of myelination is that the neuron produces a trophic substance which is transported throughout the axon and subsequently influences the surrounding glial cell in the formation and maintenance of myelin (Singer, 1968; Singer and Steinberg, 1972; Brady and Quarles, 1973). Singer and Steinberg (1972) observed Wallerian degeneration when axonal transport was blocked by colchicine, and suggested the transport of a neurotrophic factor necessary for myelin maintenance. Brady and Quarles (1973) theorized the presence of an activator substance produced by the neuron to be myelinated, which would result in a change in the membrane-membrane interaction between the neuron and the surrounding glial cell, inducing myelination.

It has been shown that the axon controls the myelinated state of both Schwann and oligodendroglial cells in vivo (Weinberg and Spencer, 1975, 1976; Aguayo, et al., 1976; Aguayo, et al., 1978). Weinberg and Spencer (1975, 1976) anastomosed the proximal end of a predominantly myelinated nerve. It was shown that the Schwann cells of the unmyelinated nerve were stimulated to form myelin by the regenerating myelinated nerve. This indicated that all Schwann cells were capable of myelination, and that this phenomenon was controlled by the type axon with which the Schwann cell associated. These authors suggested the presence of a

membrane glycoprotein involved in cell-cell recognition, present only in myelinated axons. An alternative suggestion was the presence of a type of a chemical signal, axonally transported, to subsequently either be inserted into the axonal membrane or released to interact with the Schwann cell, stimulating myelination.

Aguayo, et al., (1976) and Aguayo, et al., (1978) also showed axonal control of myelination, and that the signal for myelin induction was common to both the central and peripheral nervous systems. Regenerating axons from a myelinated nerve induced myelination in transplants of unmyelinated nerve segments from both central and peripheral nervous systems. However, it was noted that the type of myelin formed depended upon the type of glial cell present.

Biochemical evidence of a neuronal protein associated with myelin has been published by several laboratories (Giorgi, et al., 1973; Elam, 1974, 1975; Autilio-Gambetti, et al., 1975; Monticone and Elam, 1975; Prensky, et al., 1975; and Matthieu, et al., 1978). Giorgi, et al. (1973) allowed neuronal incorporation and transport of proteins, and subsequently isolated the myelin. They reported neuronal labeling of some of the major myelin proteins and of minor high molecular weight proteins.

The work of Giorgi, et al. (1973) was contradicted in part by Elam (1974, 1975), Prensky, et al. (1975), and

Autilio-Gambetti, et al. (1975). These workers found high molecular weight axonally transported proteins associated with myelin. However, no neuronally produced proteins appeared associated with the major myelin proteins. They concluded that the neuron did not significantly contribute to the proteins and glycoproteins that were definitely known to be myelin components. The neuronally labeled high molecular weight proteins were suggested to be either incorporated into myelin or to be axolemmal contaminants of the isolated myelin.

Matthieu, et al. (1978) addressed the problem of axolemmal contamination of isolated myelin by developing a new myelin isolation technique which yielded myelin-enriched fractions and axolemmal-enriched fractions. The neuronally labeled and transported high molecular weight proteins appeared in both fractions. This indicated a possible axolemmal location for these proteins, but was inconclusive due to the appearance of these proteins in the myelin-enriched fraction as well.

In order to determine if neuronally labeled high molecular weight proteins were integral membrane proteins or were loosely associated with membranes, Elam (1978) treated isolated myelin with ethylenediamine tetraacetate (EDTA). The proteins were released from the myelin and isolated with the EDTA wash. This demonstrated that they

were not integral membrane proteins. It was not resolved if they were dissociated from the myelin membrane or the axolemmal membrane.

These studies support the hypothesis that a trophic protein may be produced by the neuron and transported along the axon. It could then: 1) associate with the axolemma for a membrane-membrane interaction, or 2) be released from the axon to interact with the myelin membrane or the glial cell itself.

Evidence for a chemical signal to be released from the axon as a diffusible agent has been reported (Hines and Garwood, 1977). They demonstrated the release of high molecular weight rapidly transported proteins from the axon into the surrounding fluid in an in vitro preparation. A comparative study by Krikorian and Hines (1977) demonstrated electrophoretically similar high molecular weight proteins released from the axons in chicken, turtle, rabbit, and frog, indicating the presence of these efflux proteins across distinct classes. A comparison of axonally released proteins from normal and dystrophic mice demonstrated an alteration in one of the released proteins, suggesting induction of myelination or myelin maintenance as a possible function for these proteins (Krikorian and Hines, 1978).

If the transported proteins released from the axon have a trophic function, it is reasonable to assume a binding of

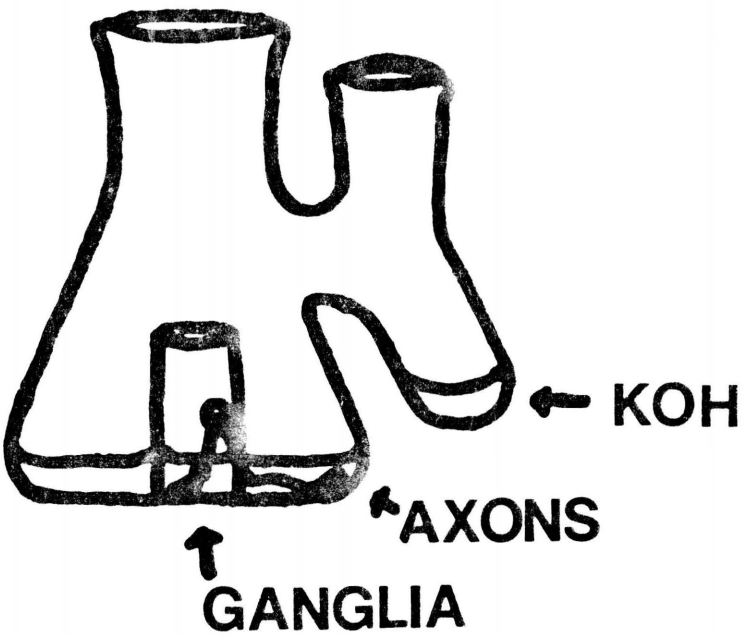
the released proteins to the myelin and/or Schwann cells. It is the purpose of this work to establish the following: 1) to show that the transported proteins released from axons are electrophoretically similar to the transported proteins isolated with myelin, and 2) to determine if the transported proteins associated with myelin are dissociable upon treatment of the isolated myelin with EDTA, or if they are integral membrane proteins. While previous workers examined transported proteins isolated with myelin in *in vivo* central nervous system preparations, this work is based on an *in vitro* peripheral nervous system preparation.

MATERIALS AND METHODS

Neuronal incorporation of proteins

Bullfrogs (Rana catesbieana) with six to eight inch legs were used. The frogs were double pithed before surgery. The standard preparation consisted of three sciatic nerves and their accompanying eighth and ninth dorsal root ganglia, with the nerve excised to the frog's knee. The preparation was placed in a modified Warburg flask (Figure 1), ganglia and roots resting in the inner well, with the nerves passing from the inner well through a hole to the outer well. Frog Ringer was made with sodium chloride, 109 mM; calcium chloride, .82 mM; sodium bicarbonate, 2.39 mM; potassium chloride, 1.88 mM; dextrose, 5.55 mM; penicillin; streptomycin, 200 units/ml, 20 ml; dissolved to one liter, and the pH titrated to 7.4 with 0.1 M sodium phosphate (Edström and Mattsson, 1972), and oxygenated. Three ml Ringer was added to the outer well, along with 100 µg/ml cycloheximide to inhibit protein synthesis by the Schwann cells. The inner well contained 0.2 ml frog Ringer, with 100 µCi ^{3}H-leucine (ICN). The side arm contained 0.2 ml of 10 % potassium hydroxide to absorb the carbon dioxide given off by the nerves. The prepared flask was attached to a Warburg manometer, immersed in a 20^{o} C water bath, and automatically shaken throughout the experiment. Manometer readings were taken at intervals

Figure 1. Modified Warburg flask, containing ganglia in the inner well, with the nerve trunks (axons) passing through to the outer well. The inner well contained 0.2 ml frog Ringer, the outer contained 3.0 ml and 300 µg cycloheximide. The side arm contained 0.2 ml of 10% KOH.


KOH
AXONS
GANGLIA

over the 24 hour period, and oxygen uptake calculated (Umbreit, et al., 1964).

At the end of the experiment, the nerves were removed from the Warburg flask and weighed. One nerve was measured for length, and placed in 10 % trichloroacetic acid (TCA) for at least 24 hours for transport studies as described later. The remaining nerves in the Warburg flask were then treated as follows. The roots, ganglia, and length of nerve in the inner well were excised and discarded. The remaining nerve length was set aside for myelin isolation. At this time the 3.0 ml solution in the outer well was collected, frozen, and labeled 'efflux.'

Myelin isolation and treatment of myelin proteins

For myelin isolation, samples of three to four nerves with ganglia excised were weighed. The samples weighed between 0.4 and 0.5 g. The nerves were frozen in liquid nitrogen, and ground to a fine powder with a mortar and pestle. The powder was placed in 21 ml of 0.29 molar (M) sucrose and homogenized in a Sorvall omnimixer. Ten ml of the homogenate were layered over seven ml of 0.79 M sucrose in nitrocellulose SW-27 centrifuge tubes and centrifuged in a Beckman L5-65 ultracentrifuge at 25,000 (25k) rpm for 45 minutes at 4° C. The myelin, which accumulated at the interface, was removed with a Pasteur pipette and diluted to

17 ml with distilled water in a nitrocellulose centrifuge tube for osmotic shock, which would release trapped axonal components from membrane micelles. The pellet at the bottom of the sucrose gradient was discarded.

The osmotically shocked myelin solution was centrifuged in the Beckman L5-65 at 25k rpm for 45 min at 4° C. Myelin was collected at the bottom of the tube. The pellet was removed, resuspended in 12 ml distilled water for a second osmotic shock, and centrifuged in the Beckman J-21B centrifuge at 16k rpm for 45 min at 4° C.

The neuronally labeled osmotically shocked myelin sample was divided into two halves. One was designated as M and frozen temporarily (-10° C). The other half was placed in 3.0 ml of 10 mM EDTA and stirred at 4° C for 14 to 16 hours. It was then centrifuged in a Beckman J-21B at 4k rpm for 45 min at 4° C. The supernatant was carefully pipetted off and dialyzed against two changes of distilled water for a total of 12 hours, then lyophilyzed. The lyophilyzed sample was labeled MW (myelin wash) and stored frozen (-10° C). The pellet was labeled washed myelin, M', and stored frozen at the same temperature until further use.

For delipidation of the myelin proteins, M and M' were thawed. Five ml ether:ethanol (3:2) was added to each sample, and homogenized in a teflon-on-glass homogenizer. The homogenates were centrifuged for 20 min in a Beckman

J-21B at 16k rpm at 4° C. The delipidation procedure was repeated on the pellet four times. The M and M' pellets were then dried with a stream of nitrogen gas.

Electrophoresis and determination of radiolabeled profiles

Efflux samples were thawed, dialyzed against at least two changes of distilled water for 16 to 24 hours, and lyophilyzed.

All samples were weighed. They were then solubilized to an approximate concentration of 100 µg/0.05 ml in a 0.1 M phosphate buffer, pH 7.2 to 7.4, containing 1 % sodium dodecyl sulfate (SDS) and 1 % beta-mercaptoethanol. The samples were placed in a water bath and sonicated until clear, to a maximum of 24 hours. Actual concentrations of the proteins were determined by the procedure of Lowry, et al. (1951). Specific activities were obtained by placing 100 µg of each sample into 10 ml of Bray's scintillation fluid and determining counts per minute (cpm) per µg sample in a Beckman LS-9000 liquid scintillation counter. The Bray's scintillation fluid consisted of 100 ml of 10 % methanol, 20 ml ethylene glycol, 60 g napthalene, 4 g 2,5-diphenyloxazole (PPO), 0.2 g 1,4-bis(2-5(5-phenyloxazolyl)) benzene (POPOP), and was dissolved to one liter with dioxane. Samples with a specific activity of less than 8 cpm/µg were discarded.

Samples with a high enough specific activity were prepared for electrophoresis by the addition of several crystals of sucrose to increase density and a drop of 1 % bromphenyl blue as a tracking dye on the gel to determine the electrophoretic front. Gels were prepared by the method of Weber and Osborn (1969). Gel buffer contained 7.8 g sodium phosphate, monobasic; 38.6 g sodium phosphate, dibasic; and 2 g SDS per liter. A 10 % acrylamide solution contained 22.2 g acrylamide and 0.6 g methylene-bis-acrylamide, dissolved to 100 ml and filtered. Gels contained 16.5 ml gel buffer, 13.5 ml acrylamide solution, 0.045 ml N,N,N',N'-tetramethylethylene-diamine (TEMED), and 22 mg ammonium persulfate. Before the gels hardened, several drops of distilled water were layered on top to form a smooth interface. The gels were made in tubes 122 mm long, with an internal diameter of 6 mm. For electrophoresis, the gel buffer was diluted 1:1 with distilled water, and placed in the two compartments of the electrophoresis apparatus. The protein samples were carefully layered on top of the gels. Electrophoresis was performed at 8 milliamperes per gel, with the positive electrode in the bottom compartment. Electrophoresis was stopped after 10 hours, the front and gel length noted for each gel for later determination of R_f values. The gels were then placed in 10 % TCA for at least 30 minutes. After removal from the TCA, they were rinsed in

distilled water once, and covered with Coomassie brilliant blue (1.25 g in 454 ml of 50 % methanol and 46 ml glacial acetic acid) for two to six hours. After staining, they were rinsed once in distilled water, and destained by diffusion in 7.5 % acetic acid-5 % methanol (Weber and Osborn, 1969). Molecular weights were determined by the concurrent electrophoresis of proteins of known molecular weights and the construction of a standard curve of these proteins and their R_f values. The standards used were DNA polymerase, 105k daltons; catalase, 61k daltons; aldolase, 40k daltons; and myoglobin, 17.2k daltons. Molecular weights were then substituted for R_f values when graphing the radiolabeled profiles and scans of the gels.

The gels were scanned on a Beckman-R112 microzone scanner at 600 nm. After scanning, they were wrapped in Parafilm, frozen, and then sliced into 2 mm segments. Each slice was incubated at 55° C in 0.2 ml Soulene-350 (Beckman) for two to four hours. If necessary for decoloration, 0.1 ml of 30 % hydrogen peroxide was added to each slice on an entire gel. The solubilized gel slices were rinsed out of the test tubes by adding one ml scintillation fluid to each. Each slice was then added to vials containing 9.0 ml scintillation fluid, which had been previously counted for two min per vial at tritium energy levels. Radioactivity for each slice was then determined on the scintillation counter

for two min per vial. Background (averaging 30 cpm) could then be subtracted to determine actual cpm per slice.

Schwann cell synthesis of myelin proteins

In order to determine Schwann cell synthesis of myelin proteins, a slightly different procedure was used. The sciatic nerve of the frog was removed to the knee, without the ganglia. The nerve was placed in a single well Warburg flask, in 3.0 ml frog Ringer containing 300 μCi ^{3}H-leucine. Manometry was carried out as above. Myelin isolation and protein treatment were identical to that of neuronal incorporation, with the exception that the myelin was not treated with EDTA. Myelin labeled by Schwann cell synthesis was designated SM.

Transport studies

To determine the transport profile, the transport nerve was removed from the TCA and pinned to a dissecting tray to dry for five to six hours. The nerve was then sliced into two mm segments and each slice was incubated at 50^{o} C in Soluene-350 for 12 to 24 hours. Radioactivity per slice was determined by placing each slice in a precounted vial containing 10 ml Bray's scintillation fluid and counting by liquid scintillation, as above.

Co-electrophoresis of efflux and isolated myelin proteins

To determine if the high molecular weight protein isolated with myelin had the same electrophoretic properties as the efflux protein, samples from each were combined and electrophoresed on the same gel. Control samples of pure efflux and pure isolated myelin were electrophoresed on separate gels at the same time.

EXPERIMENTAL RESULTS

Viability

In order to determine viability of the nerves throughout the experiment, oxygen uptake was carefully monitored. Figure 2 shows the graph of oxygen uptake of the nerves and ganglia in three vials, which averaged 48.8 ± 6.6 µl O_2/g/hr at 20° C.

A second parameter of viability monitored was rapid transport of neuronally labeled proteins. Since the transport was allowed for 24 hours, the front was not visible. However, Hines and Garwood (1977), using shorter time periods, showed normal isotope distribution and transport velocities of this preparation. An isotope distribution from a typical preparation in these experiments shows a high level of incorporation throughout the length of the nerve (Figure 3). Since the nerve axons rested in cycloheximide, the incorporation shown is due solely to neuronal protein synthesis and transport. The ganglion is at zero mm, the roots to the left, and the nerve to the right. At 30 mm, the branches of the eighth and ninth ganglia form a common nerve, producing a visible rise in radioactivity. The nerve subsequently branches again at 60 mm.

Neuronal labeling of isolated myelin

In order to determine if neuronally labeled and

Figure 2. Each curve represents oxygen uptake of a single flask containing three sciatic nerves with ganglia over 24 hrs at 20° C.

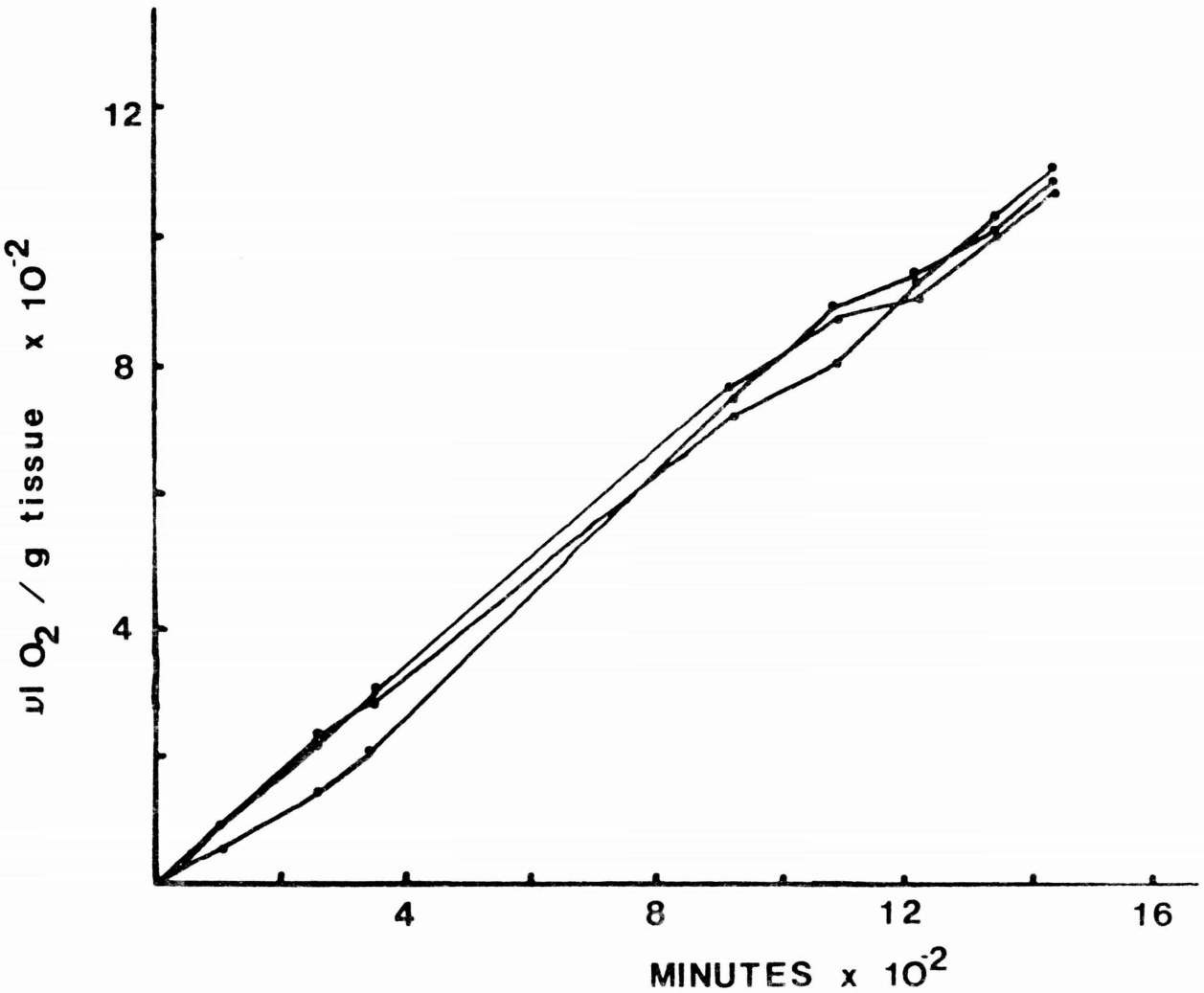
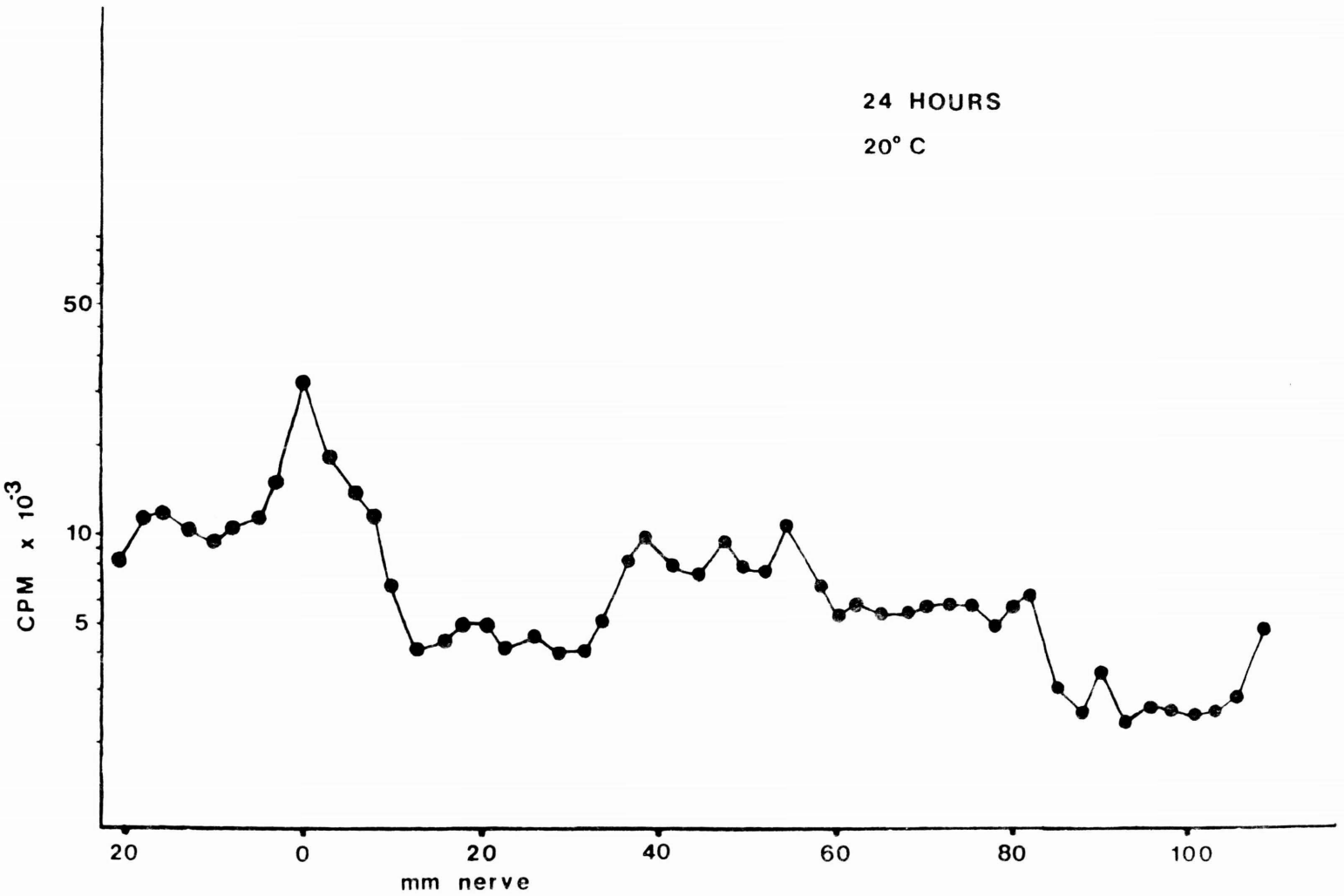

12
8
4
µl O2 / g tissue x 10-2
4
8
12
16
MINUTES x 10-2

Figure 3. Rapid transport profile, showing the distribution of labeled protein in the sciatic nerve after incubation at 20° C for 24 hours. Note the ganglia at zero mm, the roots to the left, and the nerve trunk to the right. At 30 mm, the branches of the eighth and ninth ganglia form a common nerve, producing a visible rise in radioactivity. The nerve subsequently branches again at 60 mm.


24 HOURS
20° C
50
10
5
CPM x 10^{-3}
20
0
20
40
60
80
100
mm nerve

transported proteins were isolated with myelin, the following experiment was performed. The ganglia were incubated in ^{3}H-leucine in the inner well of a Warburg flask, with the axon resting in the outer well. Cycloheximide was placed in the outer well to prevent protein synthesis by the Schwann cells. As a result, any labeled proteins observed should be neuronal in origin. The isolated myelin was examined on SDS-PAGE gels, and the gels were subsequently counted for radioactivity. The radiolabeled profile was normalized by expressing the observed cpm as a per cent of the total number of cpm placed on the gel. Molecular weight was substituted for R_f values. A typical densitometric scan of neuronally labeled myelin is shown in Figure 4A. The radiolabeled profile as determined by liquid scintillation counting of the same gel is shown in Figure 4B. The major myelin proteins, P_0, P_1, and P_2, at 40k, 28k, and 19k daltons respectively, are labeled. A minor protein band around 100k daltons also appears highly labeled.

The radiolabeled profiles from several experiments of neuronal incorporation into myelin were averaged by calculating the average incorporation in R_f values of 0.025 intervals. Figure 5 shows the averaged radiolabeled results. Incorporation is seen in the major myelin proteins, and in two high molecular proteins around 100k and 62k daltons. Average deviation lines are drawn for each average point

Figure 4. Densitometric scan (A) and radiolabeled profile (B) of proteins isolated from myelin. Labeled proteins represent those associated with myelin after neuronal incorporation of ^{3}H-leucine and rapid axonal transport. Both scan and radiolabeled profile are from the same gel.

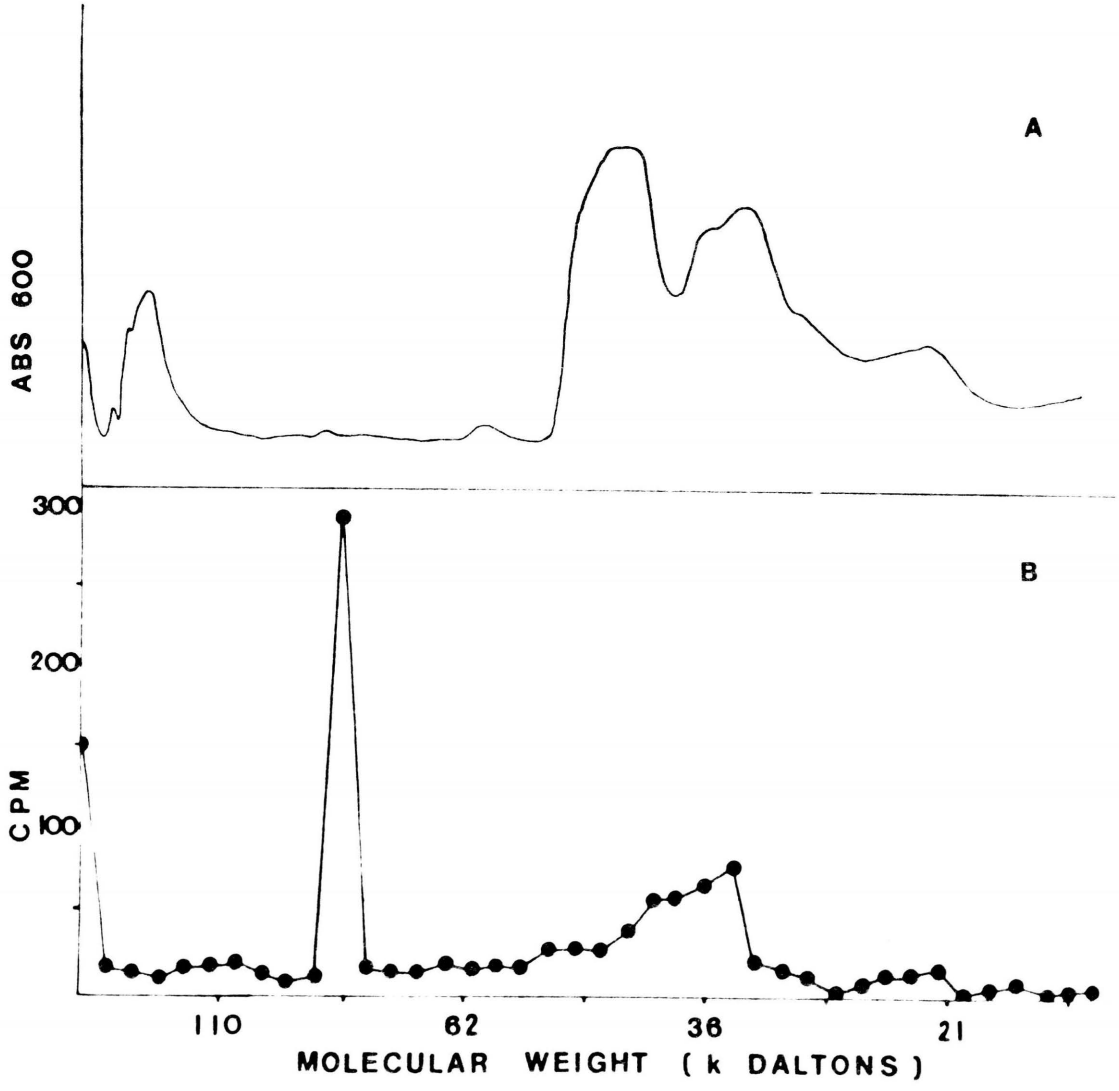
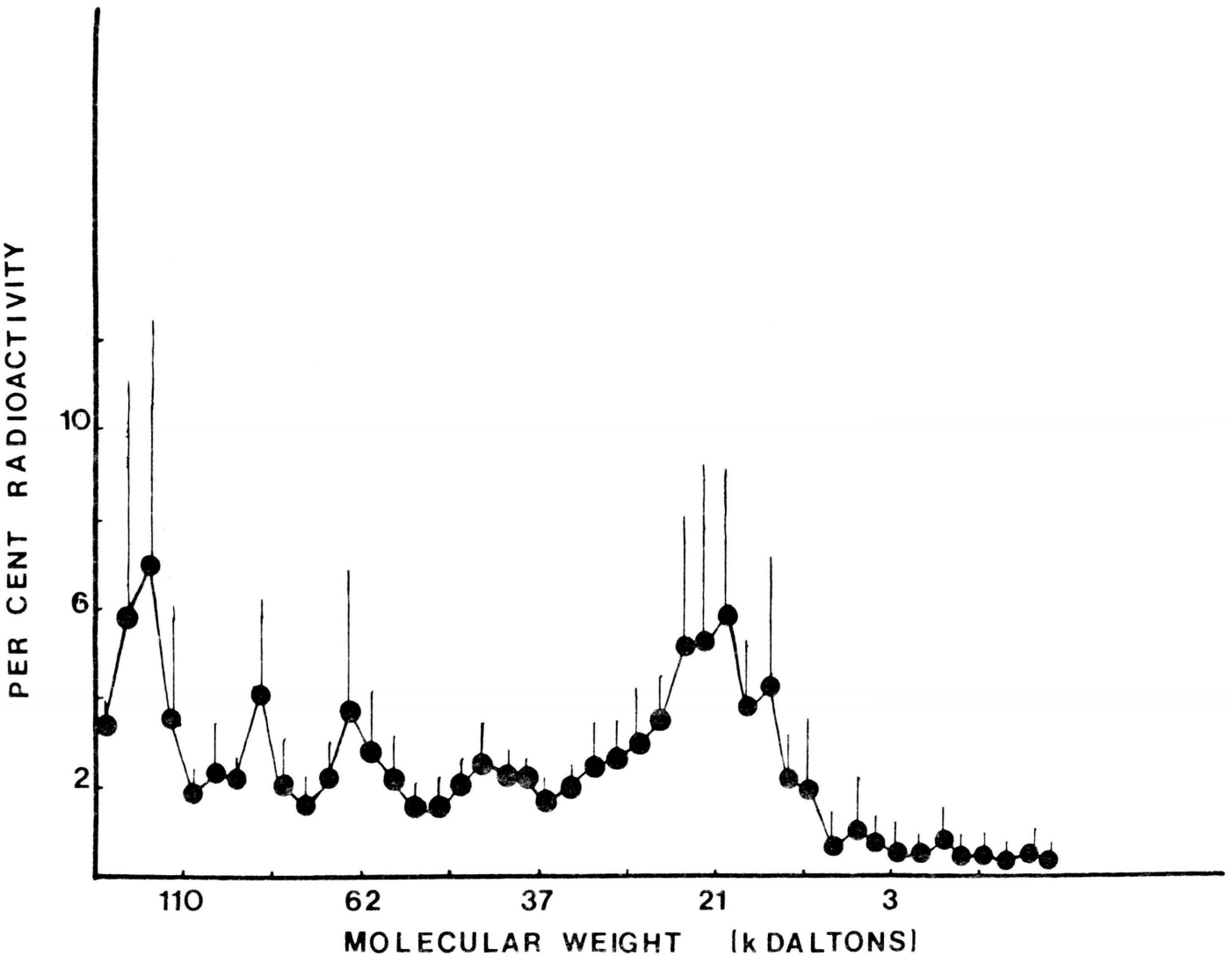

A
ABS 600
B
300
200
100
CPM
110
62
36
21
MOLECULAR WEIGHT (k DALTONS)

Figure 5. A radiolabeled profile of proteins isolated from myelin. Labeled proteins represent those associated with myelin after neuronal incorporation of ^{3}H-leucine and rapid axonal transport. This figure represents an average of eight gels and is expressed as a per cent of total radioactivity.


PER CENT RADIOACTIVITY
10
6
2
110
62
37
21
3
MOLECULAR WEIGHT (k DALTONS)

(top half only).

Schwann cell synthesis of myelin proteins

In order to determine which myelin proteins were labeled through Schwann cell synthesis, the following experiment was performed. The sciatic nerve, minus ganglia, was incubated in ^{3}H-leucine in the outer well of a Warburg flask. All labeled proteins were therefore of Schwann origin, and could be compared with the neuronally labeled myelin. Myelin was isolated and examined on SDS-PAGE gels, and examined for radioactivity. A typical densitometric scan of myelin labeled by Schwann cells is shown in Figure 6A. The radiolabeled profile as determined by liquid scintillation counting of the same gel is shown in Figure 6B. The incorporation of ^{3}H-leucine appears only in the major myelin proteins. No incorporation is noted in the high molecular weight proteins. The radiolabeled average of several experiments of Schwann cell synthesis of myelin proteins is shown in Figure 7.

Since it was unexpected to find the major myelin proteins labeled by the neuron, a comparison of the specific activities of the myelin proteins labeled by the neurons and the Schwann cells was necessary. As is seen in Table 1, neuronally labeled myelin has a specific activity of 15 ± 5 cpm/µg, while Schwann cell labeled myelin has a specific

Figure 6. Densitometric scan (A) and a radiolabeled profile (B) of proteins isolated from myelin. Labeled proteins represent those synthesized by the Schwann cells of sciatic nerves, minus ganglia. Both scan and radiolabeled profile are from the same gel.

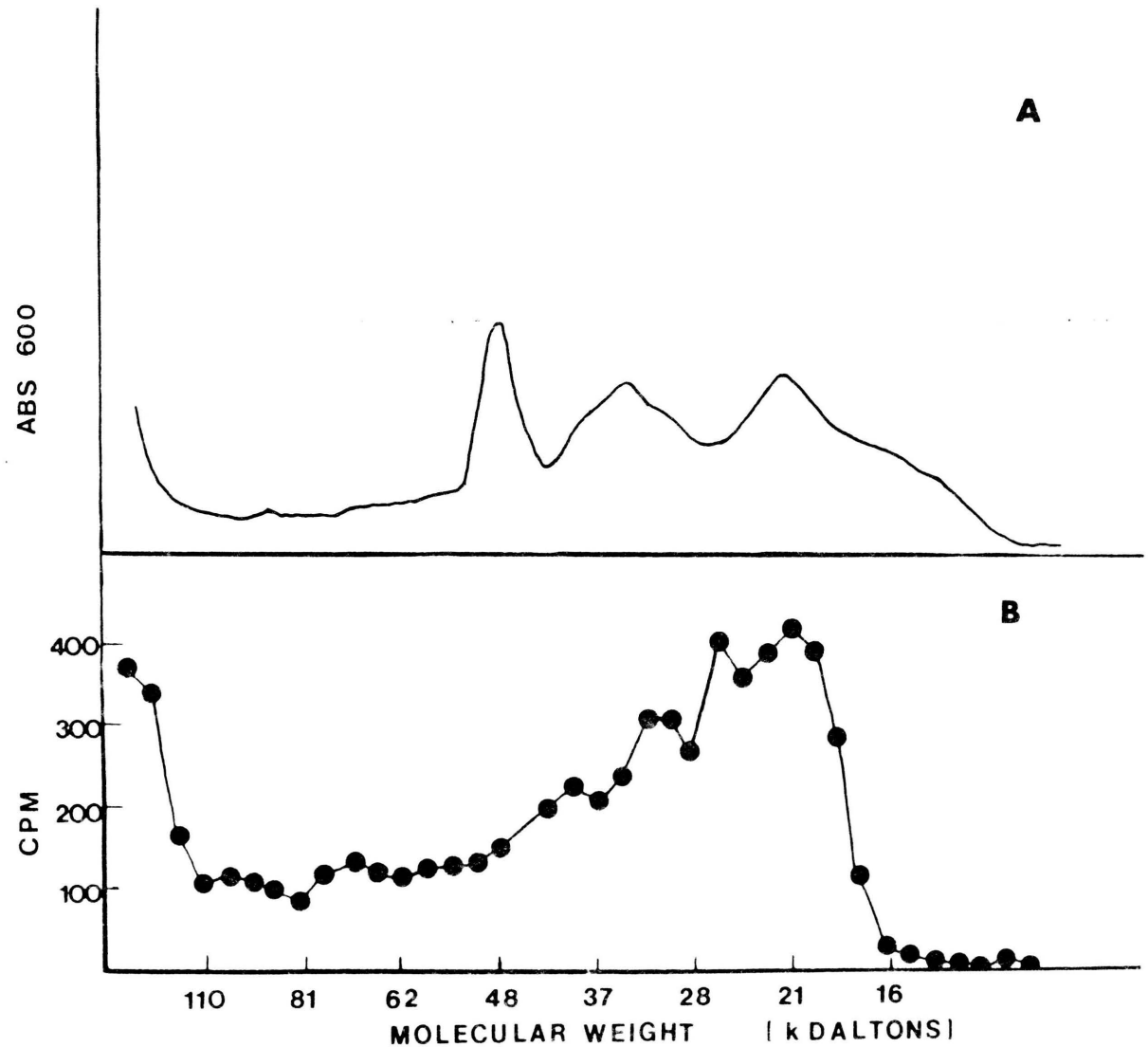

A
ABS 600
B
400
300
200
100
CPM
110
81
62
48
37
28
21
16
MOLECULAR WEIGHT [k DALTONS]

Figure 7. Radiolabeled profile of proteins isolated from myelin. Labeled proteins represent those synthesized by the Schwann cells of sciatic nerves, minus ganglia. This figure is an average of five gels and is expressed as a per cent of total radioactivity on the gel.

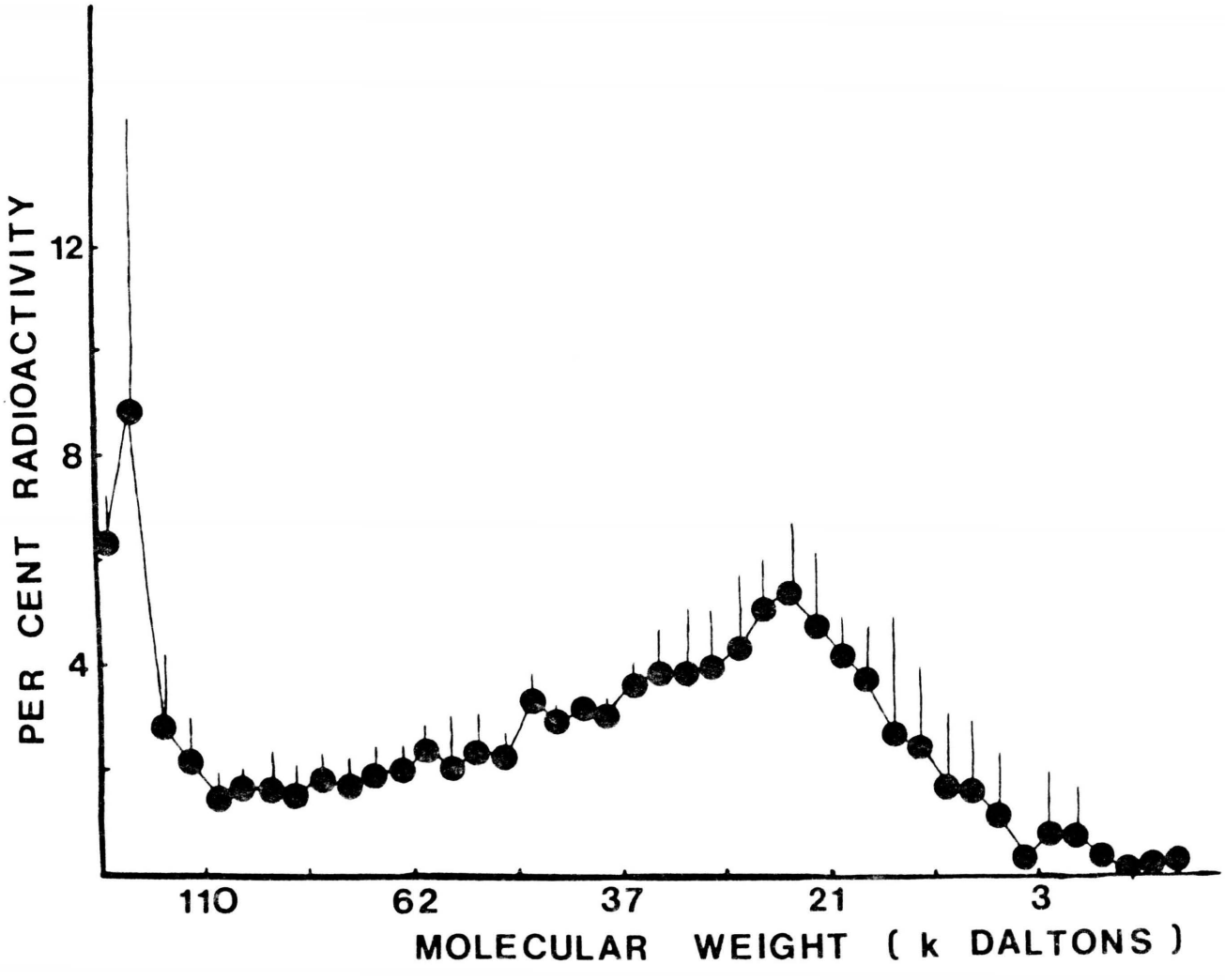

PER CENT RADIOACTIVITY
12
8
4
110
62
37
21
3
MOLECULAR WEIGHT (k DALTONS)

Table 1. Average specific activities of proteins examined.

TABLE I

Specific activity of proteins examined

Protein	cpm/ug
Neuronal labeling of isolated myelin	15.0 ± 6.8*
Neuronally labeled myelin washed with EDTA	15.5 ± 4.9*
EDTA wash of neuronally labeled myelin	9.9 ± 0.8*
Schwann cell labeling of isolated myelin	199.5 ± 96.1*
Neuronally labeled efflux	1857.5 ± 549.0*

* Average deviation

activity of 200 ± 96 cpm/μg. Even assuming that two-thirds of the neuronally labeled proteins appear in the major myelin proteins, the incorporation is only one-twentieth of the label produced by the Schwann cells.

Neuronally labeled efflux proteins

Since it has previously been shown that neuronally labeled proteins are transported and released from the axon (Hines and Easton, 1971; Hines and Garwood, 1977), it was decided to examine the solution in the outer well of the Warburg flask for neuronally labeled protein efflux, and to compare this efflux to the neuronally labeled proteins isolated with myelin. The solution in the outer well of preparations labeled by the ganglia was examined on SDS-PAGE gels. The gel scan is shown in Figure 8A, with the corresponding radiolabeled profile in Figure 8B. The efflux consists mainly of high molecular weight proteins, with labeling appearing around 100k daltons. Figure 9 shows an average of several radiolabeled efflux profiles. The majority of the radioactivity appears around 100k daltons.

Treatment of myelin with EDTA

The neuronally labeled myelin and the efflux proteins exhibit a similar labeled band on SDS-PAGE gels. It has been shown that the reduction of Ca^{++} levels disrupts

Figure 8. Densitometric scan (A) and radiolabeled profile (B) of rapidly axonally transported proteins released from sciatic nerves. The radiolabeled tag is ^{3}H-leucine.

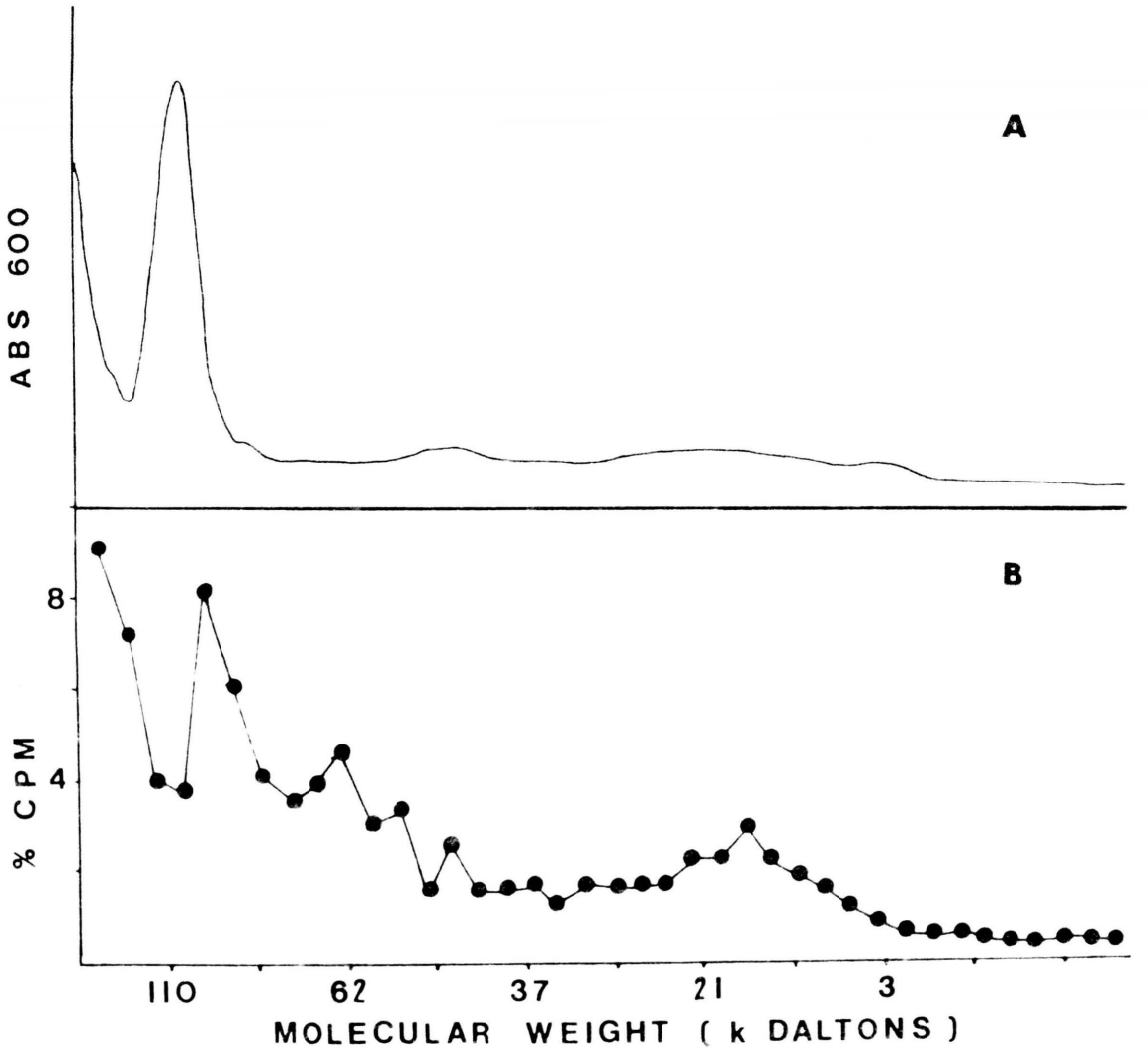
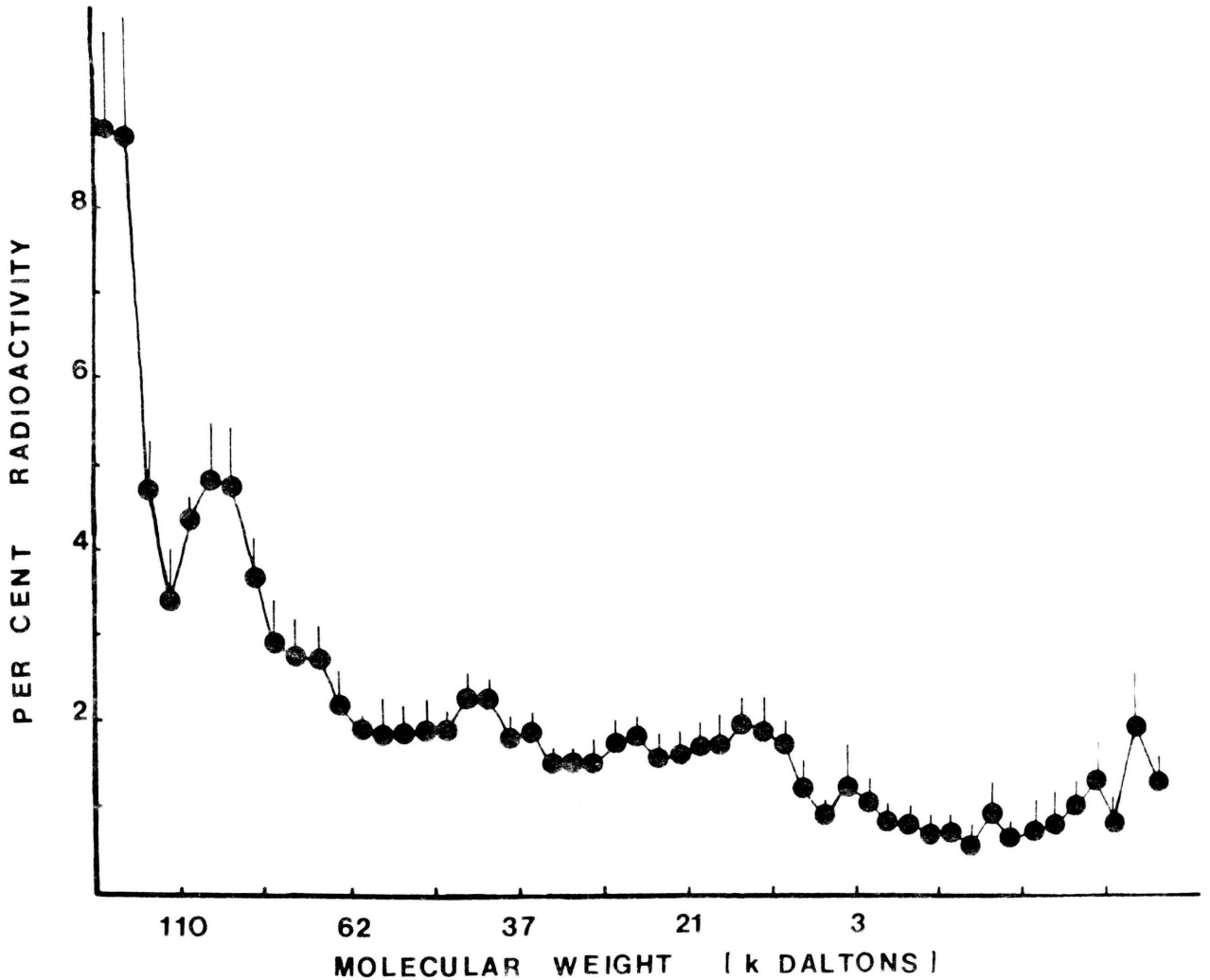

A
ABS 600
B
8
4
% CPM
110
62
37
21
3
MOLECULAR WEIGHT (k DALTONS)

Figure 9. Radiolabeled profile of rapidly axonally transported proteins released from sciatic nerves, after labeling with ^{3}H-leucine. This figure represents an average of nine gels and is expressed as a per cent of total radioactivity.


PER CENT RADIOACTIVITY
8
6
4
2
110
62
37
21
3
MOLECULAR WEIGHT [k DALTONS]

axon-myelin interactions in tissue culture (Blank, et al., 1974), and that the presence of a chelating agent facilitates the isolation of an axolemmal-enriched fraction from brain (DeVries, et al., 1976). It was therefore decided to examine the bonding of the neuronally labeled proteins isolated with myelin. Integral membrane proteins, such as the major myelin proteins, would not be dissociable. Since the efflux proteins are released from the axon, if they bind to the myelin, it would be logical to assume that they are not integral membrane proteins, and would be dissociable under the appropriate conditions. Therefore, the neuronally labeled myelin was washed with EDTA to determine if the neuronally labeled proteins isolated with myelin are dissociable. The washed myelin was then electrophoresed. The densitometric scan in Figure 10A shows the electrophoretic profile of the EDTA-washed myelin, with the corresponding radiolabeled profile in 10B. Figure 11 shows the average radiolabeled profile from several experiments. The major myelin proteins remain labeled, as does the peak around 62k daltons. However, the peak near 100k daltons has been greatly reduced. A comparison of the per cent radioactivity found the R_f range 0.125-0.225, corresponding to the molecular range around 100k daltons. The EDTA-washed myelin was reduced by 1.5 % cpm units, which is 40 % of the peak height around 100k daltons.

Figure 10. Densitometric scan (A) and radiolabeled profile (B) of proteins isolated from myelin. Labeled proteins represent those associated with myelin after neuronal incorporation of ^{3}H-leucine and rapid axonal transport. The isolated myelin was then washed with 10 mM EDTA for 14 to 16 hrs. Both scan and radiolabeled profile are from the same gel.

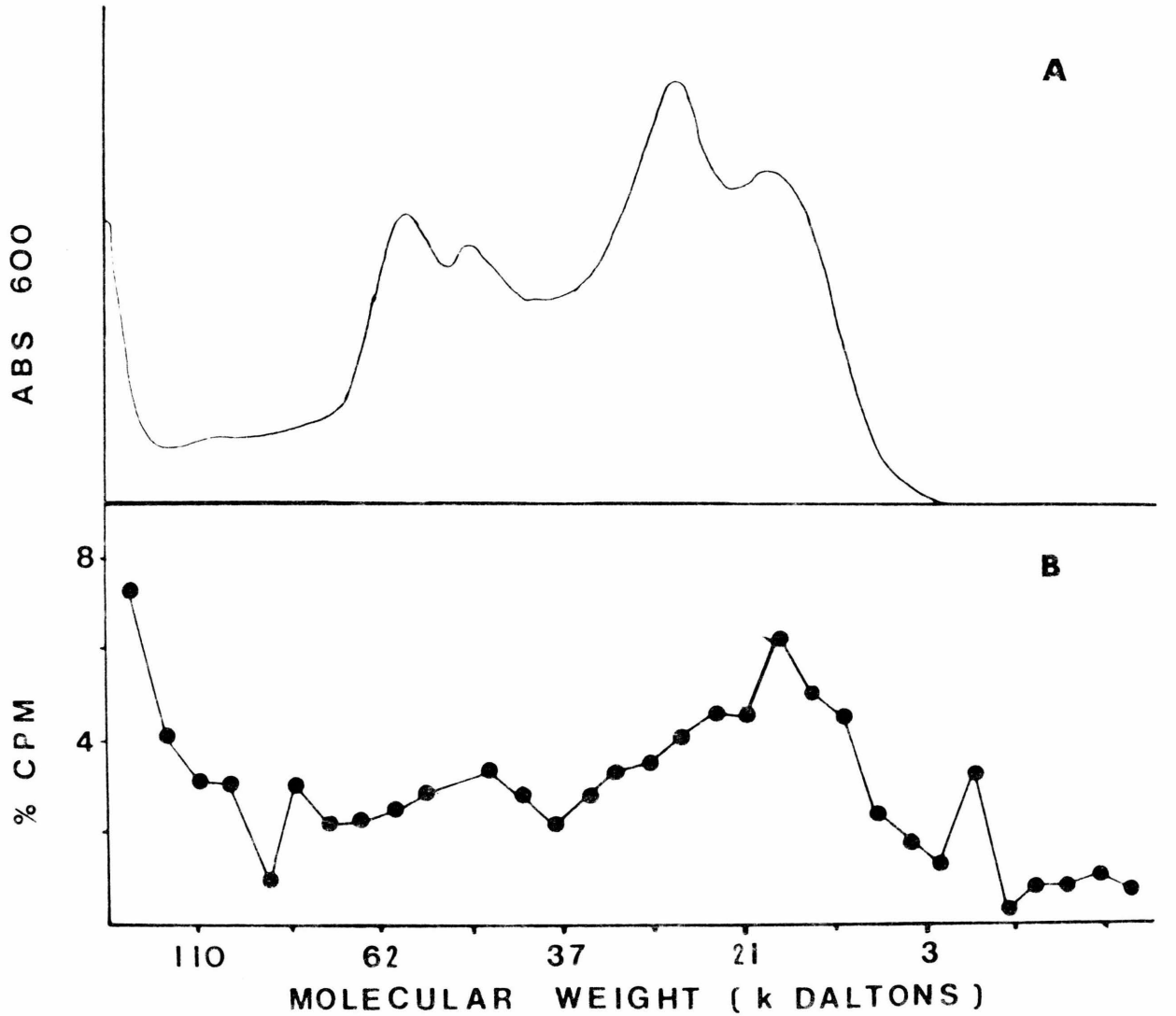
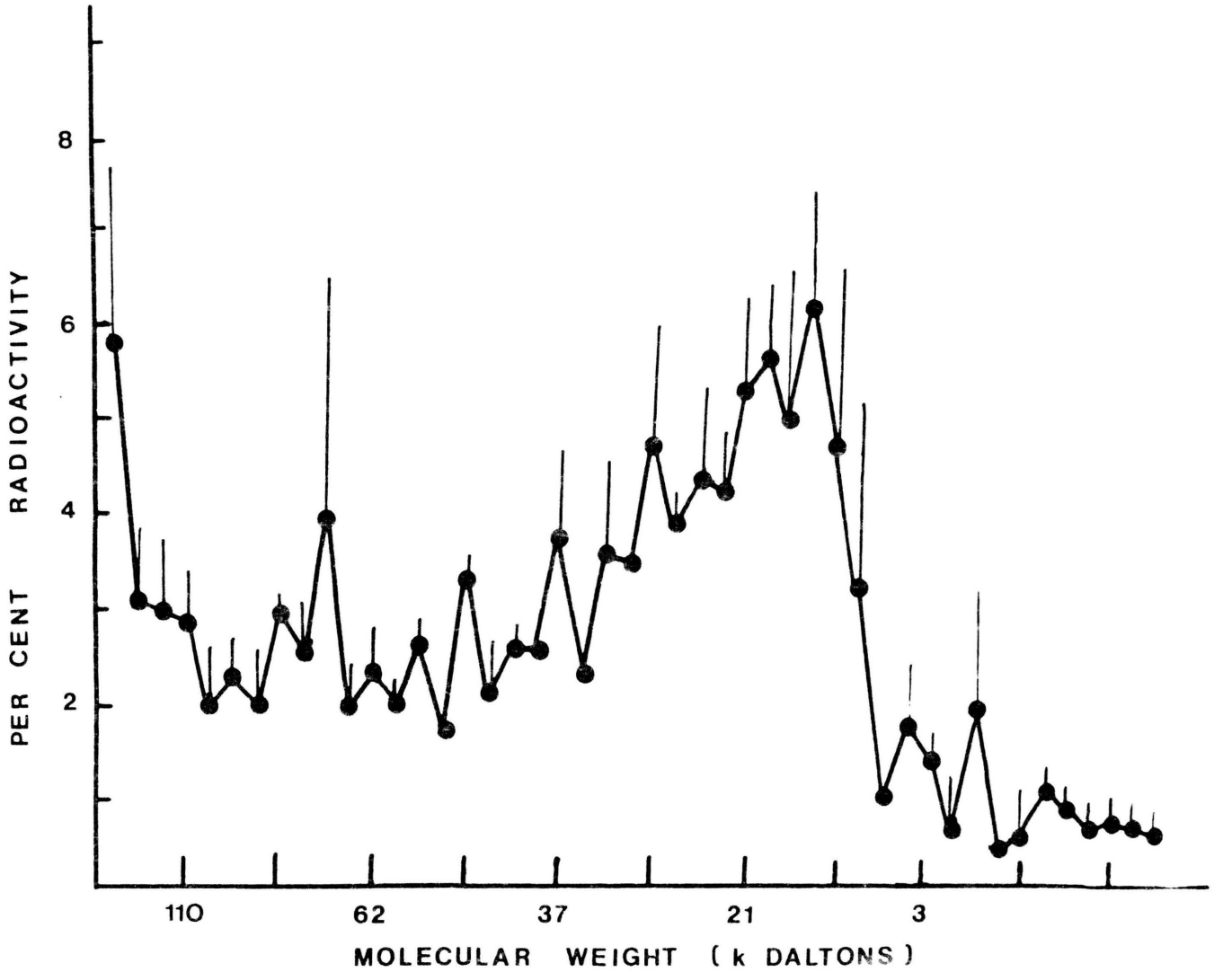

A
ABS 600
B
8
4
% CPM
110
62
37
21
3
MOLECULAR WEIGHT (k DALTONS)

Figure 11. Radiolabeled profile of proteins isolated from myelin. Labeled proteins represent those associated with myelin after neuronal incorporation of ^{3}H-leucine and rapid axonal transport. The isolated myelin was then washed with 10 mM EDTA for 14 to 16 hrs. This figure represents an average of eight gels and is expressed as a per cent of total radioactivity.


PER CENT RADIOACTIVITY
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MOLECULAR WEIGHT (k DALTONS)

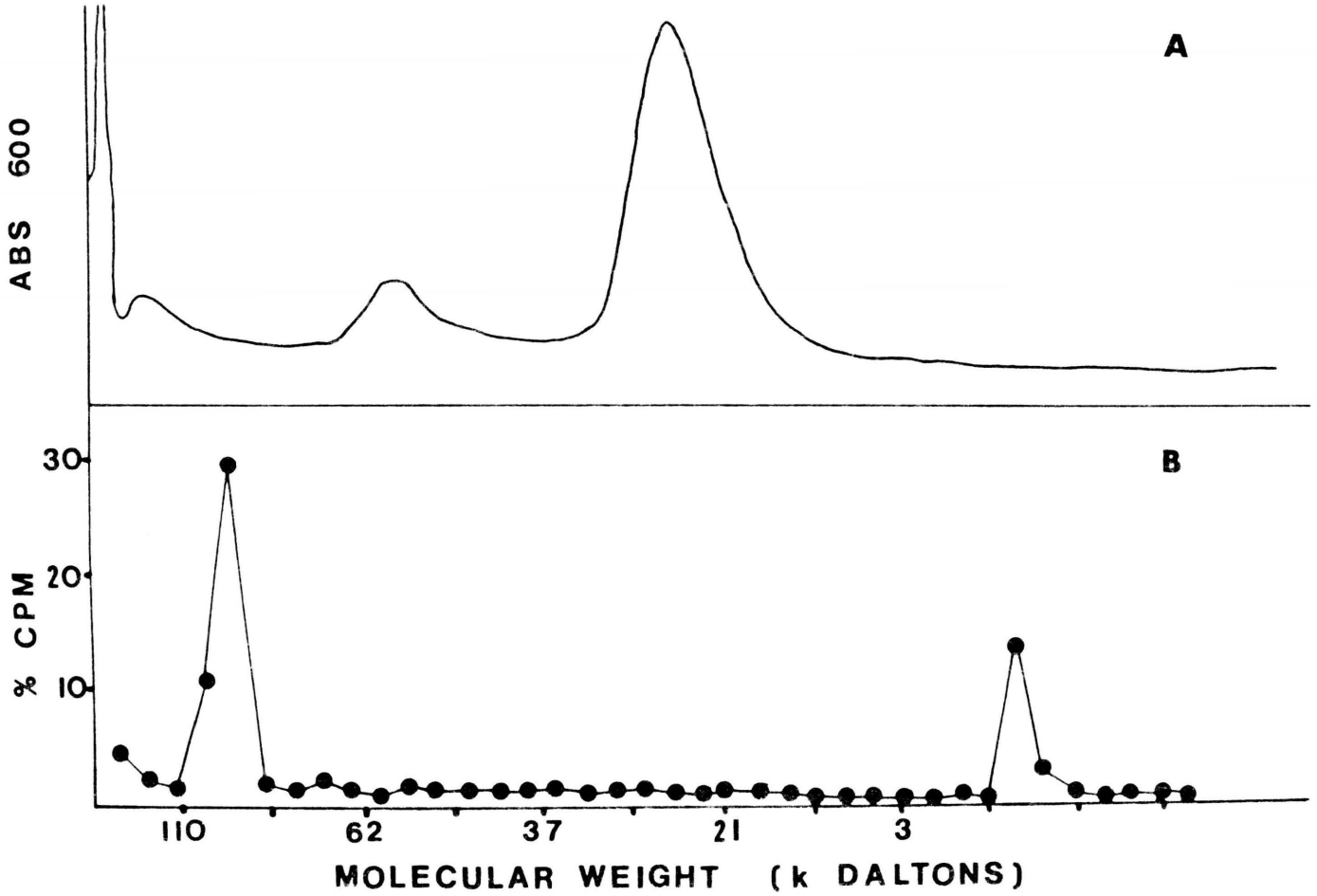
The EDTA-wash was then electrophoresed to determine which proteins were dissociated. The electrophoretic profile is shown in Figure 12A, and the radiolabeled profile in 12B. One of the major myelin proteins shows dissociation, but no labeling by the neuron. Figure 13 shows the average of several radiolabeled profiles of EDTA-wash gels. Note the major radioactive peak appearing around 100k daltons. This corresponds to the reduced peak in the EDTA-washed myelin, and to the efflux. The peak at the far right corresponds to the electrophoretic front, and is believed to be composed of breakdown products, possibly tritiated water.

Figure 14 compares the myelin labeled through neuronal incorporation (C), the neuronally labeled efflux protein (B), and the protein released from neuronally labeled myelin by treatment with EDTA (A). An electrophoretically similar radiolabeled peak appearing at 100k daltons in all three types of experiments is noted by arrows.

Co-electrophoresis of isolated myelin proteins and efflux proteins

In order to determine if the neuronally labeled protein appearing in the 100k dalton range with isolated myelin co-electrophoreses with the neuronally produced protein released from the nerve, an efflux sample was mixed with

Figure 12. Densitometric scan (A) and radiolabeled profile (B) of proteins released from isolated myelin after washing with 10 mM EDTA for 14 to 16 hrs. Myelin was labeled through neuronal incorporation of ^{3}H-leucine and rapid axonal transport. Both scan and radiolabeled profile are from the same gel.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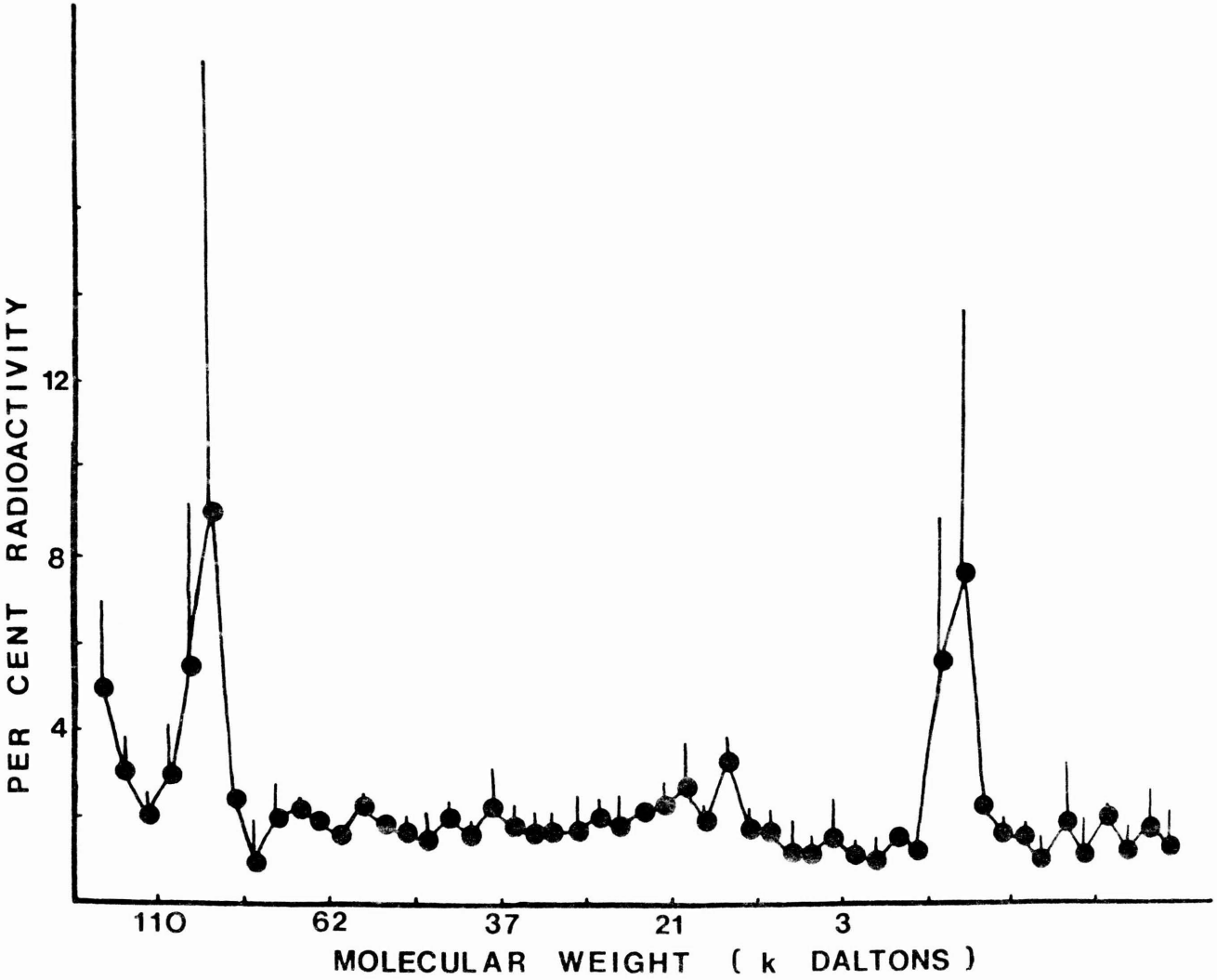
MOLECULAR WEIGHT (k DALTONS)

Figure 13. Radiolabeled profile of proteins released from isolated myelin after washing with 10 mM EDTA for 14 to 16 hrs. Myelin was labeled through neuronal incorporation of ^{3}H-leucine and rapid axonal transport. This figure represents an average of eight gels and is expressed as per cent total radioactivity.


PER CENT RADIOACTIVITY
12
8
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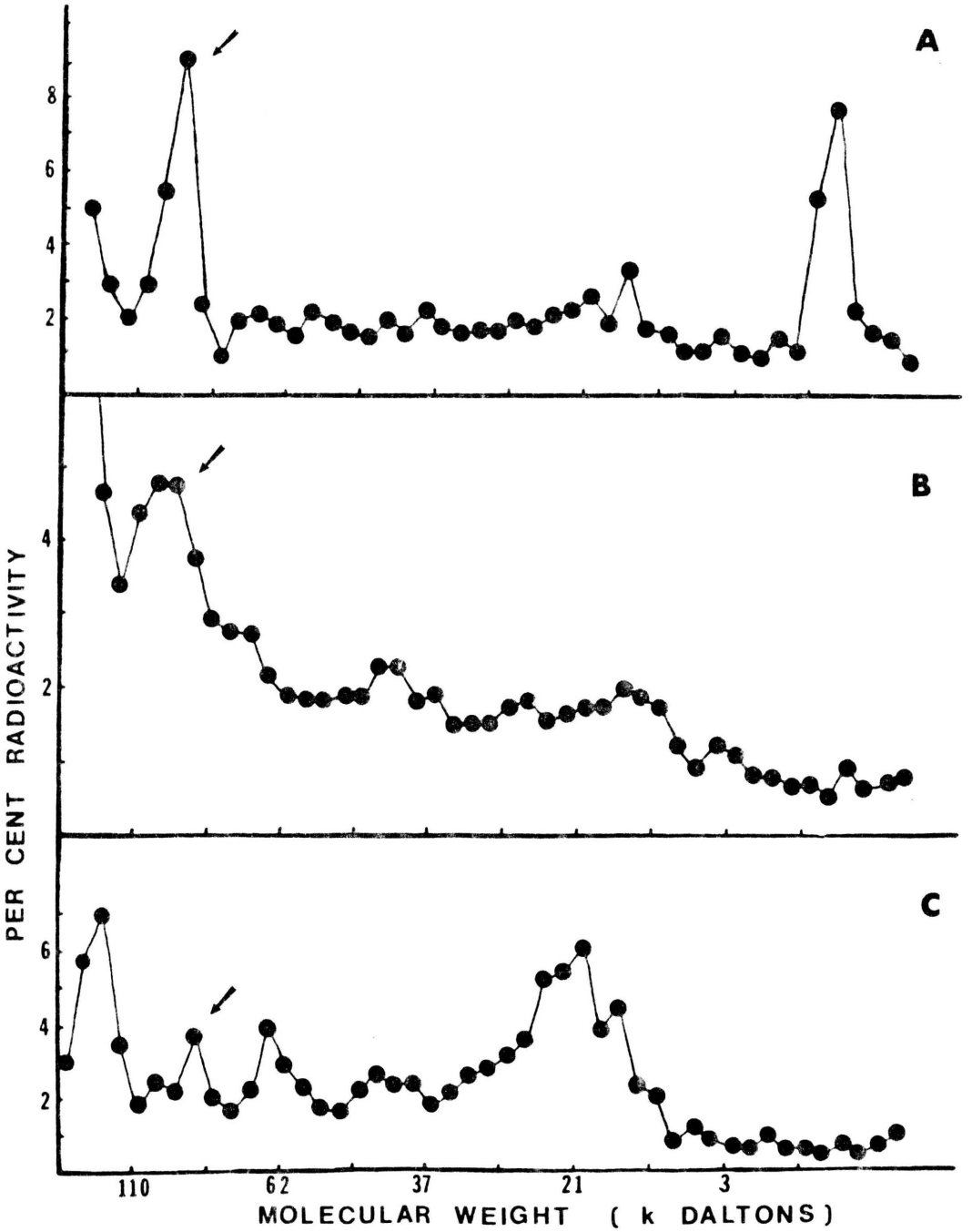
MOLECULAR WEIGHT (k DALTONS)

Figure 14. (A) Radiolabeled profile of proteins released from isolated myelin after washing with 10 mM EDTA for 14 to 16 hrs.
(B) Radiolabeled profile of rapidly transported proteins released from sciatic nerves.
(C) Radiolabeled profile of isolated myelin.
Proteins were labeled through neuronal incorporation of ^{3}H-leucine and distributed in the nerve by rapid axonal transport.
An electrophoretically similar radiolabeled protein is visible in all three (arrows).


A
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C
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PER CENT RADIOACTIVITY
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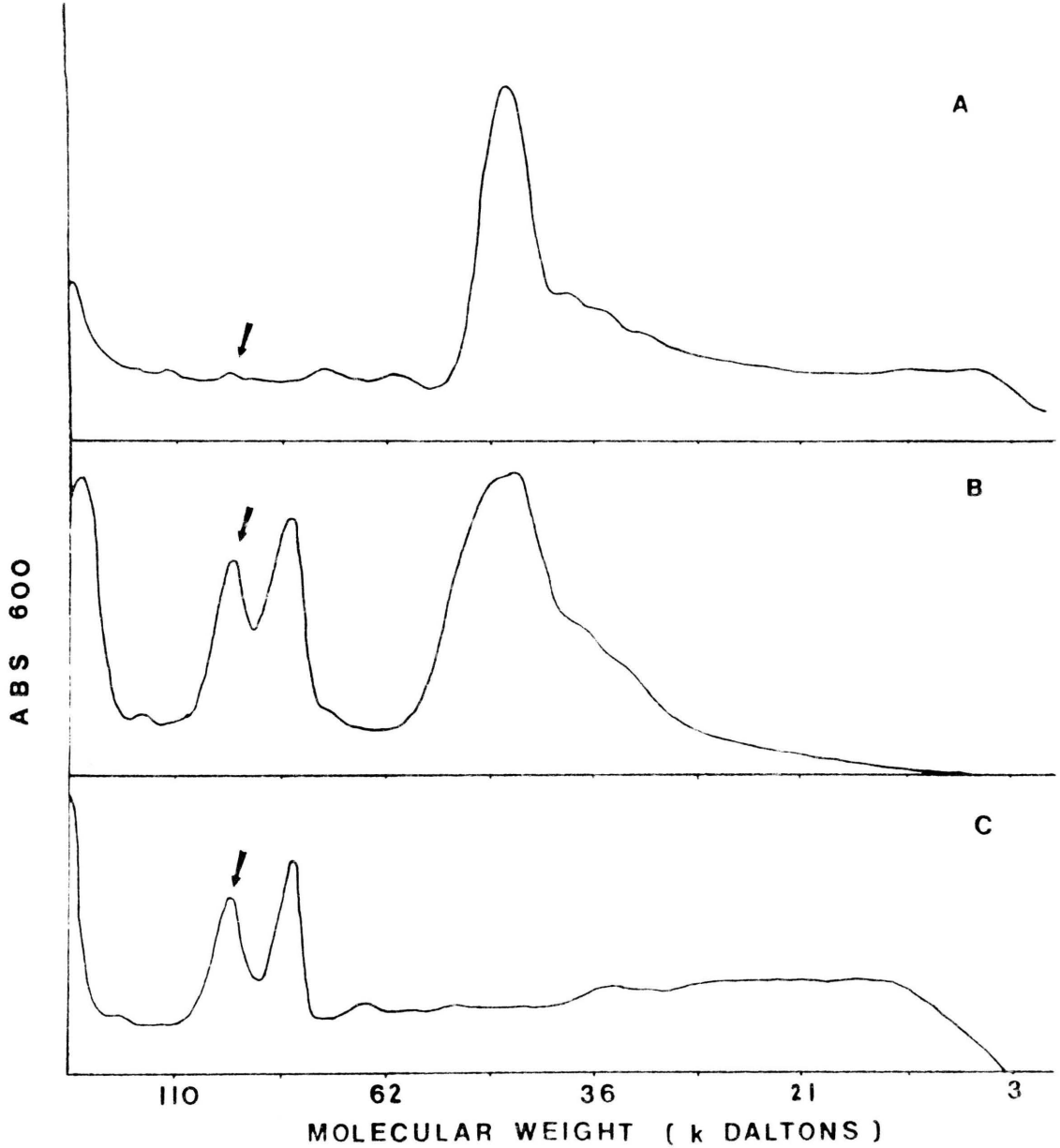
MOLECULAR WEIGHT (k DALTONS)

isolated myelin and electrophoresed on one gel. On separate gels, but electrophoresed simultaneously, were control samples of efflux protein and isolated myelin. If the protein in the 100k dalton region were the same in both samples, one band in the 100k dalton region would be visible. If not, separate bands probably would be visible. Figure 15 shows the results of the co-electrophoresis. Pure efflux shows a protein band around 100k daltons (C), and a plasma protein band (Edwards, et al., 1979), at a slightly lower molecular weight. Pure isolated myelin also shows a minor protein band around 100k daltons (A), as well as the major myelin proteins. The efflux and isolated myelin proteins electrophoresed on the same gel show a protein band in this same region of 100k daltons, with no visible shoulders, suggesting the presence of a single band.

Electrophoretic variability

Several points should be noted concerning the use of electrophoresis in this project. The determination of molecular weight by electrophoresis may vary ± 15 % (Barton, personal communication). In many of the experiments, in order to place an adequate number of cpm on the gel, the number of µg necessary overloaded the ideal electrophoretic system. This procedure of overloading gels has been used by other workers (Prensky, et al., 1975; Elam, 1978; De Vries,

Figure 15. (A) Densitometric scan of isolated myelin.
(B) Densitometric scan of isolated myelin and of rapidly transported proteins released from nerve on the same gel.
(C) Densitometric scan of rapidly transported proteins released from nerve.
An electrophoretically similar protein is visible around 100k daltons on all three scans (arrows).


A
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MOLECULAR WEIGHT (k DALTONS)

et al., 1978). However, overloading tends to cause the protein bands to spread more, and could even cause an apparent shift in molecular weight. The subsequent slicing of these gels magnifies shifts, contributing to the average deviations seen in the data. The counting of gel slices, with the high amount of quenching present, also contributes to the average deviations visible in the data. However, these are the techniques currently in use in this area, and the data obtained may be compared to that in the literature. Variability was also introduced with the isotope used. It was noticed that the percentage of tritiated water increased with time, and although precautions were taken, the specific activities of the proteins examined decreased in proportion to the age of the isotope.

DISCUSSION

The data presented here demonstrate that neuronally synthesized proteins are transported axonally and isolated with myelin. Figures 4 and 5 show that neuronally produced and transported proteins isolated with myelin appear in the high molecular weight region of 100k daltons, as well as in the major myelin proteins. Schwann cell labeling of myelin proteins appears only in the major myelin proteins, as seen in Figures 6 and 7.

The labeling of the major myelin proteins through neuronal incorporation was unexpected, since previous workers showed that neuronally synthesized and transported proteins only appeared in the high molecular weight proteins, not in the major myelin proteins (Elam, 1974, 1975; Autilio-Gambetti, et al., 1975; Prensky, et al., 1975; and Matthieu, et al., 1978). However, these previous studies used in vivo central nervous system preparations, while these data are based on an in vitro peripheral nervous system preparation. It was calculated that the labeling of the major myelin proteins by the neuron was only one-twentieth that of the Schwann cell labeling. This could easily be accounted for by the segment of nerve in the outer well extending from the hole between the wells to the cycloheximide-Ringer solution. Diffusion of ^{3}H-leucine from the inner well to this segment of nerve through the extracellular spaces would make isotope

available to Schwann cells not soaking in cycloheximide solution, and could account for the labeled major myelin proteins.

Studies of the neuronally labeled and transported proteins released into the surrounding fluid show the presence of a radiolabeled protein with a molecular weight around 100k daltons. This protein is electrophoretically similar to the neuronally incorporated high molecular weight protein associated with myelin. It is possible that these two neuronal proteins may be the same protein, since they have a common origin, are axonally transported, co-electrophorese, and have a molecular weight of about 100k daltons.

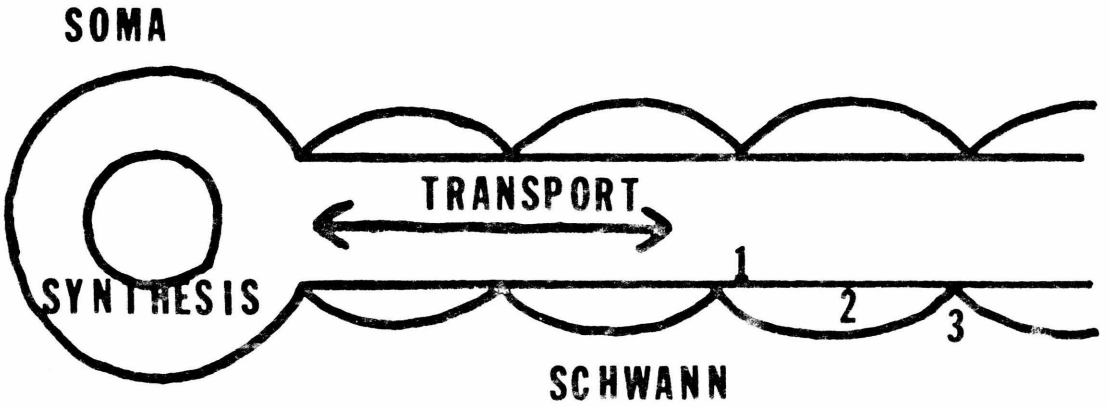
It has been previously suggested that the neuron may produce a diffusable trophic signal released from the axon to interact with either the Schwann cell or myelin (Weinberg and Spencer, 1975, 1976; Hines and Garwood, 1977). It is possible that the high molecular weight proteins in question may act as this trophic signal and appear isolated with myelin. If the efflux protein were released to interact with the Schwann cell or myelin, one would hypothesize a dissociable bonding to the membrane affected, as in the manner of hormone bonding to cell membranes, causing a membrane alteration or enzyme activation to induce further changes in the cell. However, if the transported protein associated with myelin were not the same as the efflux

proteins, but was integral membrane protein, it would be tightly bound to the membrane.

Treatment of the neuronally labeled myelin with EDTA had no effect on the major myelin proteins, suggesting that they are integral membrane proteins. However, the labeled protein of 100k daltons was released from the myelin by the chelating agent, indicating dissociable binding. This reversible binding supports the suggestion that the neuronally labeled high molecular weight protein associated with myelin could serve a trophic function, and may be the same as the efflux protein examined.

It has been suggested that the neuronal protein associated with myelin may be axolemmal, and isolated with the myelin fraction (Elam, 1974, 1975, 1978; Autilio-Gambetti, et al., 1975; Prensky, et al., 1975). This suggestion cannot be verified until improved myelin isolation techniques are developed. However, an axolemmal location would not rule out a possible trophic function for the protein in question. A theoretical model (Figure 16) illustrates possible interactions between neuronally incorporated and transported proteins and the surrounding Schwann cells. The 100k dalton protein is synthesized in the neuron cell body and transported along the axon. It is then released along the axon into the extracellular space as a diffusible signal. It could then bind to any of three sites: 1) the external

Figure 16. The 100k dalton protein is synthesized on the neuron cell body and transported along the axon. It is then released along the axon into the extracellular space as a diffusible signal. It could then bind to any of three sites: 1) the external axolemmal membrane; 2) the myelin membrane; or 3) the external Schwann cell membrane.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axolemmal membrane; 2) the myelin membrane; or 3) the external Schwann cell membrane. If the binding site were axolemmal, it could identify that particular axon as one to be myelinated, stimulating the surrounding Schwann cell by membrane-membrane interactions. The binding to the myelin or Schwann cell membrane would be a more direct interaction for myelin maintenance or activation.

The use of immunological procedures would be very important for further studies of the high molecular weight proteins. It would be of great value to determine if the neuronally labeled protein associated with myelin is immunologically cross-reactive with the efflux protein. A histochemical localization of these proteins would also determine binding sites. The influence of these proteins in tissue culture of myelinated and unmyelinated cells could answer the question of a trophic function and identify the site of interaction.

SUMMARY

1. An investigation was conducted to study the incorporation of neuronally labeled and transported proteins into myelin, and to compare these proteins with those released from the axon and those labeled by the Schwann cells themselves.

2. The following has been demonstrated:

   a. Neuronally synthesized and transported proteins are isolated with myelin.

   b. One of the proteins released from the axons is electrophoretically similar to one of the neuronally synthesized proteins isolated with myelin.

   c. The myelin proteins synthesized by Schwann cells do not show a protein band electrophoretically similar to that of the released neuronal proteins.

   d. Treatment of the neuronally labeled myelin with EDTA releases the protein that is electrophoretically similar to the axonally released proteins, indicating dissociable binding, and similarity between the proteins.

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