Labelling by axonal transport of myelin-associated proteins in the rabbit visual pathway

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After intraocular injections of [^3H]leucine, six regions of the visual pathway of adult rabbit were used to study the spatio-temporal pattern of the slow anterograde axonal transport of radioactive proteins associated with the particulate fraction, the water-soluble fraction and the myelin fraction. Unlike other fractions, myelin-associated labelled proteins represented a time-constant (for a given region) percentage of total tissue radioactivity. This percentage increased from the first half to the second half of the optic nerve and remained high in the chiasma and tract. The peak specific radioactivity of myelin decreased in the same direction. Myelin proteins were separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and the labelling patterns obtained in different regions and at different survival times were compared. At the peak of myelin radioactivity of a given region the label was typically associated with four protein bands, L1, L2, L3 and L4, of 40 000, 44 000, 62 000 and 68 000 mol.wts. respectively. The basic protein, the proteolipid protein and the W1 component (mol.wt. 51 000–53 000) of the Wolfgram proteins were not significantly labelled. The radioactivity associated with the W2 component (mol.wt. 60 000) of the Wolfgram proteins could be derived from the closely migrating L3 component. At shorter survival times no clear labelling pattern could be detected. At longer survival times radioactivity was almost totally localized around band L3. The results presented underline the importance of choosing appropriate experimental conditions to obtain a consistent labelling pattern of myelin-associated proteins and to investigate the possible mechanism responsible for this phenomenon.

Intraocular injection of radioactive amino acids allows the study of the anterograde transport of labelled proteins along the optic pathway (Droz, 1975; Grafstein, 1975). Myelin extracted from the optic pathway under these conditions also contains labelled proteins (Giorgi *et al.*, 1973; Elam, 1974, 1975, 1978; Autilio-Gambetti *et al.*, 1975; Prensky *et al.*, 1975; Giorgi, 1978; Matthieu *et al.*, 1978; Haley & Ledeen, 1979; Giorgi & DuBois, 1980). No conclusive evidence is available to explain this latter phenomenon and the following three possibilities remain to be investigated. Labelled axonal proteins could become associated with myelin lamellae

during subcellular fractionation (cross-contamination). Labelled axolemmal proteins structurally related to the myelin sheath could remain associated with myelin preparations (co-purification). Labelled amino acids, derived from the turnover of axonal proteins, could become available to oligodendrocytes that synthesize myelin proteins (amino-acid reutilization). Each of these possibilities bears some interest for our understanding of myelin biology. Methods for myelin purification (Norton, 1977) would be improved by the elimination of known cross-contaminations. The characterization of specialized axolemmal proteins structurally related to myelin (Schnapp & Mugnaini, 1978) would shed light on axoglial interactions underlying myelination. The demonstration that oligodendrocytes reutilize axonal amino acids, as Schwann cells do with axonal lipids (Droz *et al.*, 1978), would shed light on the ability of neurons to regulate their own myelin sheath (Spencer, 1979).

Abbreviations used: N1 and N2, two equal halves of the optic nerve; T, optic tract; LGB, lateral geniculate body; SC, superior colliculus.

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We are carrying out a series of studies designed to explain the labelling by axonal transport of minor myelin-associated proteins. Before doing this, we found it necessary to define the spatio-temporal pattern of labelling in the adult rabbit. In the present paper we describe such a pattern and we demonstrate the importance of adopting precise experimental conditions (survival time and region of the optic pathway) to obtain an optimal and consistent labelling of myelin-associated proteins.

Experimental

Materials

L-[4,5-³H]Leucine (sp. radioactivity 100 Ci/mmol, 1 mCi/ml) was from The Radiochemical Centre, Amersham, Bucks., U.K. Acrylamide, *NN'*-methylenebisacrylamide, sodium dodecyl sulphate and *NNN'*-tetramethylethylenediamine were purchased from Serva Feinbiochemica G.m.b.H. and Co., Heidelberg, Germany. Polyacrylamide was from BDH Chemicals, Poole, Dorset, U.K. EDTA sodium salt, Coomassie Brilliant Blue R250 and the oligomer peptide standards for molecular-weight determination were from Fluka A.G., Buchs, Switzerland. Cytochrome *c*, carbonic anhydrase, pepsin ovalbumin and bovine serum albumin were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. 2,5-Diphenyloxazole and 1,4-bis-(5-phenyloxazol-2-yl)benzene were from E. Merck, Darmstadt, Germany. Soluene-350 was from Packard Instruments Int., Zurich, Switzerland.

Animals and surgery

Adult albino rabbits (about 3.5 kg body wt.) were used for all experiments. For intraocular injections, the animals were lightly anaesthetized with 1 ml of Nembutal (sodium pentobarbitone; 60 mg/ml) diluted to 3 ml with iso-osmotic saline and slowly injected intravenously. The eye was locally anaesthetized with 2% xylocaine. [³H]Leucine was injected into each eye with a no. 20 needle connected to a delivering microsyringe through nylon tubing filled with liquid paraffin. The needle was inserted through the sclera (2 mm outside the cornea) until its tip appeared behind the lens. The delivery of the labelled compound lasted 3–4 min and the needle was left in position for a further 2 min to allow intraocular pressure to normalize. The general anaesthesia lasted 1–2 h. The animals were fed *ad libitum* and kept under a 12 h light/dark cycle until they were killed with an overdose of Nembutal. The optic nerve was cut 1–2 mm behind the eye and six regions of the optic pathways were dissected as shown in Fig. 1. The dissection was completed about 10 min after death. Samples were frozen in liquid N₂ and stored at –45°C. The material studied at each post-injection time interval was obtained from two

Fig. 1. *Regions of the optic pathway studied*
Abbreviation used: Ch, chiasma. The superior colliculus, which is not shown in the Figure, was separated from the inferior colliculus by a horizontal cut. Lengths of segments are given in cm (± 0.05). The broken line represents the maximum intraocular length of optic axons. The head of the optic nerve is at the centre of a band inside which most retinal ganglion cells are localized (Vaney & Hughes, 1976).

rabbits. Thus each region of the pathway (at each time interval) was studied in four pooled pieces of tissue (with the exception of the two chiasmata).

Subcellular fractionation

All operations were carried out at 0–4°C. Tissue samples were homogenized in 0.8 M-sucrose with an all-glass homogenizer (30 strokes after tissue disruption). Samples (4 ml of about 5% homogenate) were overlaid by 1 ml of 0.32 M-sucrose and centrifuged at 125 000 *g* for 45 min in a Beckman SW-50 rotor. The material present at the interface was dispersed in 40 ml of water and left to stand for 1 h. This water-shocked crude myelin fraction was centrifuged at 12 000 *g* for 30 min. The pellet was resuspended in 0.8 M-sucrose containing 1 mM-EDTA adjusted to pH 7.2 with NaOH. This suspension was homogenized, centrifuged, water-shocked and re-centrifuged as described above (except that both sucrose solutions contained EDTA). The final pellet represented the myelin fraction. This method of myelin preparation was essentially as described by Giorgi *et al.* (1973). In some experiments myelin was prepared, for comparison, by the method of Norton & Poduslo (1973).

The material left in the centrifuge tube after the first centrifugation step was dispersed and the

medium was adjusted to 0.32M-sucrose. After centrifugation at 160 000g for 3 h in a Beckman 50 rotor, the supernatant represented the water-soluble fraction and the pellet the non-myelin particulate fraction.

Determination of radioactivity

Portions of the myelin fraction were freeze-dried, weighed, wetted with a few drops of water and solubilized with Soluene-350. Their radioactivity was determined in 10 ml of toluene containing 0.5% 2,5-diphenyloxazole and 0.03% 1,4-bis-(5-phenyloxazol-2-yl)benzene with a Searle Delta 300 spectrometer (efficiency about 40%). Portions of the initial homogenate and of the two non-myelin fractions were treated with final concentration of 10% trichloroacetic acid and centrifuged. The pellets were dissolved in Soluene-350 for radioactivity determination as described above.

Polyacrylamide-gel electrophoresis

Myelin proteins were separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis as described by Laemmli (1970), with the following modifications. Proteins were dissociated by resuspending wet myelin in the sample buffer and heating at 40°C for 2 h. Separation was carried out in a vertical gel slab containing a 7–15% continuous polyacrylamide gradient. The electrode buffer was at pH 8.8. Proteins migrated overnight with a current of 20 mA (50 V).

Gel slabs were stained in 0.2% Coomassie Blue, 10% acetic acid and 10% ethanol and destained by diffusion in the same mixture without stain. The densitometric trace of gel strips was obtained at 550 nm in a Beckman 25 spectrophotometer and stored in a Nova 2 Data General Computer. Gel strips were sliced and fractions were incubated for 4 h in Soluene-350 at 40°C for radioactivity determination as described above. Radioactivity data were transferred to the computer to obtain results in the form of histograms superimposed on the densitometric tracing.

For the molecular-weight determination of minor myelin proteins, myelin samples were separated by gel electrophoresis in parallel with the following commercial samples of known molecular weight: cytochrome c, 12 000; carbonic anhydrase, 30 000; pepsin, 36 000; ovalbumin, 45 000; bovine serum albumin, 67 000. For this computation the myelin basic protein was regarded as mol.wt. 18 500 and the proteolipid protein as mol.wt. 24 000 (Braun & Brostoff, 1977). Comparable results were obtained when a mixture of commercial oligomer peptides of molecular weight ranging between 14 000 and 71 000 was used.

Other determinations

Protein determination was carried out by the method of Lowry *et al.* (1951) after dissolving the sample in 0.1% sodium dodecyl sulphate.

Results

Protein radioactivity associated with total tissue and the myelin fraction

The spatio-temporal pattern of radioactivity of total tissue protein is illustrated in Fig. 2. The amounts of radioactivity are indicated as d.p.m., not as protein specific radioactivity, because this value would not provide information on the degree of labelling of protein associated with optic nerve fibres. Information on the protein specific radioactivity of myelin can be derived from data in Table 1. The range of survival times chosen was suitable to study the slow phase of axonal transport (Karlsson & Sjöstrand, 1971). Slowly transported proteins moved along the optic pathway as a broad wave with peaks approximately at 4–5 days in N1, 5 days in N2, 11 days in the chiasma, 12–13 days in T, 13 days in LGB and more than 13 days in SC. The broadness of the radioactivity waves can be explained (cf. Fig. 1) by the wide range of intraocular lengths of optic fibres (0–1.5 cm; Vaney & Hughes, 1976), compared with the relatively short length of the optic nerve and tract (1.5 and 0.9 cm respectively). Because of this situation, the curves obtained from contiguous segments of the pathway overlapped considerably. The relatively high amounts of protein radioactivity found at short survival times (1–7 days) in LGB and particularly in SC have been described by Karlsson & Sjöstrand (1972) and indicate the existence of a phase of axonal transport with a speed that is intermediate between the rapid and the slow phases (Karlsson & Sjöstrand, 1971; Willard *et al.*, 1974).

Preliminary control experiments with delipidated myelin, as well as subsequent experiments on myelin proteins separated by gel electrophoresis, showed that more than 90% of the myelin radioactivity was localized in proteins. The spatio-temporal pattern of myelin-associated radioactivity was very similar to that of total tissue in all regions (Fig. 2). Quantitatively, this corresponded to the fact that the percentage of total tissue radioactivity associated with myelin remained essentially constant throughout the range of survival time studied (Fig. 3). The behaviour of myelin-associated radioactivity differed considerably from that of the soluble and particulate fractions, which underwent marked (and opposite) time-course changes. A low correlation between the percentage of radioactivity in myelin and survival time was established with the rank

Fig. 2. Radioactivity of total tissue proteins and of myelin-associated proteins

Values, obtained at nine survival times after intraocular injection of [³H]leucine (50 μCi) in six regions of the optic pathway (cf. Fig. 1), are averages of triplicate determinations of samples obtained from tissue pooled from two animals. Abbreviation used; Ch, chiasma.

Table 1. Parameters concerning tissue and myelin recovered from six regions of the optic pathway

Values refer to material pooled from two animals for each experiment and are means for nine experiments ± S.D. Values of peak specific radioactivity are approximate because they are based on myelin peak radioactivity derived from the curves of Fig. 2 and on the average values of myelin dry weight from this Table. Myelin had 0.197 ± 0.009 mg of protein/mg dry wt. (mean ± 1 S.D.) with no significant differences between regions. The percentage of total tissue protein recovered with myelin was 39% in the optic nerve and 55% in the optic tract. The statistical significance of differences between regions in the percentage of radioactivity associated with myelin is discussed in the Results section. Abbreviation used: Ch, chiasma.

Regions	Tissue wet wt. (mg)	Myelin dry wt. (mg)	Myelin radioactivity (% of tissue d.p.m.)	Peak specific radioactivity of myelin (d.p.m./mg dry wt.)
N1	39.2 ± 4.8	4.9 ± 0.8	10.6 ± 3.0	44 000
N2	37.8 ± 3.5	6.6 ± 0.9	13.7 ± 3.6	24 000
Ch	27.5 ± 1.6	6.0 ± 0.9	15.1 ± 3.1	14 000
T	85.1 ± 13.6	20.6 ± 3.7	14.2 ± 1.8	11 000
LGB	372.3 ± 44.2	54.3 ± 11.7	9.3 ± 1.6	2200
SC	508.6 ± 56.8	53.8 ± 8.0	5.6 ± 0.5	960

correlation test of Spearman (Siegel, 1956). Thus it was possible to calculate, for each region, an average value for the percentage radioactivity in myelin. This

value, independent of survival time, differed throughout the optic pathway (Table 1). It was about 10% in N1, 9% in LGB and 5% in SC. No statistical

Fig. 3. Comparison between the radioactivity of myelin-associated proteins and that of the other two subcellular fractions

Values were obtained from the same experiments shown in Fig. 2. ●, Non-myelin particulate fraction; ○, water-soluble fraction; △, myelin fraction. The lateral geniculate body and the superior colliculus were selected to demonstrate the difference between myelin and non-myelin fractions because these two regions, owing to their distance from the eye, displayed a wider range of temporal changes of the non-myelin fractions. The same phenomenon was, however, present in all regions of the optic pathway. Other subcellular fractions, obtained as by products of myelin preparation (cf. the Experimental section) accounted for the remaining total tissue radioactivity, whose recovery varied from 83% to 98%.

Fig. 4. Separation of myelin-associated proteins by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

Myelin was prepared from the optic chiasma and tract and proteins were separated along a continuous 7–15% polyacrylamide gradient. No substantial differences were found between myelin samples obtained from different regions of the optic pathway. The origin is on the left. The total length of the gel was 8.0cm. Abbreviations used: BP, basic protein; PLP, proteolipid protein; W1, lower molecular-weight Wolfgram protein; W2, higher molecular-weight Wolfgram protein; L1, L2, L3 and L4, protein bands that become labelled by axonal transport (cf. Fig. 5). After calibration of the gel with two different sets of molecular-weight markers, the following apparent molecular weights were found: L1, 40000; L2, 44000; W1, 51000–53000 (doublet); W2, 60000; L3, 62000; L4, 68000.

difference existed among the percentage values obtained for N2, chiasma and T, when a two-tailed Student's *t* test was applied ($2P=0.5$). The average of the pooled values of these three regions (14.3%) was relatively high and differed significantly from that of N1 ($0.02 > 2P > 0.01$) and from that of LGB ($2P < 0.001$).

Characterization of myelin-associated proteins by gel electrophoresis

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis along a continuous 7–15% polyacrylamide gradient produced a good separation of higher molecular-weight components associated with myelin, whereas the basic protein and the proteolipid protein migrated close to each other (Fig. 4). Among minor components, two were recognized as Wolfgram proteins on the basis of their molecular

weight, according to the characterization of Nussbaum *et al.* (1977). Their apparent molecular weight was 60000 for W2 and 51000–53000 for W1. The latter was present as a doublet.

When myelin was extracted from the optic chiasma and tract at 13 days after the intraocular injection of [^{3} H]leucine (peak of myelin-associated radioactivity) a characteristic radioactivity pattern was obtained from protein separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Fig. 5a). The same radioactivity pattern was obtained when myelin was extracted from the optic nerve 5 days after injection. Therefore this pattern was typical of myelin preparations obtained when the peak of the slow phase of axonal transport reached a given region of the optic pathway (cf. Fig. 3). The radioactivity was consistently found associated with four protein bands (L1, L2, L3 and L4), the higher amounts of radioactivity being always associated with bands L2 and L3. These protein bands had the following apparent molecular weights: L1, 40000; L2, 44000; L3, 62000; L4, 68000. WP1 was not radioactive. WP2 migrated too close to L3 to establish, under the present experimental conditions, whether it was radioactive. The basic protein and the proteolipid protein did not appear to be radioactive. No substantial difference, in both the

Fig. 5. Radioactivity associated with myelin proteins separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

The gels were cut into subsequent strips of 2 mm in the proximal and distal regions, and into strips of 1 mm in the intermediate-molecular-weight region for a better localization of labelled bands. (a) Myelin prepared from the optic chiasma and tract 13 days after intraocular injection of [^3H]leucine ($70\mu\text{Ci}$); (b) myelin prepared from the optic chiasma and tract 2 days after injection; (c) myelin prepared from the first half of the optic nerve 13 days after injection.

densitometric tracing and the radioactivity pattern, was found when myelin was prepared as described by Norton & Poduslo (1973).

No definable radioactive pattern was obtained when myelin was extracted before the peak of slow axonal transport had reached the region of the optic

pathway studied. Fig. 5(b) shows the gel radioactivity distribution obtained when myelin was extracted from the optic chiasma and tract 2 days after injection. No protein band had a radioactivity value clearly higher than the background. In this region, the radioactive pattern shown in Fig. 5(a) began to appear with a certain clarity only at 7–9 days after injection.

The adoption of a too long survival time also affected the gel radioactivity pattern typically obtained around the peak of slow axonal transport. When myelin was extracted from the first half of the optic nerve 13 days after injection, myelin-associated radioactivity was almost totally limited to protein bands L3 and W2 (Fig. 5c). On the other hand, the protein radioactivity pattern of myelin extracted from this region 3–4 days after injection was that typical of survival times corresponding to the peak of the slow axonal transport (Fig. 5a).

Discussion

The aim of the present study was to define the experimental conditions with which one can obtain maximal labelling of minor protein components associated with optic pathway myelin after intraocular injection of radioactive amino acids in the adult rabbit. The information provided here will be used for subsequent studies (see below) designed to explain how radioactivity finds its way into the myelin fraction.

The rate of transport of proteins along the optic nerve and tract demonstrated here is well in agreement with that reported previously by Karlsson & Sjöstrand (1971). Considering an average intraocular portion of optic axons of 0.75 cm (Fig. 1), the distance between retina and the lateral geniculate body of 3.55 cm is covered in about 16 days, which corresponds to the slow phase of axonal transport with a rate of 2 mm/day. The present detailed spatio-temporal study confirms the suggestion, previously based on few experimental points (Giorgi *et al.*, 1973), that myelin-associated proteins become labelled in conjunction with the slow phase of axonal transport. This is in agreement with similar studies carried out in the goldfish optic pathway (Elam, 1975, 1978). The peak specific radioactivity of myelin decreases from the first half of the optic nerve to the superior colliculus (Table 1). Thus if myelin with high specific radioactivity is needed, one should use the first half of the optic nerve 4 days after injection. If a larger amount of myelin is needed, one should use the chiasma pooled with the optic tract 13 days after injection. The specific radioactivity of this latter material is still compatible with the determination of radioactivity associated with bands after separation by gel electrophoresis (Fig. 5). Such analysis is not possible

with myelin extracted from the lateral geniculate body and the superior colliculus, both of which may contain large amounts of unlabelled myelin (i.e. not related to the optic pathway). Further reasons to use the chiasma–tract preparation are the facility of homogenization in the absence of the dura mater, and the possibility of diffusion of labelled precursors along the initial portion of the optic nerve after intraocular injection (Chihara, 1979; Haley *et al.*, 1979). However, there are reasons to believe that the myelin-associated radioactivity described here is not due to such a phenomenon. In fact, significant amounts of trichloroacetic acid-soluble radioactivity were only found at short survival times (Sjöstrand & Karlsson, 1969) and in regions close to the eye (Chihara, 1979; Haley *et al.*, 1979), whereas protein radioactivity described here builds up at long survival times and in all regions of the optic pathway (Fig. 2). Furthermore, myelin preparations obtained from the optic nerve and those obtained from the optic tract have the same radioactivity pattern (at survival times close to the peak of slow transport). Known myelin proteins should be clearly labelled in the optic nerve (but not in the optic tract), if extra-axonal diffusion of labelled amino acids was responsible for the labelling of myelin-associated proteins.

The problem of adopting correct experimental conditions does not concern only the amount of myelin-associated radioactivity. The present study demonstrates the importance of choosing a defined region of the optic system and a rather precise survival time after intraocular injection, to study the labelling of defined protein components associated with the myelin fraction. In fact the adoption of a survival time sufficiently remote from the peak of the slow axonal transport can affect the type of radioactivity pattern of proteins separated by gel electrophoresis to the point of obtaining no definable pattern (too short survival times; Fig. 5b) or a different pattern (too long survival times; Fig. 5c). This latter gel radioactivity pattern, where essentially only two protein components are labelled, may be due to the longer half-life of these proteins compared with the other ones that are labelled at the peak of the slow axonal transport. Pooling together the optic nerve and tract would correspond to the adoption of an incorrect survival time and it would modify the pattern of radioactivity of proteins separated by gel electrophoresis. This problem becomes a serious one when developing animals are used and optic nerves and tracts are pooled to obtain enough tissue (Autilio-Gambetti *et al.*, 1975; Prensky *et al.*, 1975; Matthieu *et al.*, 1978; Haley & Ledeen, 1979). In fact, in the optic pathway of developing rabbits it is almost impossible to define the spatio-temporal pattern of the slow axonal transport, because its rate corresponds roughly to

that of axonal elongation due to growth (Hendrickson & Cowan, 1971). Differences in gel radioactivity patterns between the present work and that of Autilio-Gambetti *et al.* (1975), Prensky *et al.* (1975) and Matthieu *et al.* (1978) are probably accounted for by the above problem, as well as by different gel electrophoresis systems. As already mentioned in the Results section, methods used to purify myelin should not be responsible for differences in gel radioactivity patterns.

The first study on the relationship between axonal transport and myelin proteins in the rabbit optic pathway (Giorgi *et al.*, 1973) reported that the proteolipid protein, as well as some minor components, become labelled several days after intraocular injection of ^3H -labelled amino acids. We now know that the labelling of the proteolipid protein was due to its co-migration with a labelled minor protein component, a phenomenon that occurs in phenol/formic acid gel electrophoresis, but not in the sodium dodecyl sulphate gel system (Giorgi & DuBois, 1980). Only traces of radioactivity associated with the basic protein have been reported (Giorgi *et al.*, 1973; Prensky *et al.*, 1975; Matthieu *et al.*, 1978). On the other hand, significant amounts of radioactivity have been found associated with protein bands defined as Wolfgram proteins (Elam, 1975; Prensky *et al.*, 1975; Giorgi, 1978; Figs. 3 and 4 in Matthieu *et al.*, 1978), which are considered true myelin components (Roussel *et al.*, 1978). The Wolfgram protein is, in fact, a family of related polypeptides with molecular weights reportedly ranging between 23 000 and 62 000 (Wiggins *et al.*, 1974; Nussbaum *et al.*, 1977; Waehneltdt & Malotka, 1980). If these proteins were really labelled by axonal transport, one would be obliged to propose that a process of amino-acid reutilization is taking place in a glial compartment specialized for the synthesis of the Wolfgram protein, as suggested by Giorgi (1978). To clarify this critical point we adopted a polyacrylamide-gel system that gave an optimal separation of the higher molecular-weight components. The 7–15% gradient system allowed us to establish that the label previously found associated with Wolfgram proteins in a phenol/formic acid-gel system (Giorgi, 1978; Giorgi & DuBois, 1980) and in other sodium dodecyl sulphate gel systems (Elam, 1975; Prensky *et al.*, 1975; Figs. 3 and 4 in Matthieu *et al.*, 1978) is in fact associated with minor protein bands that are different from the two immunologically related Wolfgram proteins of mol.wts. 54 000 and 62 000 (Nussbaum *et al.*, 1977). We are still not sure whether the radioactivity associated with L3 (specially in the type of experiment described in Fig. 5c) is also derived from the closely migrating W2 component. Gel autoradiography after intraocular injection of [^{35}S]methionine would clarify this point. It should also be noted that

the classification of our protein bands W1 and W2 is based on the close similarity in molecular weight with those characterized by Nussbaum *et al.* (1977). However, according to Waehneltdt & Malotka (1980) W1 and W2 are the doublet that we call W1 (molecular-weight difference 2000–3000). On the other hand the molecular weights of W1 and W2 calculated from their amino-acid composition (Nussbaum *et al.*, 1977), assuming three methionine residues, suggest a molecular-weight difference of 8000–9000. Amino-acid analysis of protein extracted from the gel would clarify this point. Thus, the present results (Fig. 5) demonstrate the absence of labelling associated with W1 only.

The possible association of tubulin (mol.wt. 56 000) and actin (mol.wt. 42 000) with myelin prepared from the mammalian brain has been reported (Gozes & Richter-Landsberg, 1978; Waehneltdt & Malotka, 1980). Contaminating cytoskeletal proteins could, therefore, account for some of the radioactivity associated with myelin under the present experimental conditions. Both L1 and L2 have molecular weights similar to that of actin. The molecular weights of L3 and L4 are too high if compared with that of tubulin.

It is generally accepted that most water-soluble proteins move along the axon with the slow phase of transport, whereas membrane-bound proteins are mainly transported by the rapid phase (Droz, 1975; Grafstein, 1975; Lorenz & Willard, 1978). Our data on temporal changes in the percentage of tissue protein radioactivity associated with the soluble and particulate fractions (Fig. 3) agree with this concept. The constant percentage associated with the myelin fraction clearly differs from that of these other two fractions. This seems to support the idea, without proving it, that myelin-associated proteins are not simply derived by cross-contamination from one or the other of these two fractions. We are currently subfractionating myelin labelled by axonal transport to characterize a possible contaminating fraction. Although our data suggest that no known myelin protein becomes labelled by axonal transport, we cannot presume that all myelin proteins have already been described (Zanetta *et al.*, 1977). Thus the possibility of amino-acid reutilization by an oligodendrocyte compartment specialized in synthesizing one or more minor myelin proteins still requires investigation. For this purpose we have injected inhibitors of protein synthesis around the optic chiasma and tract between 5 and 13 days after intraocular injection of label, to check for changes in the labelling pattern of myelin-associated proteins. By using high-resolution autoradiography we have checked whether compact myelin lamellae or peripheral glial loops are labelled. Immunocytochemical methods will ultimately be necessary to investigate the idea that myelin-associated proteins labelled by

axonal transport may represent axolemma components of the paranodal axoglial junctions (Schnapp & Mugnaini, 1978) co-purifying with the myelin fraction.

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