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The Identity of a Myelin-Like Fraction Isolated from Developing Brain

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1. A myelin-like membrane fraction was isolated from developing rat brain by a new method. 2. The chemical composition and morphology of the fraction are described. 3. The myelin-like fraction is similar to myelin in characteristic enzyme activity but differs in the absence of basic protein and cerebrosides. No similarity to other subcellular fractions was observed. 4. It is suggested that the myelin-like fraction is a stage in the formation of compact myelin from glial plasma membrane. 5. 'Early' myelin consists of the myelin-like and compact myelin fractions from developing brain.

During the early stages of myelination in both the central and peripheral nervous system electron micrographs indicate that the first myelin membranes are only loosely wound around the axon; later compact myelin lamellae form with the appearance of a clear intraperiod line (Peters & Vaughan, 1970). Histological changes in myelination are also seen by light microscopy (Wolman, 1957; Duckett & Blunt, 1967). Thus although myelin rings in the kitten optic nerve can be first detected by lipid stains at about 10 days after birth (Banik, Blunt & Davison, 1968) osmiophilia does not develop until about 25 days after birth. Since the whole nerve was conspicuously deficient in cerebrosides and contained a high proportion of phospholipid with short-chain fatty acids it was considered possible that the 'early' myelin formed during the first few weeks of myelination had a different composition from that of mature myelin (Davison, Cuzner, Banik & Oxberry, 1966). Biochemical studies also suggested that myelin undergoes changes in composition during development. Horrocks, Meckler & Collins (1966) demonstrated a difference in the lipid composition of myelin isolated from developing mouse brain compared with that in the mature animal. Changes have also been reported in the chemical composition of 'early' myelin isolated by centrifugation from rat (Cuzner & Davison, 1968; Eng & Noble, 1968), mouse (Horrocks, 1968), rabbit (Dalal & Einstein, 1969) and human (Eng, Chao, Gerstl, Pratt & Tavaststjerna, 1968) brain during development. The phospholipid (particularly phosphatidylcholine)/cholesterol ratio was higher and relatively little cerebroside was found in developing compared with mature myelin. As maturation proceeds the composition of the myelin gradually becomes similar to that of the adult brain (Cuzner & Davison, 1968).

Since even in the initial stages of myelination electron micrographs indicate that some compact as well as loose myelin is present (Bass, Netsky & Young, 1969) it seemed possible that two types of myelin were present in the crude myelin isolated by centrifugation. Thus purified myelin prepared by osmotically shocking and density-gradient centrifugation gave a preparation qualitatively similar to mature myelin (Eng & Noble, 1968; Banik & Davison, 1969), and during purification a second myelin-like fraction could be isolated from the crude myelin (Banik & Davison, 1969). The myelin-like fraction had a relatively high phospholipid/cholesterol ratio and contained little cerebroside and thus resembled plasma membranes (Ashworth & Green, 1966) in lipid composition rather than adult myelin. Examination of the myelin-like fraction by electron microscopy showed it to contain predominantly single membrane vesicles, but small amounts of unidentified fragments were also seen; for this reason we have used an alternative and improved procedure for preparing this fraction and purified myelin. Evidence is presented for the purity and identity of both and some results on the metabolism of lipid and protein in each of the myelin fractions are reported.

EXPERIMENTAL

Materials. Wistar rats of either sex were used throughout this study. Animals up to 20 days of age were decapitated and young adult rats were lightly anaesthetized with

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chloroform before exsanguination. Brains were quickly removed into precooled beakers, weighed in ice-cold beakers and immediately homogenized.

Preparation of subcellular fractions. The initial isolation of crude mitochondrial pellet was carried out essentially by the technique of De Robertis, de Iraldi, Lores Arnaiz & Salganicoff (1962) except that the brain was homogenized in a tight-fitting Teflon–glass homogenizer (clearance 0.15–0.23 mm) with four up-and-down strokes at a speed of 1500 rev./min for 2 min. The mitochondrial pellet was suspended (33% wt. of original tissue/v) in 0.32 M-sucrose solution containing 10 μM-Ca^{2+} (except when determination of $Na^+ + K^+$-stimulated adenosine triphosphatase, EC 3.6.1.3, was performed) in a loose-fitting Teflon–glass homogenizer. The suspension was layered over a discontinuous gradient of 1.4–0.8 M-sucrose solution containing 10 μM-Ca^{2+} [incubated for 1 h at 37°C and then stored at 0°C as described by De Robertis *et al.* (1962) and Lapetina, Soto & De Robertis (1967)] and centrifuged at 53000*g* for 2 h (MSE 50, rotor 2417). This led to the separation of five distinct layers, which have been designated A, B, C, D and E by De Robertis *et al.* (1962). The upper A layer containing crude myelin was treated with 9 vol. of water and left overnight at 0°C. The osmotically shocked myelin was then centrifuged at 12500*g* for 10 min to give the purified myelin fraction (Suzuki, Poduslo & Norton, 1967). The supernatant was removed, care being taken not to disturb the purified myelin pellet. Centrifugation of this supernatant at 78000*g* for 1 h gave a pellet containing the myelin-like fraction. If the crude myelin is not osmotically shocked only one major band (at 0.65 M-sucrose) can be obtained on separation on a continuous density gradient. Under the same conditions but after osmotic shock a pellet of myelin-like material (density greater than 1 M-sucrose) can be separated.

Electron microscopy. Pellets were fixed in buffered osmium tetroxide solution, pH 7.3 (Palade, 1952), and embedded in Araldite, and ultra-thin sections were prepared.

Enzyme assay. The method of Kurihara & Tsukada (1967) was employed and the conditions were as described by Banik & Davison (1969). In the determination of 2′:3′-cyclic nucleotide 3′-phosphohydrolase all samples were suspended in an equal volume of 1% (v/v) Triton X-100 in water. The tubes were kept in ice for 60 min and shaken occasionally.

Determination and separation of proteins. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) and as modified by Hess & Lewin (1965). Crystalline bovine serum albumin was used as a standard.

Preparation of fractions for disc gel electrophoresis. Each fraction was diluted with excess of cold water and centrifuged at 100000*g* for 30 min. The pellets were resuspended in a large volume of water and the above process was repeated two more times. This allowed complete removal of cytoplasmic proteins and was also found necessary for extraction of basic protein, for sucrose interfered during the initial extraction.

Proteins were separated by disc electrophoresis on polyacrylamide gel at room temperature by three different methods. (1) For complete resolution of the membrane protein, fractions were solubilized in 5 M-urea in phenol–acetic acid–water (2:1:1, w/v/v) and portions containing 300 μg of protein in a total volume of 0.03 ml were applied directly on to freshly prepared gel columns under 75% (v/v) acetic acid solution. The gels were prepared and electrophoresis was performed according to the technique of Takayama, MacLennan, Tzagoloff & Stoner (1964). Electrophoresis was carried out at 5 mA/gel for 1 h and this was found to minimize the tailing and concavity of the bands observed by Cotman & Mahler (1967). (2) Each fraction was freeze-dried and extracted with 100 vol. of 0.1 M-HCl for 1 h at 0°C. The suspension was centrifuged for 1 h at 0°C at 37000*g*, filtered on Whatman no. 54 filter paper and dialysed against 0.1 M-acetic acid solution at 0–4°C for 3 days. The dialysis residue was freeze-dried. The separation and identification of myelin basic protein was carried out by polyacrylamide-gel electrophoresis by the method of Martenson, Deibler & Kies (1969) at pH 10.6, in which system only highly basic proteins (including histones) migrate into the gel. Other proteins extracted by acid migrate out of the gel. Electrophoresis was at 2.5 mA/gel for 6 h, and only four gels were run at a time. (3) The acid-extracted proteins from each fraction were further characterized by polyacrylamide-gel electrophoresis with 1 M-acetic acid as electrolyte. In this system most of the proteins entered the gel but were excluded when electrophoresis was carried out at alkaline pH (see 2). However, the characteristic proteins from each membrane fraction were easily distinguished, since myelin encephalitogenic basic proteins ran as two distinct bands, as observed by Martenson, Deibler & Kies (1970). The conditions described by Martenson *et al.* (1970) were modified by running the proteins for a period of 60 min at 2.5 mA/gel to prevent migration of some of the bands out of the gel to the cathode.

Lipid extraction and analysis. Portions of the aqueous suspensions of the original fractions were mixed with 5 vol. of chloroform–methanol (2:1, v/v); this procedure gave the same results as when 19 vol. of chloroform–methanol (2:1, v/v) was used. The mixtures were kept at 0–4°C until the resulting two phases were clear, with a protein interface. The upper (aqueous) phases were aspirated and discarded, leaving the protein interface floating over the chloroform-rich solution. These chloroform extracts were washed twice more with 'synthetic upper phase' containing citrate (Folch, Lees & Sloane-Stanley, 1957). Finally the washed lipid extracts were filtered into measuring cylinders, made up to suitable volumes with chloroform–methanol (2:1, v/v) and analysed. This procedure was found to give quantitative extraction of lipids.

When ganglioside values were required portions of the original aqueous fractions were mixed with sufficient chloroform–methanol (1:1, v/v) to form a single phase. The mixtures were filtered and residues washed with chloroform–methanol (2:1,v/v). The composition of the combined filtrates and washings was adjusted to give final mixtures of 5 parts of chloroform–methanol (2:1, v/v) to 1 part of water. The resulting two phases were separated. The lower (chloroform) layers were washed once with 'synthetic upper phase' containing citrate (as above) and twice with 'synthetic upper phase' made up of chloroform–methanol–water (3:48:47, by vol.). The washed lipid extracts (chloroform layers) were filtered to remove proteolipid protein, made up to known volumes and analysed.

The combined upper phase, containing gangliosides

together with non-lipid contaminants, was concentrated to about one-third of the original volume on a rotary evaporator under reduced pressure at 45°C. The concentrated solutions were dialysed at 0–4°C for 4 days with at least five daily changes of water. The volumes of the dialysed solutions were measured, and portions were used for direct assay of ganglioside *N*-acetylneuraminic acid.

Separation and analysis of individual phospholipids. Lipid samples containing known amounts of lipid phosphorus were chromatographed on silica gel G (E. Merck A.-G., Darmstadt, Germany) plates (500μm thick) in chloroform–methanol–water (24:7:1, by vol.). Bands were located with Bromothymol Blue (0.4%, w/v, in 0.01M-KOH) and the plates allowed to dry. The dry phospholipid bands were scraped into stoppered tubes and lipids eluted from the powder by shaking with 10ml of chloroform–methanol–water (7:7:1, by vol.) followed by centrifugation. Portions of the clear solutions were removed for phosphorus assay. Recovery of phosphorus from the plates ranged from 85% to 100%.

Analytical methods. Lipid phosphorus was determined by the method of Martland & Robison (1926) after washing of the dry lipid residues in a sand bath with 60% (w/v) $HClO_4$–5M-H_2SO_4 (1:1, v/v).

Cholesterol was determined by the method of Davison, Dobbing, Morgan & Payling-Wright (1958).

For determination of cerebroside total lipid extracts were subjected to t.l.c. on silica gel G (E. Merck A.-G.) in chloroform–methanol–water (24:7:1, by vol.). Lipid bands were located by spraying with Bromothymol Blue. The cerebroside double bands were scraped into stoppered tubes and lipids were eluted from the powder by shaking with 5ml of chloroform–methanol–water (65:35:4, by vol.) followed by centrifugation; 3ml portions of the resulting solution were removed gently, care being taken not to disturb the settled powder. Bromothymol Blue was removed from the clear extracts by partition against 0.1M-KCl followed by exhaustive washing with 'synthetic upper phase' until the resultant (lipid) chloroform layers became colourless. After evaporation of the solvent the galactose content of the material was determined by reaction with anthrone (Radin, 1958).

Ganglioside *N*-acetylneuraminic acid was determined by the method of Svennerholm (1957) as modified by Miettinen & Takki-Luukkainen (1959).

Metabolic studies. (1) Lipids. In short-term experiments on lipid metabolism 16-day-old rats were given intraperitoneal injection of 12.5μCi of [1-^{14}C]acetate in 0.9% NaCl solution. Rats were killed 10min and 24h after injection. The brains were removed and fractionated as described above. Protein-free total lipid extracts were prepared and portions evaporated to dryness in counting vials. The dried lipids were dissolved in 20ml of 0.5% 2,5-diphenyloxazole, 0.03% 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene and 3% (v/v) ethanol in toluene, and radioactivity was determined by scintillation counting (87% efficiency). Radioactivity was determined in individual lipids, localized by iodine vapour after t.l.c., by removing silica gel zones directly into counting vials, adding 20ml of the same scintillation counting mixture and counting directly after thorough mixing. Recovery was over 85% and the efficiency of counting was as above.

(2) Proteins. In the long-term protein-metabolism experiments 11-day-old rats from two litters were given subcutaneous injection of equivalent to 0.2μCi of [1-^{14}C]-leucine/g body wt. in 0.9% NaCl solution. At intervals from 17h onwards animals from each litter were killed and the brains removed. In the first experiment subcellular fractions were prepared as described above, but in a second experiment the method was modified by washing the crude mitochondrial pellet three times with 5vol. of 0.32M-sucrose solution and sedimenting at 15000*g* for 15min; this procedure was to remove microsomal contamination.

Preparation of protein for radioactivity determination. Samples were treated with an equal volume of cold 10% (w/v) trichloroacetic acid solution containing L-leucine (0.5%). The suspension was centrifuged and the precipitate washed with 5% (w/v) trichloroacetic acid containing L-leucine (0.5%). The procedure was repeated twice, and the resultant precipitate was extracted with ethanol–diethyl ether (1:1, v/v) and resedimented three times. The protein residue was heated at 90°C in 5% (w/v) trichloroacetic acid for 15min and the residue washed with 95% ethanol followed by anhydrous diethyl ether. After being dried *in vacuo* the residue was dissolved in formic acid (98%, w/v) and 0.2ml of this solution was removed into a vial for counting of radioactivity according to the method of Hall & Cocking (1965) as described by Agrawal, Bone & Davison (1970). The efficiency of counting was 70±0.7%.

RESULTS

Although in this study a different method from that of Banik & Davison (1969) was employed for the isolation of the myelin-like fraction from developing brain, the lipid composition and yields by both methods were comparable. The myelin-like preparation from 16-day-old rats contained relatively more phospholipid (particularly phosphatidylcholine) and much less cerebroside than mature myelin. The purified myelin fraction had a higher phospholipid/cholesterol ratio than mature myelin and in the present study proportionately less cerebroside was found (Table 1). The amount of ganglioside *N*-acetylneuraminic acid found in the myelin-like fraction of 16-day-old rat brain was 52.5μg/g of original tissue as opposed to the purified myelin value of 21.6μg/g of original tissue. The corresponding values for cholinergic and non-cholinergic nerve endings were 76.8 and 42.8μg/g of original tissue respectively. In terms of μg of ganglioside *N*-acetylneuraminic acid/mg of protein the myelin-like fraction gave the highest value of 25.7, more than twice that for purified myelin (10.9); the values for cholinergic and non-cholinergic nerve endings were 7.0 and 8.0 respectively. This high ganglioside content of the myelin-like material may in some way be related to the glycolipid-rich cell coat thought to be part of the glial plasma membrane (Lehninger, 1968). No difference was seen in the ganglioside pattern separated from the myelin fractions by t.l.c. with the system described by Suzuki *et al.* (1967).

Table 1. *Composition of rat myelin, the myelin-like fraction and rat liver plasma membrane*

The A layer of the De Robertis fraction (De Robertis *et al.* 1962) was separated into pure myelin and a second myelin-like fraction. The results for liver plasma membrane lipids of Skipski *et al.* (1965) and Stahl & Trams (1968) for ganglioside were recalculated in terms of molar ratios and the values for liver plasma membrane protein are those of Pfleger, Anderson & Snyder (1968). Results are given as means (±S.D. where appropriate) of the numbers of analyses given in parentheses. No DNA was detected in 16-day-old rat myelin and myelin-like fractions; less than 0.12mg of RNA/μg wet wt. of brain was found in the purified myelin fraction and none in the myelin-like fraction.

	Composition (mol/mol of cholesterol)				
		Adult rat fractions		16-day-old rat fractions	
	Adult rat liver plasma membrane	Pure myelin	Myelin-like	Pure myelin	Myelin-like
Cholesterol	100	100 (7)	100 (2)	100 (2)	100 (2)
Cerebroside	0	43	13	22	2
Total phospholipid	124	123	164	139	191
Phosphatidylcholine	46	37	75	57	94
Ethanolamine phospholipid	20	50	52	43	43
Sphingomyelin	23	17	28	14	17
Minor phospholipids*	19	9	8	0	4
N-Acetylneuraminic acid	0.2	3	—	3.9	14.4
Protein (mg)	308	64	154	111	174
Protein (mg/g wet wt. of tissue)	0.88±0.24 (13)	10.2 (9)	3.1 (7)	2.1±0.4 (5)	2.1±0.4 (5)
Lipid phosphorus (μmol/g wet wt. of tissue)	—	19.6 (9)	3.3 (7)	3.2±0.4 (5)	2.9±0.5 (5)
N-Acetylneuraminic acid (μg/g wet wt. of tissue)	—	65	—	21.6	52.5

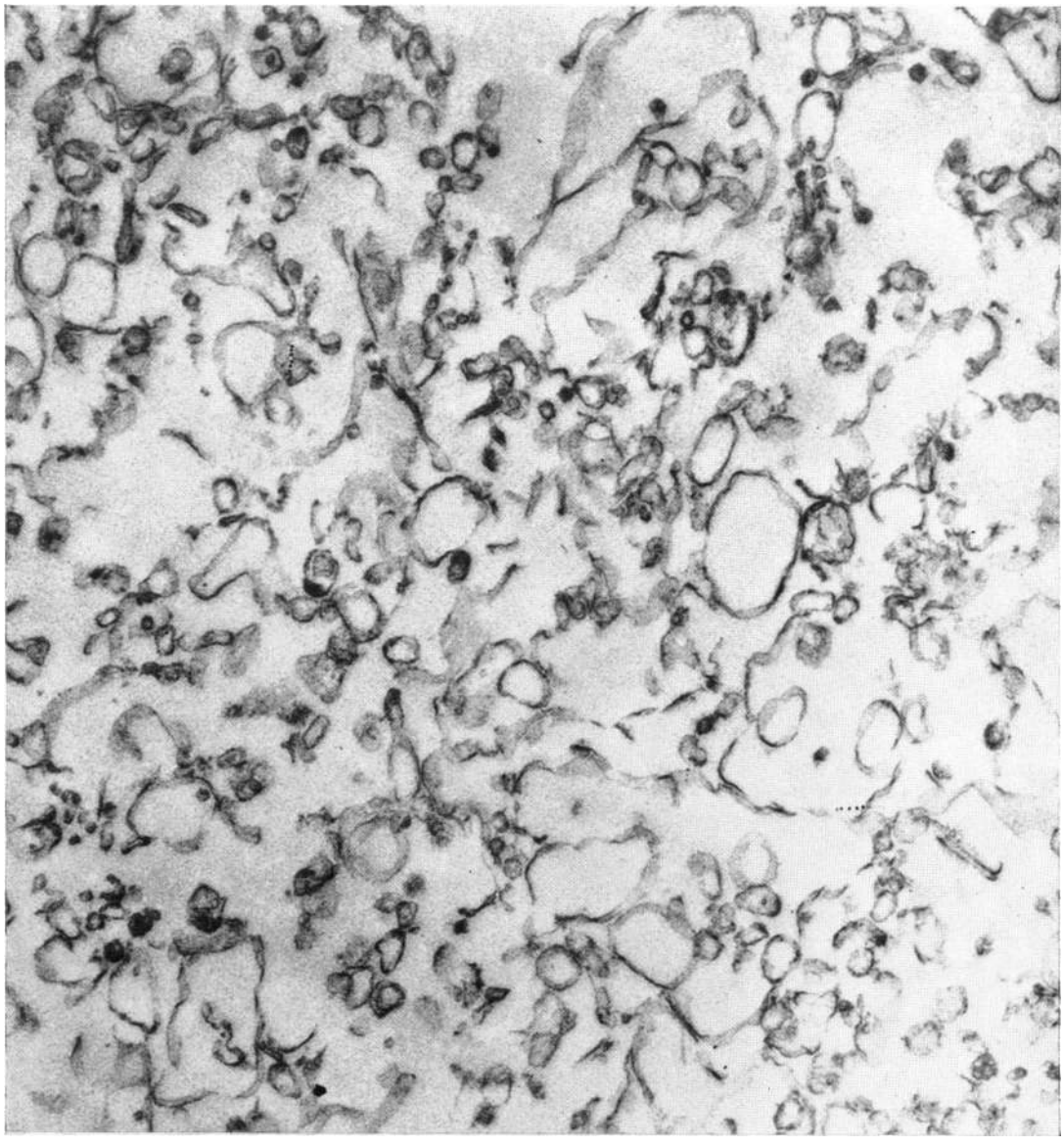
* Inositol and serine phospholipid.

Table 2. *Specific activities of certain 'marker' enzymes in myelin and in subcellular fractions of brain from 15-day-old rats*

Methods are as described by Banik & Davison (1969). The numbers of samples analysed are shown in parentheses. Results for other fractions are from Banik & Davison (1969).

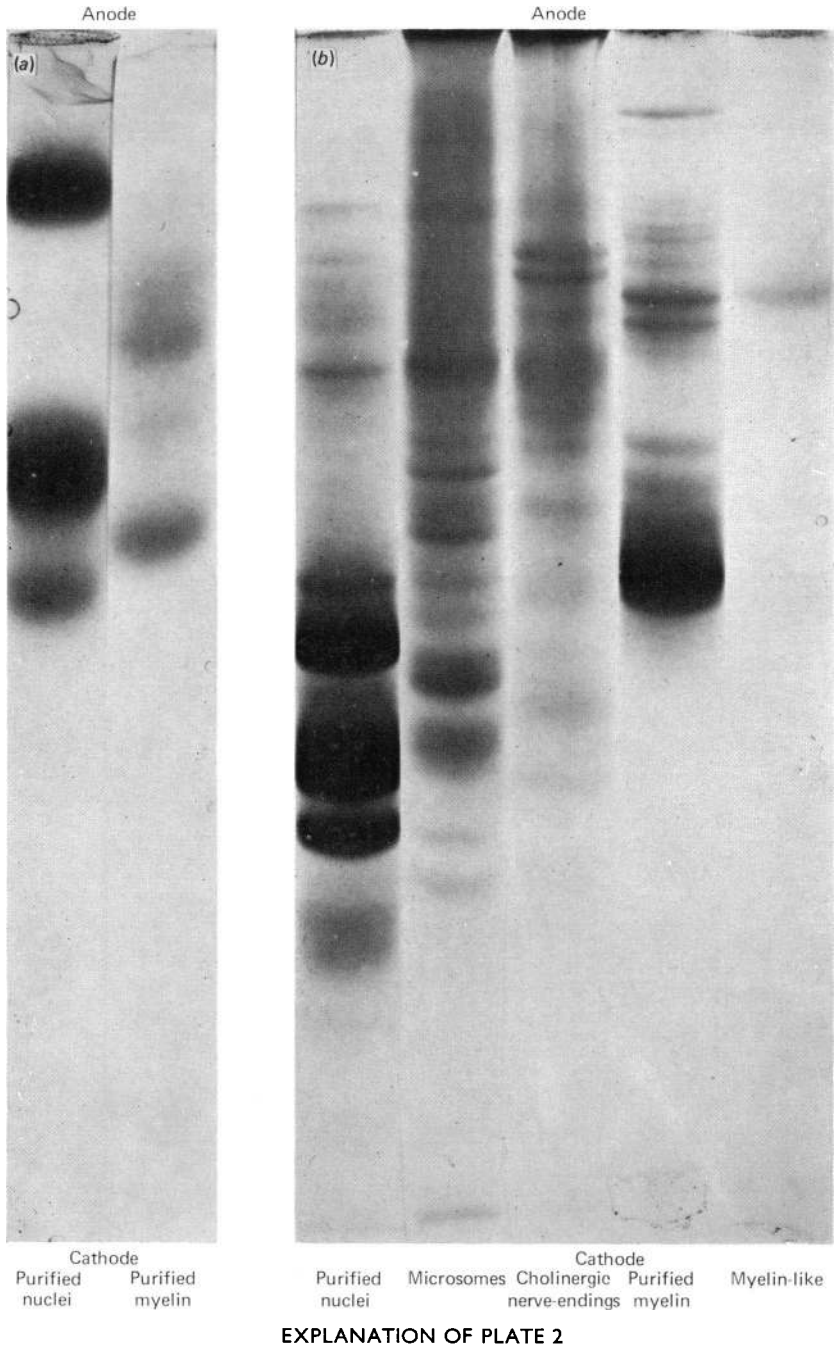
		Specific activity (μmol of substrate/h per mg of protein)		
Enzymes	Typical localization	Myelin	Second myelin-like fraction	Other fractions
Succinate dehydrogenase (EC 1.3.99.1)	Inner mitochondrial membrane	0.74 (2)	0.09 (2)	11.90 (mitochondria) 7.00 (nerve endings)
Acetylcholinesterase (EC 3.1.1.7)	Nerve ending particles	4.0 (2)	3.4 (2)	5.2 (nerve endings) 8.9 (microsomes)
5′-Nucleotidase (EC 3.1.3.5)	Plasma membranes	0.52 (2)	0.45 (2)	0.90 (microsomes)
Leucine aminopeptidase (EC 3.4.1.1)	Myelin	1.38 (2)	1.26 (2)	0.51 (microsomes) 0.32 (nerve endings)
2′:3′-Cyclic nucleotide 3′-phosphohydrolase	Myelin	116 (3)	90 (3)	19.7 (microsomes) 10.8 (nerve endings) 166 (purified myelin) 54.2 (myelin-like)

Characterization of the myelin-like and purified myelin fractions with enzyme markers gave (Table 2) similar results to those reported previously (Banik & Davison, 1969) and again indicated that both fractions had high phosphohydrolase and leucine aminopeptidase activities. Electron micrographs of the myelin fractions showed the myelin-like material contained predominantly



EXPLANATION OF PLATE 1

Myelin-like membrane fraction. The preparation was prepared (see the text) and stained in 1% (w/v) phosphotungstic acid in ethanol. Magnification × 32000. We are grateful to Dr N. A. Gregson and Mr G. S. Stephens of Guy's Hospital for preparing this photograph.



EXPLANATION OF PLATE 2

Gel electrophoresis of acid-extractable protein isolated from myelin and brain subcellular fractions. Equal amounts of protein (200 μg) from each fraction after solubilization in 8 M-urea were applied to each gel. The first two gels (*a*) shown were run under alkaline conditions (Martenson *et al.* 1969) and the remaining five (*b*) in 1 M-acetic acid (Martenson *et al.* 1970). We are grateful to Miss P. M. Turnbull (Charing Cross Hospital) for photography.

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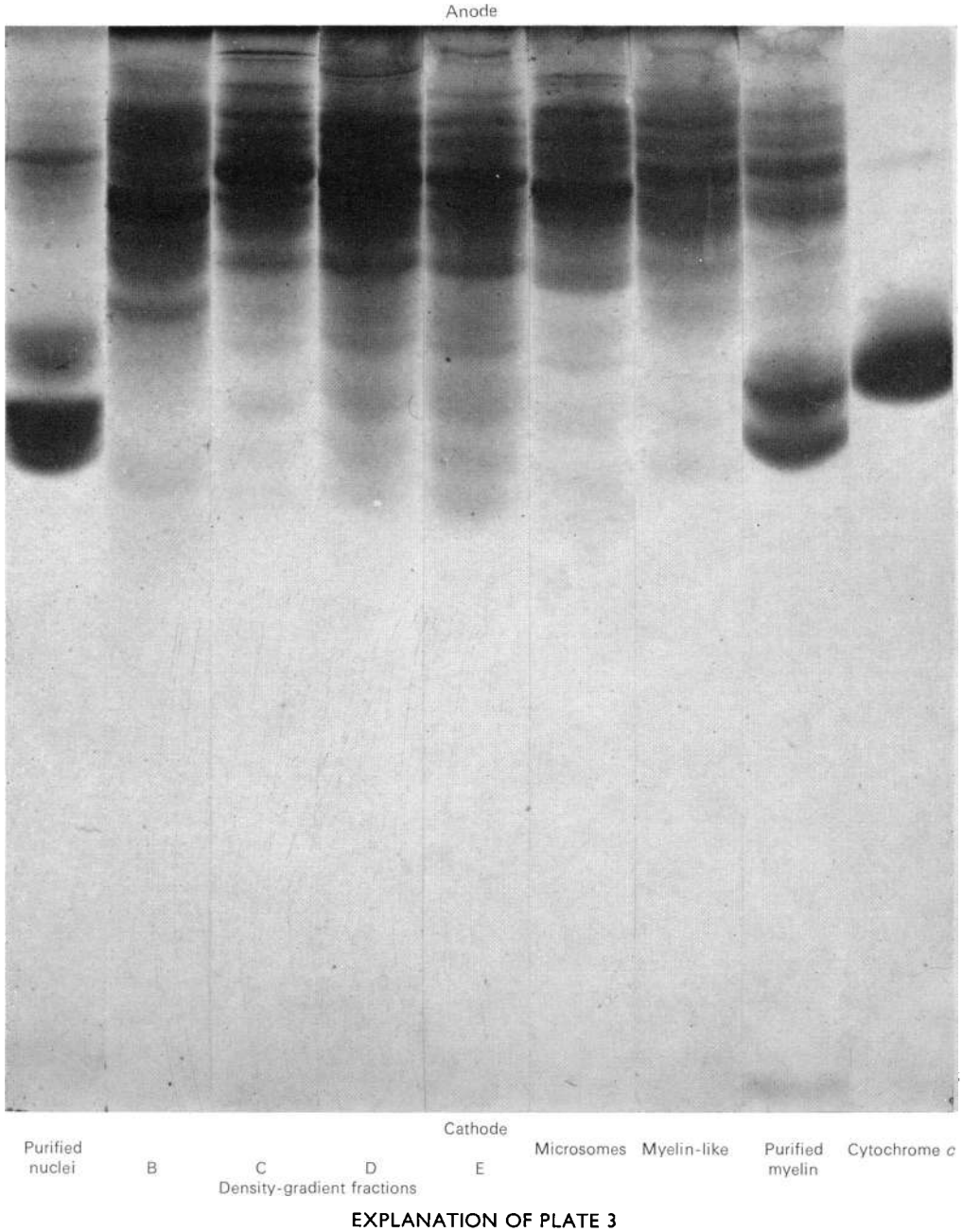

EXPLANATION OF PLATE 3

Gel electrophoresis of myelin and brain subcellular fractions. The samples were solubilized in 5M-urea in phenol–acetic acid–water (2:1:1, w/v/v) and equal amounts (300μg) of protein from each fraction were applied to each gel. Duplicate samples were run at the same time and the electrophoretic pattern was reproducible. Cytochrome *c* (20μg) was used as a standard. Conditions for electrophoresis were as described by Takayama *et al.* (1964). Subcellular fractions were prepared as described in the text, the letters indicating the mixed membrane (B), cholinergic (C) and non-cholinergic nerve endings (D) are as identified by De Robertis *et al.* (1962).

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single membrane vesicles (Plate 1), but less contaminating material was evident than in the preparation obtained by Banik & Davison (1969).

Proteins of myelin, myelin-like and subcellular fractions of the developing brain. Electrophoresis of the acid-extractable proteins from the subcellular fractions at an alkaline pH (10.6) gave characteristic patterns only for the purified myelin and nuclear fractions (Plate 2*a*) comparable with those observed by Martenson *et al.* (1969) for adult rat brain. No stainable band was observed for the other subcellular fractions. To resolve the acid-extractable proteins, the electrophoresis of each fraction was repeated under extreme acid conditions (1M-acetic acid). This method gave a more heterogeneous pattern of bands than did electrophoresis at alkaline pH. The basic encephalitogenic protein still appeared in purified myelin as two bands, but there were many other well-separated bands in the gel and the encephalitogenic protein was absent from the myelin-like preparation (see Plate 2*b*). When all the subcellular fractions were solubilized in 5M-urea in phenol–acetic acid–water (2:1:1, w/v/v) and subjected to electrophoresis in 10% (v/v) acetic acid as described by Takayama *et al.* (1964) a very complex electrophoretic pattern for layers B, C, D, E and microsomes was observed (see Plate 3). The purified myelin fraction was characterized by the unique presence of the basic encephalitogenic protein which was absent from the myelin-like and other subcellular fractions, thus demonstrating that the myelin-like fraction is not grossly contaminated with myelin. Except for the absence of the encephalitogenic basic protein the electrophoretic patterns of both purified myelin and the myelin-like fraction were remarkably similar. No such close similarity was apparent with the other subcellular fractions including that of purified nuclei (see Plate 3).

Developmental changes. Crude myelin was separated into two fractions during maturation of the rat brain. From about 8 days after birth onwards the amount of myelin-like material isolated from each brain increased slightly and then declined as myelination proceeded (Fig. 1). In contrast the amount of purified myelin increased exponentially during at least the first year of life, a finding in agreement with that of Suzuki *et al.* (1967) and Suzuki, Poduslo & Poduslo (1968). From our studies it therefore seemed possible that the appearance of the myelin-like fraction was related to the overall process of myelination.

Metabolism of myelin and the myelin-like fractions. Since these two fractions are clearly closely related it was important to distinguish between two possibilities. First, the myelin-like fraction may be a precursor of adult-type myelin, or alternatively it may be a separate form of myelin. Experiments were designed, with labelling with radioactive isotopes, to distinguish between these possibilities.

Lipid metabolism. In the first series of experiments 16-day-old rats were given [^{14}C]acetate by intraperitoneal injection. Radioactivity in the individual lipids of the two myelin fractions and subcellular fractions were determined thereafter. Even 10min after injection the extents of labelling of lipid in the crude, purified myelin and myelin-like fractions were similar (Table 3). This similarity was not due to incorporation of ^{14}C into any single

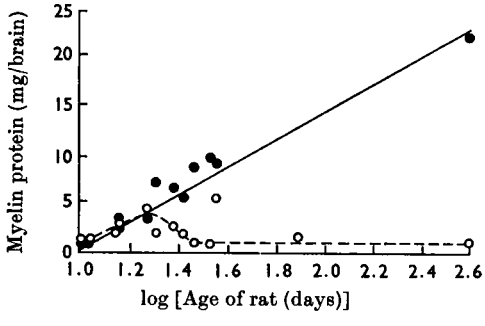

Fig. 1. Increase in myelin and myelin-like protein during maturation of rat brain. Myelin fractions were prepared by the method described in the text and also by the technique of Banik & Davison (1969). The age of the adult rats has been taken as 400 days. Myelin increment (●) is shown as a solid line and the increase in the myelin-like (○) protein is shown as a broken line.

Table 3. *Incorporation of* ^{14}C *from* [^{14}C]*acetate into the lipids of myelin and myelin-like fractions isolated from developing rat brain*

The rats (16 days old) were given 12.5μCi of [^{14}C]acetate by intraperitoneal injection. Techniques for determination of subcellular and radioactivity fractionation are described in the text. Results are given as means (±S.D. where appropriate) of the numbers of determinations given in parentheses.

	Total radioactivity (d.p.m./mg of protein)		
Time after injection	Crude myelin	Purified myelin	Myelin-like
10min	3914±417 (3)	4300±779 (3)	5103±964 (3)
24h	2793 (2)	4794±398 (3)	2664±708 (4)

Table 4. *Incorporation of* ^{14}C *from* [^{14}C]*acetate into the individual lipids of the myelin fractions of brain from 16-day-old rats*

Rats were injected intraperitoneally with 12.5 μCi of [^{14}C]acetate, and killed after 10 min. Radioactivities in individual lipids are shown as percentages of total lipid radioactivity in each fraction.

	Radioactivity (%)		
Lipid	Crude myelin	Purified myelin	Myelin-like
Cholesterol	35.5	39.0	38.9
Minor glycolipids*	8.6	7.5	4.9
Cerebrosides	2.8	5.9	2.9
Ethanolamine glycerophospholipid	4.8	5.9	7.6
Phosphatidylcholine	25.5	18.5	27.3
Sphingomyelin	9.4	5.1	3.3
Minor phospholipids	—	4.3	4.2

* Cerebroside esters and galactosyl glycerides.

Table 5. *Metabolism of myelin and subcellular fraction protein after incorporation of* [1-^{14}C]*leucine into the brain from 11-day-old rats*

Two experiments were carried out on 13 and 16 rats respectively. Conditions were as described in the text, but in the second experiment the crude mitochondrial pellet was washed three times with 0.32 M-sucrose and recentrifuged to remove all traces of microsomes. Because of the considerable loss of myelin the microsomal pellet suspended in 0.32 M-sucrose was layered over a 0.8 M-sucrose gradient. After centrifuging for 30 min at 53000 *g* at 2°C myelin was recovered at the top interface, and purified myelin was reshocked with 18 ml of ice-cold water.

	Mean specific radioactivity (d.p.m./mg of protein)							
Time after injection (days)	Whole brain	Nuclei	Microsomes +supernatant	Purified myelin	Myelin-like	Cholinergic nerve endings (C)	Non-cholinergic nerve endings (D)	Mitochondria (E)
1	2731	2503	2950	2531	2441	2348	3795	4605
	2262	2568	2891	2481	2526	2417	2460	3541
8	1430	1383	1161	1666	1506	1530	1499	1151
	1274	1238	1650	1587	1393	1593	1630	1636
16	694	—	—	541	543	965	844	229
	684	609	339	586	588	845	789	699
23			522 (microsomes) 362 (supernatant)					
	358	—	—	429	439	—	—	—

lipid species, for on separation of lipids by t.l.c. radioactivity was found in all lipids in proportion to their concentration in the respective myelin fractions (Table 4). Thus similar amounts of ^{14}C were detected in cholesterol of all three myelin preparations, but more ^{14}C was found in the phosphatidylcholine of the myelin-like and the crude myelin fractions than in the purified myelin (Table 4). When the experiment was repeated on rats allowed to survive for 24 h, radioactivity in the total lipid of the myelin-like fraction had fallen slightly but there was no increase in ^{14}C of the purified myelin fraction. Since these experiments showed no precursor–product relationship metabolism of labelled myelin proteins was next examined.

Protein metabolism. Two experiments were performed: in the second the crude mitochondrial pellet was repeatedly washed to remove microsomes and the purified myelin pellet was also rewashed. The results show an equal incorporation of [^{14}C]leucine into both purified myelin and the myelin-like fractions 1 day after injection (Table 5). Subsequently the specific radioactivity of each myelin fraction declined at a remarkably similar rate. This again therefore suggests that the two fractions are closely related and that there is no precursor–product relationship.

DISCUSSION

After differential centrifugation of nervous tissue in 0.32M-sucrose solution a myelin preparation can be isolated by centrifugation of the mitochondrial fraction over an appropriate density gradient. If this crude myelin from developing rat brain is layered over a continuous sucrose density gradient only one major band is separable, but if the crude myelin is first subjected to water-shock treatment it can then be separated into two fractions of different density. On separation on a continuous density gradient the less-dense fraction appears to be similar to myelin whereas the second has a significantly higher density. This density difference was utilized by Banik & Davison (1969), who centrifuged a layer of water-shocked crude myelin in 0.32M- over 0.85M-sucrose solution at 53000*g* for 1h at 2°C, collecting myelin at the interface and myelin-like material as a pellet.

In the present paper, rather than employing the separation procedure of Gray & Whittaker (1962) for the isolation of crude myelin, we have used the fractionation scheme described by De Robertis *et al.* (1962). For the separation of the myelin and myelin-like fractions we have taken advantage of differences in sedimentation characteristics of each after osmotic shock of crude myelin. Thus to sediment the myelin-like fraction it was necessary to centrifuge at 75000–100000*g* for 1h, whereas myelin could be deposited by centrifugation at 12500*g* for 10min. This difference could be related to the physical properties of each fraction, for electron microscopy indicates that although the myelin has the normal lamella structure the myelin-like fraction predominantly contains single small membrane vesicles (Plate 1).

Analysis of the myelin-like material isolated by the present method shows it to contain only traces of cerebroside and a high phospholipid/cholesterol ratio (Table 1); we consider this to reflect the high degree of purity of our fraction. This is further confirmed by the absence from the myelin-like material of the basic encephalitogenic protein when the acid extractable (0.1M-hydrochloric acid) protein was subjected to disc electrophoresis at extreme alkaline (pH 10.6) and acid conditions (pH 3.0).

Identity of the myelin-like fraction. The possibility that the myelin-like form is due to contamination of a small myelin fraction with membranes derived from another subcellular system has been considered previously to be unlikely (Banik & Davison, 1969) and the experiments now reported confirm this view. Eng & Noble (1968) have also isolated two fractions from crude myelin from developing rat brain and on the basis of protein content conclude that contamination by microsomes is an unlikely source of their light myelin fraction, but since our sample was obtained from fresh brain and the myelin-like fraction was more dense than myelin it is unlikely that these are similar fractions. This is supported by our demonstration of the absence of the basic encephalitogenic protein from our myelin-like fraction.

Since the myelin-like fraction is only obtainable from crude myelin as a result of dilution in ice-cold water, it seems probable that it is intimately connected with myelin. During the initial stages of myelination loose myelin membranes appear to be continuous with the multi-lamellae form of the sheath (Peters & Vaughan, 1970; Caley & Maxwell, 1968); we postulate that osmotic shock releases the loose single myelin lamellae from compact myelin and that the former are broken down to small vesicles, as is known to happen on isolation of liver plasma membranes (Coleman & Finean, 1966). The close relationship between the myelin-like fraction and myelin is seen from the high specific activity of leucine aminopeptidase and the 2′:3′-cyclic nucleotide 3′-phosphohydrolase together with the low activity of enzyme 'markers' for other subcellular fractions. The separated protein patterns of both myelin fractions show some similarities (Plate 3) when electrophoresis is carried out after solubilizing these fractions in 5M-urea in phenol–acetic acid–water (2:1:1, w/v/v). Similarity of both myelin fractions is also seen in the labelling experiments (Tables 3, 4 and 5). Such results are difficult to assess (Davison, 1968) in the developing brain, but there is no indication of a clear-cut precursor–product relationship between the myelin-like and myelin fractions. Nevertheless the quantity of the myelin-like material obtainable by fractionation is greatest at the time when synthesis of myelin is most rapid (Fig. 1) and only relatively small amounts of the myelin-like fraction can be separated from the adult brain.

'Early' myelin. It has previously been suggested that the myelin-like fraction is principally derived from oligodendroglial plasma membrane (Davison *et al.* 1966; Banik & Davison, 1969), but the proposal that myelin derived from the glial plasma membrane pushes round the axon has always been a difficult one to accept (Peters & Vaughan, 1970). As shown in Table 1, analysis shows that the myelin-like fraction resembles plasma membranes in its lipid composition (Ashworth & Green, 1966), and it therefore seemed possible that the myelin-like material could have been regarded as a precursor membrane system that would act as a framework for the synthesis of mature myelin, but our isotope experiments do not support this contention. In addition, the gel-electrophoresis pattern of myelin and the myelin-like fractions are remarkably similar except for the absence of the basic protein from the myelin-like material (Plate 3).

The protein pattern of both myelin fractions differs from that of other subcellular fractions. Moreover turnover after incorporation of radioactive carbon from [^{14}C]leucine into the proteins of other subcellular fractions differs from that of both myelin fractions (Table 5). The observation that the protein pattern from the myelin fractions is entirely different from that of other subcellular fractions including nerve-ending particles (see Plate 3) suggests that the myelin-like fraction is not of neuronal origin. This is further substantiated by the high phosphohydrolase activity of the myelin-like fraction, indicating close similarity to myelin. It may be concluded that the myelin-like material is a modified form of oligodendroglial plasma membrane. There is evidence that the compositions of both myelin and the myelin-like fractions change in the maturing brain (Table 1); the same phenomenon has also been observed by Eng & Noble (1968), Suzuki *et al.* (1967,1968), Dalal & Einstein (1969) and Horrocks (1968). We therefore propose that the myelin-like material is a form of myelin in continuity with the glial plasma membrane and with compact myelin. 'Early' myelin therefore includes a mixture of both the myelin-like and myelin fractions. We suggest that compact myelin contains cerebroside and the basic encephalitogenic protein, possibly localized in the inter- and intra-period lines (Kornguth, Anderson & Scott, 1966; Adams, Bayliss, Hallpike & Turner, 1970; C. E. Lumsden, S. R. Aparicio, J. Dickinson & K. M. Jones, unpublished work).

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