Journal of Neurochemistry, 1973, Vol. 20, pp. 1589 to 1603. Pergamon Press. Printed in Great Britain.

MYELIN SUBFRACTIONS IN DEVELOPING RAT BRAIN: CHARACTERIZATION AND SULPHATIDE METABOLISM

JOYCE A. BENJAMINS, KAREN MILLER and G. M. MCKHANN

Department of Neurology, Johns Hopkins School of Medicine, Balitmore, MD 21205, U.S.A.

(*Received* 25 *October* 1972. *Accepted* 12 *January* 1973)

Abstract—Centrifugation of isolated myelin on discontinuous sucrose gradients resulted in a separation into three bands and a pellet. The three bands were morphologically identical to myelin, whereas the pellet consisted primarily of vesicular membranes. These four fractions differed from one another in their lipid-to-protein ratios and in molar ratios of cholesterol:phospholipid:galactolipid. All of the fractions contained proteins typical of myelin, although the proportions of the proteins varied, with the pellet being the lowest in basic protein and proteolipid protein. High activity of 2′,3′-cyclic nucleotidase and low activity of cerebroside sulphotransferase further distinguished these fractions from the microsomal fraction. Distribution of radioactive sulphatide in the subfractions at 15 min after intracranial injection of radioactive sulphate indicated that newly-labelled sulphatide first appeared in the lipid-poor fractions, followed by the lipid-rich fractions; results of pulse-chase experiments also suggested this relationship. Several days or weeks after the injection of radioactive sulphate, most of the radioactive sulphatide was in the lipid-rich fractions.

WE HAVE previously studied the introduction of sulphatide into the myelin membrane by investigating (1) the transport of sulphatide to myelin by cytoplasmic lipoproteins (HERSCHKOWITZ, MCKHANN, SAXENA and SHOOTER, 1968*a*) and (2) the relationship between protein synthesis and sulphatide accumulation in myelin (BENJAMINS, HERSCHKOWITZ, ROBINSON and MCKHANN, 1971). To examine the biochemical mechanism of myelin formation in more detail, we have isolated fractions of myelin which differ in lipid-to-protein ratios. We have used sulphatide labelling in these fractions to determine whether the lipid-poor membranes are precursors of the lipid-rich membranes.

Myelin is characterized by a high lipid-to-protein ratio which facilitates its separation from other subcellular membranes on density gradients. A number of investigators have reported isolation of myelin membranes of varying densities from relatively purified preparations of myelin (NORTON and AUTILIO, 1966; CUZNER and DAVISON, 1968; ADAMS and FOX, 1969; ENG and BIGNAMI, 1972; MCMILLAN *et al.*, 1972). The fractions differ slightly in lipid and protein composition one from another, but evidence for metabolic relationships between the fractions is lacking. Myelin isolated from young animals has a lower lipid:protein ratio, lower proportions of galactolipids and basic proteins, and higher proportions of phospholipids and high-molecular-weight proteins than myelin isolated from adult animals (NORTON, 1971; MORELL *et al.*, 1972). A myelin-like fraction isolated by AGRAWAL *et al.* (1970) has several properties similar to myelin from young animals, characteristics suggesting that it may be a precursor membrane to myelin, but again a metabolic relationship

Abbreviation used: SDS, sodium dodecyl sulphate.

has not been clearly established. In the present study we have isolated myelin membranes of varying densities from brains of rats at several ages, and the labelling of sulphatide in these membranes suggests that they are metabolically related.

MATERIALS AND METHODS

Materials and animals

Sodium [^{35}S]sulphate (400 mCi/mmol) and 3′-phosphoadenosine-5′-phospho [^{35}S]sulphate ([^{35}S]PAPS; 950 mCi/mmol) were purchased from New England Nuclear Corp. (Boston, Mass.). Bacterial alkaline phosphatase (EC 3.1.3.1), egg albumin and adenosine-2′,3′-monophosphate (2′,3′ cyclic AMP) were from Sigma Chemical Co. (St. Louis, Mo.). 1- Amino-2-naphthol-4 sulphonic acid-dry mixture (sulphite-bisulphite) was purchased from Fisher Scientific Co. (Pittsburgh, Pa.). Lipid standards were obtained from Applied Science Laboratories (State College, Pa.).

Sprague–Dawley rats (Charles River Breeding Labs., Wilmington, Mass.) were used. Litters were reduced to 10 animals during the first week after birth to ensure more consistent growth rates. Animals were injected intracranially with 20–200 μCi of Na_2[^{35}S]sulphate in 30 μl of 0·15 M NaCl. For chase experiments, unlabelled sodium sulphate was injected intracranially (1% w/v, 30 μl) and intraperitoneally (7% w/v, 0·5 ml).

Preparation of myelin and microsomal fractions

Myelin was isolated from the brains of rats of all ages by a slight modification of a procedure of NORTON (1971). The tissue was homogenized in 9 vol of 0·32 M sucrose, then diluted with another 10 vol of 0·32 M sucrose; 18 ml of this suspension was layered over 18 ml of 0·72 M sucrose in a 40-ml tube and the preparation was centrifuged in the Beckman SW 27 rotor for 30 min at 75,500 *g* (25,000 rev./min). We removed the myelin bands from the interface by aspiration; each band was transferred to a 50 ml tube, diluted with 45 ml of deionized water, and suspended by repeated aspiration with a disposable pipette. After 30 min, the tubes were centrifuged at 37,000 *g* (17,500 rev./min) in a Sorvall for 20 min. The resulting pellet was osmotically shocked 2–5 times by resuspending the pellet in 50 ml of deionized water as already described and then centrifuging at 12,100 *g* (10,000 rev./min) for 10 min. To prepare myelin subfractions, we first isolated the myelin as already described. After two osmotic shocks, three pellets were pooled and suspended in 6 ml of deionized water. Sucrose was added to bring its final concentration to 0·32 M. We prepared discontinuous gradients in 13 ml tubes by layering 3 ml each of 0·72, 0·63 and 0·50 M sucrose, and 3·0 ml of the myelin suspension (1–2 mg of protein) were placed on top of the gradient. The tubes were centrifuged for 30 min at 75,500 *g* (25,000 rev./min) in the Beckman SW 41 rotor. Three distinct bands (*A*, *B*, and *C*) were collected by aspiration. Each of these fractions was resuspended in 12 ml of water by repeatedly drawing the samples into a disposable pipette; the pellet *D* was resuspended in 12 ml of water with a glass homogenizer. All four fractions were then centrifuged at 37,000 *g* (17,500 rev./min) for 15 min; this washing step was repeated. The procedure has been used routinely with myelin from 17-day-old rats; with rats of different ages, we adjusted the amount of myelin layered on the 3-step gradient to 1–2 mg of protein, the same amount applied in preparations from 17-day-old rats. About 50–60 per cent of the protein applied to the 3-step gradient was recovered in the fractions.

Microsomal fractions were prepared from the same homogenates used to prepare myelin. Ten ml of the homogenate (1 g of tissue in 19 ml of 0·32 M sucrose) were centrifuged at 17,300 *g* (12,000 rev./min) for 1 h; the resulting supernatant fluid was centrifuged at 100,000 *g* (40,000 rev./min) for 1 h to give a microsomal pellet.

Analytical procedures

Procedures for extraction of lipids and the counting of radioactivity in sulphatide have been described in detail (BENJAMINS *et al.*, 1971). Total galactolipid in the washed lipid extract was measured by an orcinol method (SVENNERHOLM, 1956); sulphatide was determined by Azure A binding (KEAN, 1968); cholesterol was measured by the method using acetic anhydride (ENG and NOBLE, 1968); and total phospholipid was determined by a modified Bartlett procedure (HERSCHKOWITZ *et al.*, 1968*b*). The activity of 2′,3′-cyclic nucleotidase was assayed as described by DRUMMOND *et al.* (1971). Each fraction was suspended in 0·6% (w/v) deoxycholate for 10 min before the assay. Cerebroside sulphotransferase activity was determined on whole or Triton-extracted fractions (FARRELL and MCKHANN, 1971).

RESULTS

In preliminary experiments, we examined the properties of myelin on various sucrose and CsCl gradients (Fig. 1). We concluded that a short centrifugation on a

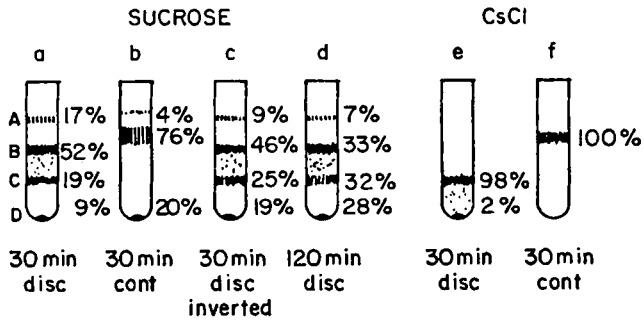

FIG. 1.—Distribution of myelin on sucrose and CsCl gradients. Samples of myelin containing 1–2 mg of protein were placed on gradients prepared as described below. The gradients were centrifuged at 75,500 *g* (25,000 rev./min) in a swinging bucket rotor. Visible bands and pellets were collected, and their protein contents were measured (LOWRY *et al.*, 1951). The percentages entered beside each gradient represent the distribution of the recovered protein. Each gradient was run in duplicate and the samples were pooled after centrifugation. Of the total protein applied, 80–95 per cent was recovered.

Tube *a*—Discontinuous 'gradient prepared by the regular procedure as described in Methods: the gradient consisted of 3 ml each of 0·72, 0·63 and 0·50 M sucrose, with the myelin in 3 ml of 0·32 M sucrose layered on top.

Tube *b*—Continuous linear gradient prepared by using equal volumes of 0·32 and 0·72 M sucrose in a gradient maker, with the myelin in 2 ml of 0·32 M sucrose layered on top.

Tube *c*—Discontinuous gradient with myelin in 3 ml of 0·72 M sucrose at the bottom overlaid with 3 ml each of 0·63, 0·50 and 0·32 M sucrose.

Tube *d*—Discontinuous gradient prepared as in *a*, but centrifuged for 2 h.

Tube *e*—Discontinuous gradient prepared with 3 ml each of 9, 12 and 15% (w/v) CsCl, with the myelin in 3 ml of 6% (w/v) CsCl layered on top.

Tube *f*—Continuous linear gradient prepared by using equal volumes of 5 and 22% (w/v) CsCl in a gradient maker with the myelin in 2 ml of 5% (w/v) CsCl layered on top.

discontinuous sucrose gradient yielded several fractions which might then be analysed for differences in morphology, composition, and sulphatide metabolism. Our decision to apply the myelin at the top of the gradient (Fig. 1a), rather than placing it at the bottom of the gradient (Fig. 1c) was based on the behaviour of the microsomal membranes under these two conditions (Table 1). We were concerned that Fraction *D* represented a mixture of microsomal and myelin membranes, since it was the most similar of the four myelin subfractions to the microsomal fraction in composition and in turnover of sulphatide. Therefore, we chose conditions which would minimize contamination of *D* by the microsomal fraction. These conditions included placing the myelin sample at the top of the gradient (Fig. 1), and washing the subfraction twice at 37,000 *g* for 15 min (Table 1). Comparison of morphology and protein patterns of subfractions before and after these two washes also indicated removal of non-myelin membranes during washing. The washes also removed sucrose which might interfere with galactolipid determinations.

Characterization of myelin subfractions

The components in each subfraction were examined by electron microscopy (Fig. 2). Fractions *A* and *B* consisted almost completely of long strands of myelin, with

TABLE 1.—RECOVERY OF RADIOACTIVE SULPHATIDE FROM MICROSOMES ON REGULAR AND INVERTED GRADIENTS

Sample	Regular			Inverted		
	Microsomes	Washed microsomes	Myelin plus microsomes	Microsomes	Washed microsomes	Myelin plus microsomes
Radioactive sulphatide						
d.p.m. applied	7000	7000	7000	46,300	46,300	46,300
d.p.m. recovered	6900	2225	2750	44,350	10,050	11,150
% of applied radioactivity						
Above A	52	7	31	—	—	—
A	34	11	1	—	—	—
B	9	4	1	—	—	—
C	2	—	—	16	5	—
Below C	1	9	5	69	13	23
D	2	—	2	11	4	1

Microsomal fractions were prepared from the brains of 17-day-old rats at 1 h after injection of $Na_2[^{35}S]$sulphate. The microsomal pellets containing radioactive sulphatide were suspended in 0·32 M sucrose by hand homogenization. The 3-step discontinuous sucrose gradients were prepared as described in Methods. Four gradients were each layered with 3 ml of the labelled microsomal suspension (300 μg of protein). Two gradients were layered with a mixture of labelled microsomal suspension (300 μg of protein) and unlabelled myelin (1200 μg of protein).

For the inverted gradients, 3 ml of 0·72 M sucrose containing the labelled microsomal suspension or the mixture of microsomes and myelin were overlaid with 3 ml each of 0·63, 0·50 and 0·32 M sucrose. The samples were centrifuged for 30 min at 75,500 *g* (25,000 rev./min) in a swinging bucket rotor. The pellets (*D*) and the regions corresponding to the sucrose interfaces (*A*, *B* and *C*, from the top) were collected, and duplicate samples were pooled. One-half of each of the samples from the microsomal gradients were used directly for the extraction of lipids and counting of radioactive sulphatide. The remaining microsomal samples and those from the gradients containing a mixture of labelled microsomes and unlabelled myelin were each diluted 10-fold with water and washed twice for 15 min at 37,000 *g* (17,500 rev./min) in a fixed-angle rotor. Radioactive sulphatide was then counted in the lipid extracts of the pellets remaining after this washing procedure. In addition, the material above the first interface (above *A*) and the material between the lower interface and the pellet (below *C*) were collected from all the gradients, and subjected to lipid extraction without the two final water washes.

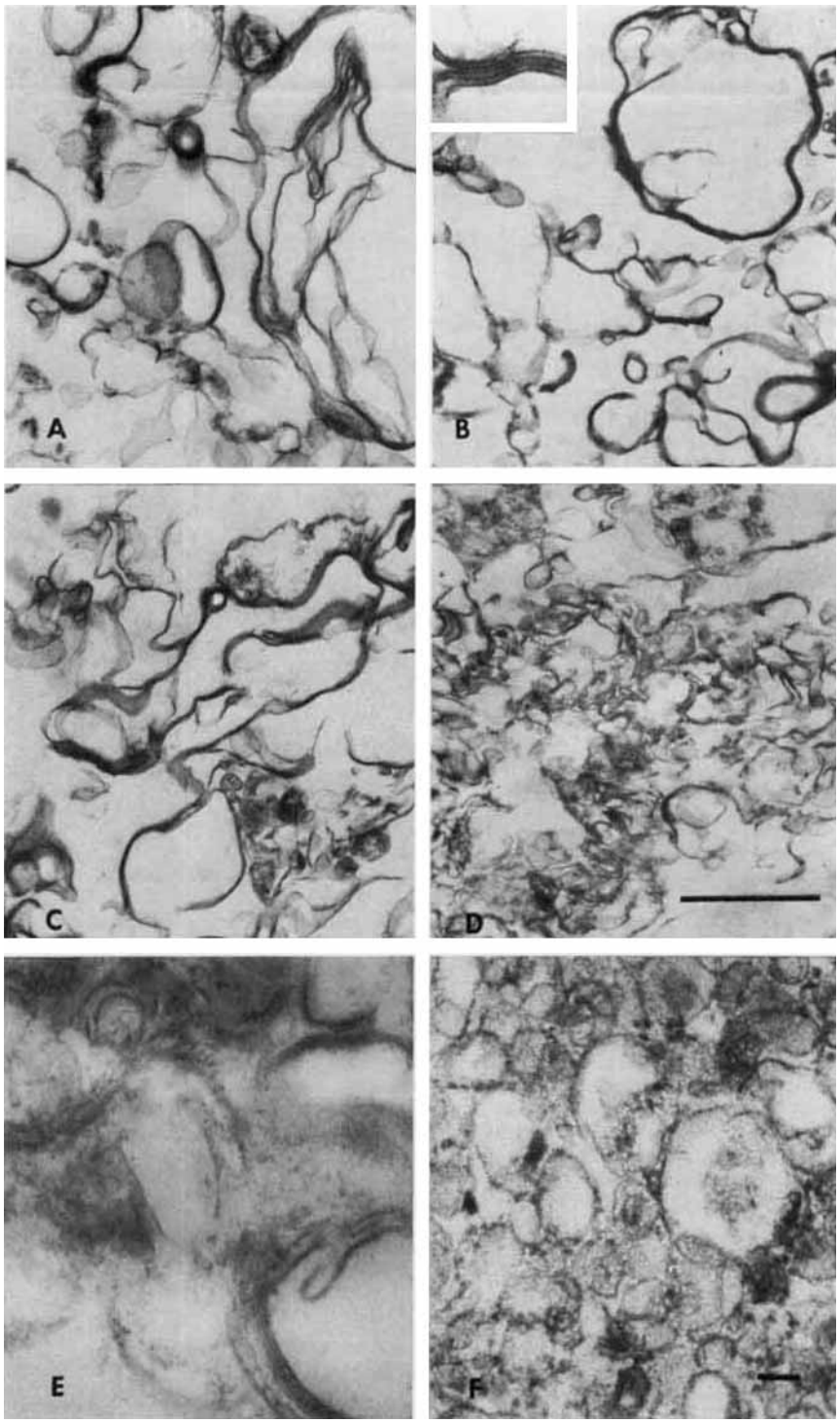

FIG. 2.—Electron micrographs of fractions from myelin: (a) fraction *A*; (b) fraction *B*; (c) fraction *C*; (d) fraction *D* (all at magnification × 18,000; calibration bar = 1·0 μm); (e) fraction *D*; (f) microsomal fraction; (b inset) fraction *B* (all at magnification × 54,000; calibration bar = 0·1 μm). Samples were suspended in 4·5% (v/v) glutaraldehyde with cacodylate buffer, and centrifuged at 100,000 *g* for 30 min. The pellets were fixed in 1% (w/v) OsO_4, dehydrated, embedded in Spur low viscosity medium, and stained with lead citrate and uranyl acetate. (Courtesy of Dr. Robert Herndon and Miss Lilliana Descalzi).

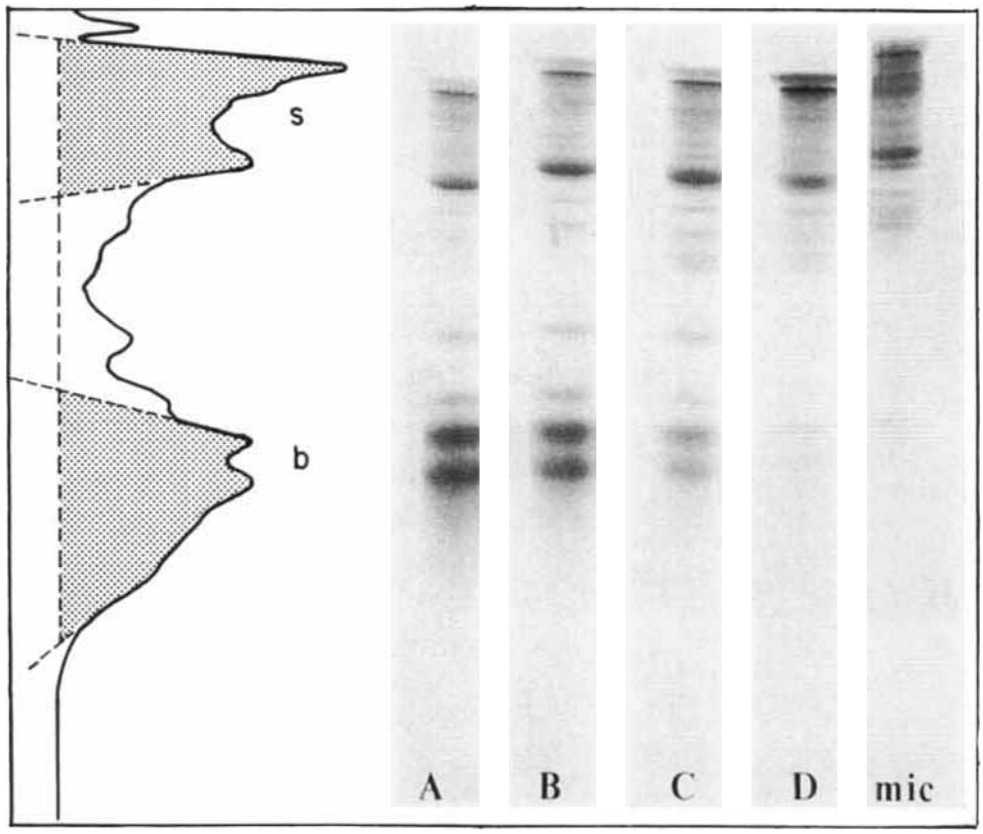

FIG. 3.—SDS acrylamide gel electrophoresis of myelin and microsomal fractions. Gels of 7·5% (w/v) acrylamide containing SDS were run at pH 8·3, as described by GREENFIELD *et al.* (1971). Fractions were delipidated with five washes in ethanol–ether (3:2, v/v) and then assayed for protein content (LOWRY *et al.*, 1951); 100 μg of delipidated protein were applied to each gel. The samples were separated at 45 V for 18 h, and stained with acid fast green. The patterns were almost identical to those described by GREENFIELD *et al.* (1971) for mouse myelin. From the top of the gel, the bands include several high molecular weight proteins, the most prominent probably the acidic protein; proteolipid protein; an intermediate protein; and two basic proteins. From left to right the gels illustrate patterns for fractions *A*, *B*, *C* and *D* from myelin, and the microsomal (MIC) fraction, all from 17-day-old rats. The gels were scanned at 650 nm; the scan shown on the left is for fraction *A*. The ratios of the areas of the peaks for the slow-moving proteins (s) to the areas of those of the two basic proteins (b) were calculated:

	Experiment 1	Experiment 2
A	1·4	1·0
B	1·9	1·2
C	2·5	2·3
D	4·5	4·8
Microsomal	8·7	8·0

somewhat larger pieces in *A* than in *B*. Fraction *C* was also primarily myelin, but occasionally axonal material and mitochondria could be found. Fraction *D* contained primarily vesicular membranes, with some myelin fragments. Unlike the microsomal fraction, fraction *D* had no ribosomes, and the vesicles were larger.

The amounts of protein recovered in each subfraction varied with age (Table 2). In fractions from 13-day-old rats, fraction *D* consistently contained the most material,

TABLE 2.—DISTRIBUTION OF PROTEIN IN MYELIN FRACTIONS PREPARED FROM RAT BRAIN AT VARIOUS AGES

Fraction	μg of protein recovered/brain		
	13 days	17 days	40 days
A	5·4–8·0	137 ± 22	416–484
B	21·4–30·0	424 ± 21	4000–4200
C	12·0–24·0	164 ± 25	1700–2270
D	28·0–32·0	124 ± 35	301–445
	μg of protein/fraction / μg of protein in all fractions		
A	9%	16%	6%
B	29%	50%	37%
C	23%	19%	31%
D	39%	15%	5%
	μg of protein in fraction / μg of protein in fraction at age 13 days		
A	1	20	67
B	1	19	185
C	1	9	123
D	1	4	12

Values for μg of protein recovered/brain are means ± S.D. for nine experiments (17-day-old animals) or individual values from two experiments.

whereas at 17 and at 40 days of age fraction *B* contained the most material. Between ages 13 and 17 days, the amount of protein recovered increased sharply in fractions *A*, *B* and *C* in comparison to that in fraction *D*, i.e. about a 20-fold increase in the former compared to a 5-fold increase in the latter. Between ages 17 and 40 days, protein recovered in fractions *B* and *C* increased 10-fold, whereas that in fractions *A* and *D* increased only 3- to 4-fold.

At all three ages that we studied the sulphatide: protein ratio was highest in fraction *A* and progressively lower in fractions *B*, *C* and *D* (Table 3). The fractions appeared to be richer in sulphatide at age 13 days than at ages 17 or 40 days. We have not yet confirmed the identity of the sulphated lipid in the 13-day fractions as sulphatide. In 17-day-old rats, the molar ratios of cholesterol: phospholipid: galactolipid differed among the subfractions (Table 4), with fraction *A* being highest in cholesterol and

TABLE 3.—DISTRIBUTION OF SULPHATIDE IN MYELIN FRACTIONS FROM RAT BRAIN AT VARIOUS POSTNATAL AGES

Fraction	13 days	17 days	40 days
		μg of sulphatide/brain	
A	6–16	31 ± 4	90–96
B	8–12	65 ± 8	458–482
C	4	22 ± 3	203–209
D	4–6	13 ± 2	36–40
Microsomal	241–251	149 ± 16	210–250
		μg of sulphatide/brain / mg of protein/brain	
A	1000–2000	250 ± 42	190–230
B	350–590	162 ± 8	108–132
C	170–370	136 ± 12	90–100
D	130–210	75 ± 15	85–95
Microsomal	24–34	15 ± 1	19–21

Values are means ± S.D. for nine experiments (17-day-old animals) or individual values from two experiments.

TABLE 4.—LIPID COMPOSITION OF MYELIN FRACTIONS FROM BRAINS OF 17-day-OLD RATS

Fraction	Cholesterol	Lipids (μmol/mg of protein) Phospholipid	Galactolipid	Total
A	1·06	1·06	0·58	2·70
B	0·92	0·99	0·49	2·40
C	0·78	0·94	0·43	2·14
D	0·52	0·64	0·19	1·33
Microsomal	0·22	0·39	0·09	0·70
		Molar ratios, cholesterol = 1		
A	1·0	1·0	0·55	
B	1·0	1·1	0·53	
C	1·0	1·2	0·55	
D	1·0	1·2	0·30	
Microsomal	1·0	1·8	0·41	

See Methods for analytical procedures. Values represent results of a single typical experiment.

galactolipids. The sum of the contents of these three classes of lipids per mg of protein was greatest in fraction *A*, and decreased in fractions *B*, *C* and *D* successively.

The protein patterns for the myelin and microsomal fractions on SDS acrylamide gels showed that fraction *A* was richest in basic proteins and proteolipid protein, with successively smaller amounts in fractions *B*, *C* and *D* (Fig. 3). The proportions of basic proteins in each fraction were quantified by scanning the gels and comparing the area of the peaks of the slow-moving proteins to that of the basic proteins (Fig. 3).

The microsomal fraction contains traces of proteins with R_f values similar to those of the myelin basic proteins. We do not yet know whether these basic proteins are in the endoplasmic reticulum or in myelin fragments sedimenting with the microsomal fraction (HERSCHKOWITZ *et al.*, 1968*a*).

The activity of 2',3'-cyclic nucleotidase, an enzyme enriched in CNS myelin (OLAFSON, DRUMMOND and LEE, 1969) was high in the myelin subfractions in comparison to the activity in the microsomal fraction (Table 5). Pretreatment of the

TABLE 5.—DISTRIBUTION OF 2'3'-CYCLIC NUCLEOTIDASE AND CEREBROSIDE SULPHOTRANSFERASE IN MYELIN FRACTIONS FROM RAT BRAINS

Fraction	2'3'-Cyclic Nucleotidase μg of P_i. $(10\ min)^{-1}$. μg^{-1} of protein		Cerebroside Sulphotransferase d.p.m. $(30\ min)^{-1}$. μg^{-1} of protein
A	1·76	2·70	410
B	3·95	3·25	573
C	3·59	3·10	490
D	3·31	—	360
Microsomal	0·36	0·13	7900

Myelin and microsomal fractions were prepared from brains of 17-day-old rats. See Methods for analytic procedures and preparation of fractions. Values represent data for individual experiments.

membranes with 0·6% (w/v) deoxycholate greatly increased the enzymatic activity in all of the fractions, but the myelin subfractions were still 6- to 10-fold higher in specific activity than the microsomal fraction. The activity of cerebroside sulphotransferase, an enzyme which is enriched in the microsomal fraction (FARRELL and MCKHANN, 1971; HERSCHKOWITZ *et al.*, 1968*a*) and characterized by a marked increase during myelination, was 15- to 20-fold higher in the microsomal fraction than in the four fractions derived from myelin (Table 5). When the fractions were treated with Triton X-100 (FARRELL and MCKHANN, 1971), about 60 per cent of the protein in the microsomal fraction was solubilized, and the specific activity of cerebroside sulphotransferase in the supernatant fraction increased 3-fold. However, with the myelin subfractions, only about 15 per cent of the protein was solubilized, and little or no sulphotransferase activity could be detected in these supernatant fractions.

Labelling of sulphatide in myelin fractions

Short-term labelling. All of the myelin fractions that we isolated had little capacity to synthesize sulphatide in comparison to the microsomal fraction. Thus, short-term experiments in which $Na_2[^{35}S]$sulphate was incorporated into brain sulphatide might indicate the capacity of the myelin fractions to accept sulphatide newly synthesized in the microsomal fraction or in some other donor membrane. Choice of a reference base with which to express results was difficult. Radioactivity of sulphatide in a given fraction indicated how many new molecules of sulphatide had been added, assuming that the donor pool was the same for all myelin fractions. Sulphatide radioactivity/μg of sulphatide indicated what proportion of the sulphatide in the membrane had been added during a given period of time. Sulphatide radioactivity/μg of protein might indicate the number of binding sites for newly synthesized sulphatide in a given

membrane. This latter assumption was, of course, subject to error if proteins from membranes other than myelin contaminated the fractions.

At 15 min after intracranial injection of radioactive sulphate, the specific radioactivity of sulphatide was 6- to 12-fold greater in the microsomal fraction than in the myelin fractions, as expected from the low sulphotransferase activity found in the latter fractions. The specific radioactivity of sulphatide followed the pattern $D>C>B>A$ (Fig. 4a); the patterns were similar when the results were expressed per μg of protein (Fig. 4c). These results indicated that the lipid-poor fractions

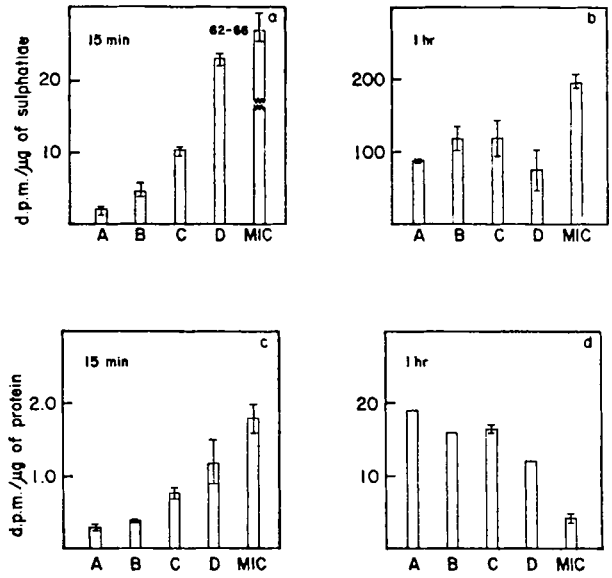

FIG. 4.—Specific radioactivities of sulphatide in myelin fractions at 15 min and 1 h after injection. Rats (17-day-old) each recived 125 μCi of $Na_2[^{35}]$sulphate intracranially in 30 μl of 0·15 M NaCl. After 15 min or 1 h the animals were killed, and the myelin and microsomal fractions from the brains were prepared. Portions of each fraction were used for measurement of protein or extraction of lipid. Sulphatide and radioactivity were measured in portions of the lipid extract. The data represent the averages from two separate experiments, with the ranges indicated.

accepted newly synthesized sulphatide to a greater extent than the lipid-rich fractions. In other words, fraction *C* might contain a higher proportion of myelin membranes in process of formation whereas fraction *A* might contain a higher proportion of more 'mature' myelin, laid down before injection of the radioactive sulphate. Another possible interpretation of these data was that the myelin fractions might be contaminated with microsomal membranes, with fraction *D* most highly contaminated and fraction *A* least contaminated. On the basis of the lipid and protein compositions, it is possible that fraction *D* is a mixture of microsomes and fragments of lipid-rich myelin membranes. However, the marked enrichment of 2′,3′-cyclic nucleotidase and the low level of cerebroside sulphotransferase in fraction *D* was comparable to that of the other myelin fractions rather than that of microsomes. Furthermore, centrifugation of microsomes layered on top of discontinuous gradients with or without

admixed myelin demonstrated that most of the microsomal membranes remained at the top of the gradient, so that fraction *A* would have been most highly contaminated by microsomes (Table 1). Finally in the water shock steps and the final water washes, the myelin fractions were centrifuged at low *g* forces so that most microsomal membranes would not sediment (RUMSBY *et al.*, 1970).

At 1 h after the intracranial injection of radioactive sulphate the specific radioactivity of sulphatide in the microsomal fraction had increased about 3-fold over its 15 min value (Fig. 4b), an indication of an almost linear rate of synthesis during this period. The specific radioactivities of sulphatide in the myelin fractions increased 8- to 20-fold, and were approaching the specific radioactivity of sulphatide in the microsomal fraction. These data showed an initial lag in transfer of newly-labelled sulphatide from the microsomal fraction to myelin, followed by equilibration between the microsomal and myelin membranes. At longer periods after injection of radioactive sulphate, the specific radioactivity of sulphatide in fractions *A*, *B* and *C* exceeded that of the microsomal fraction, while that of fraction *D* remained lower. This observation was confirmed by pulse-chase experiments (Fig. 5); injection of

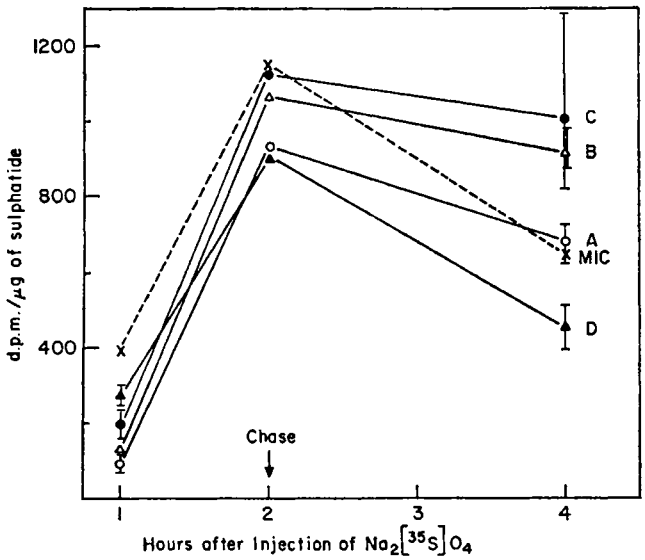

FIG. 5.—Specific radioactivity of sulphatide in myelin and microsomal fractions following a chase with unlabelled sulphatide. Experimental details were as described for Fig. 4, except that each animal received 200 μCi of $Na_2[^{35}S]$sulphate. For chase experiments, animals received 0·5 ml of Na_2SO_4 (7%, w/v) intraperitoneally and 30 μl of Na_2SO_4 (1%, w/v) intracranially. These animals were killed at 2 h after the chase.

unlabelled sulphate at 2 h after the injection of radioactive sulphate caused a sharp drop in the specific radioactivity of microsomal sulphatide during the next 2 h. The specific radioactivity of fraction *D* closely followed the drop in microsomal sulphatide, an indication that the sulphatide in fraction *D* was turning over very rapidly. The specific radioactivities of sulphatide in fractions *A*, *B* and *C* remained constant or dropped only slightly, an indication of little loss of newly-labelled sulphatide from these membranes.

Long-term labelling. DAVISON and GREGSON (1966) and PRITCHARD (1966) have reported that $Na_2[^{35}S]$sulphate injected intraperitoneally into rats at age 5 days (before myelination has started to any great extent) enters a pool of sulphatide which is subsequently incorporated into the myelin membrane. We repeated this type of experiment by using intracranial rather than intraperitoneal injection to minimize recycling of $Na_2[^{35}S]$sulphate in serum, and obtained essentially the same results. Total sulphatide radioactivity in the myelin fractions increased markedly between ages 12 and 40 days (Table 6). About 30 per cent of the labelled sulphatide could be

TABLE 6.—DISTRIBUTION OF LABELLED SULPHATIDE IN MYELIN FRACTIONS FROM RAT BRAIN AFTER INJECTION OF $Na_2[^{35}S]$SULPHATE AT 5 DAYS OF POSTNATAL AGE

	Sulphatide radioactivity					
	d.p.m./brain			d.p.m./μg of sulphatide		
Fraction	12 d	19 d	40 d	12 d	19 d	40 d
Homogenate	108,000	96,000	75,000	—	—	—
A	1178	3920	3800	74	115	41
B	2627	8980	16,600	328	107	36
C	1002	3980	3500	251	87	17
D	484	420	485	121	21	13
Microsomal	26,000	6650	2410	106	43	10

Rats at age 5 days each received 40 μCi of $Na_2[^{35}S]$sulphate intracranially. Six animals were killed at 12 days of age and three each at 19 and 40 days. The brains of animals of the same age were pooled. Myelin and microsomal fractions were prepared as described in Methods. Lipids were extracted from each fraction for measurement of sulphatide content and counting of $[^{35}S]$ radioactivity. At 40 days the d.p.m. of sulphatide/μg of protein were 8·6 in *A*, 4·2 in *B*, 1·6 in *C*, 1·3 in *D*, and 0·21 in the microsomal fraction.

accounted for by the microsomal and myelin fractions as we isolated them; for example at age 19 days, the combined microsomal and myelin fractions contained about 24,000 d.p.m. of radioactive sulphatide, while sulphatide radioactivity in the total homogenate was 96,000 d.p.m. The radioactive sulphatide which eventually ended in the myelin may have arisen entirely from the microsomal fraction or from some other membrane, perhaps the oligodendroglial plasma membrane, which might serve as a reservoir of labelled sulphatide. Recycling of radioactive inorganic sulphate might be great enough to supply some newly labelled sulphatide during myelination. Whatever the explanation for the accumulation of radioactive sulphatide in myelin at many days after the injection of the precursor isotope, we would have expected myelin synthesized near the time of injection to contain sulphatide of a higher specific radioactivity than that formed later. From our short-term labelling experiments with the myelin fractions, we predicted that fraction *A* would contain the lowest proportion of newly-formed myelin and the highest proportion of more 'mature' myelin, laid down early in myelination. Thus we expected to see the most sulphatide radioactivity/μg of protein or sulphatide in fraction *A*, followed successively by fractions *B*, *C* and *D*. This situation was in fact what we observed in rats at ages 19 days and 40 days following injection of radioactive sulphate at 5 days (Table 6).

The total sulphatide radioactivity in each of the myelin fractions increased as a function of postnatal age in a pattern similar to that of the increase in protein content

of each fraction; that is, fraction *D* showed little increase in total sulphatide radioactivity; fractions *A* and *C* increased somewhat more rapidly, whereas fraction *B* showed a marked increase between ages 19 and 40 days. These increases were consistent with the hypothesis that the *D* fraction was enriched in fragments of membrane adjacent to the oligodendroglial cell; the amounts in fractions *A* and *C* should have increased at about the same rate, and increased only as long as oligodendroglial cells were initiating myelination. When initiation of myelination had stopped, lamellae would continue to be formed; fraction *B* might contain the bulk of the intermediate membranes, and thus would continue to increase more rapidly than the other fractions between ages 19 and 40 days.

DISCUSSION

Separation of membranes into fractions on density gradients is a powerful tool, but one that may yield misleading results through artifacts. We have isolated four membrane fractions from a myelin preparation, but obviously each of these fractions is still heterogeneous. A continuum of membranes differing in size, density, composition and subcellular origin comprises the original myelin preparation layered on the discontinuous gradient. We have operationally separated this continuum into four fractions. The lipid: protein ratios suggest that part of this separation is attributable to density properties of the membranes, since fraction *A* (the top band) has a higher lipid: protein ratio than fractions *B* and *C*. Prolonged centrifugation of the gradient resulted in progressive loss of material from fractions *A* and *B* into fractions *C* and *D* (Fig. 1, a and d), an observation indicating some of the membranes do not reach their isopycnic density after 30 min of centrifugation.

We chose to examine labelling of sulphatide in the myelin fractions for several reasons. First, we wanted to determine whether the fractions differed from each other in uptake and turnover of one of their components. Furthermore, sulphatide is markedly enriched in myelin compared to other readily identified subcellular fractions such as mitochondria and synaptosomes, so contamination by these membranes would have a minimal effect on our results. Finally, increased synthesis of sulphatide is closely related to the onset of myelination (McKhann and Ho, 1967; Silberberg *et al.*, 1972) and probably to differentiation of oligodendroglial cells (Fry, Lehrer and Bornstein, 1972).

The pattern of labelling of sulphatide in the subfractions suggests that fractions *D* and *C* may contain a higher proportion of myelin in process of formation than fractions *B* and *A*. Although it is impossible with biochemical techniques to pin-point the morphological location of this sulphatide labelling, we suggest that fractions *D* and *C* may be enriched in fragments of myelin originally near the oligodendroglial cell, whereas fractions *B* and *A* may be enriched in fragments originally near the axon. Alternatively, the newly-forming myelin may be near the external, internal and lateral cytoplasmic loops, as postulated by Hirano (1968) and Morell *et al.* (1972), with the mature myelin more distant from these loops. Fractions *D* and *C* appear to contain more short fragments of myelin than fractions *B* and *A*. If the myelin membrane were more susceptible to fragmentation at its forming end, then fractions *D* and *C* would contain a higher proportion of newly-forming myelin per unit of membrane than the longer strands in fractions *B* and *A*. The above interpretation is based on the assumptions that oligodendroglial cells synthesize sulphatide very

actively compared to other cell types in brain, and that most of the labelled sulphatide in the fractions is contributed by myelin or related membranes of oligodendroglial origin. Two likely sources of contamination in the subfractions are microsomal and axonal membranes. In our method of preparation, fractions *A* and *B* would be most likely to contain microsomal membranes; however, this contamination should be small (Table 1). Also, the morphology, lipid and protein composition and high activity of 2',3'-cyclic nucleotidase indicate minimal contamination by microsomes. Axonal membranes would be found primarily in fraction *D*, based on the observations of DE VRIES, NORTON and RAINE (1972) who isolated axons from a myelin fraction following water shock. In their study, the axons formed a pellet in 0·88 M sucrose while the myelin floated. Thus we would expect myelin with increasing amounts of axonal material attached to have an increasing density. Fraction *C* might contain axonal material if the osmotic shock and two centrifugations at 12,000 *g* do not detach and remove this material; fraction *D* would contain axonal material free of myelin. In the electron micrographs of fraction *C*, we saw an occasional myelin figure enclosing undisrupted axonal material. Fraction *D* contained primarily single vesicular membranes whose origin was not readily identified, and occasional fragments of myelin. Thus an alternative explanation of our observations may be that fractions of increasing density have increasing amounts of axonal material, and that the myelinated axons contribute labelled sulphatide of high specific radioactivity to the fractions. We know there is a small amount of axonal material in *C* and probably in *D*; the question is how much of the material is axonal, and whether the labelled sulphatide is primarily in the axonal or myelin components.

If axons do contribute significantly to the properties of fractions *C* and *D*, we find it difficult to explain the high activity of 2',3'-cyclic nucleotidase in these fractions since DE VRIES, NORTON and RAINE (1972) reported very low levels of this enzyme in their axonal preparations. Of course, the enzyme may have been lost or inactivated in their procedure. We find a rapid turnover of sulphatide in fraction *D*. If this property is due to axonal contamination, the axons would have to contribute sulphatide of very high specific radioactivity, since axons have low levels of sulphatide (DE VRIES *et al.*, 1972).

We think it unlikely that axonal material in fractions *C* and *D* has the capacity to synthesize sulphatide, since cerebroside sulphotransferase was equally low in all myelin fractions and not increased in fractions *C* and *D*. The highly labelled sulphatide may have come from the neuronal cell body, but this is not consistent with our findings of several-fold lower activities of cerebroside sulphotransferase in grey matter and isolated neurons when compared to white matter and isolated oligodendroglia from calf brain (unpublished observations).

Heterogeneity of the fractions may also contribute to the differences in protein patterns of the subfractions. Thus, the high molecular weight proteins on the gels may be partly of axonal origin. WAEHNELDT and MANDEL (1972) reported that their myelin fractions lost high molecular weight proteins after extensive purification. However, MORELL *et al.* (1972) supported the idea that some of the high molecular weight proteins are intrinsic to the myelin membrane since myelin isolated and highly purified by two different methods still showed multiple bands of high molecular weight proteins. Both groups used SDS gels, but different pore sizes and electrophoretic conditions. We have observed that our myelin preparations have a simpler

pattern on the Triton-urea gels of LIM and TADAYYON (1970) than on the SDS gels used by MORELL *et al.* (1972), but we have not yet used the conditions of WAEHNELDT and MANDEL (1972). Until further information is available, we are unable to say whether heterogeneity or simplicity of protein pattern is an artifact of the acrylamide gel system, or a reflection of the purity of the myelin preparation.

While this study was in progress, several papers reporting the isolation and characterization of three myelin fractions from adult rat brain have appeared (MCMILLAN *et al.*, 1972; DANIEL, KAUFMAN and DAY, 1972). In agreement with our results with sulphatide, DANIEL *et al.* (1972) observed in 40-day-old animals that the myelin fraction lightest in density contained phospholipids most highly labelled with radioactive phosphate which had been injected into the animals at age 11 days. The lipid composition of the three fractions described by MCMILLAN *et al.* (1972) differed primarily in the amount of cholesterol/mg of protein, whereas we found the greatest differences in galactolipid content. Our fraction of lightest density *A* had the highest lipid:protein ratio, followed by fractions *B* and *C*, whereas the lightest and the densest fractions isolated by MCMILLAN *et al.* (1972) had identical lipid:protein ratios. The major differences in the methods of preparation are: (a) use of an osmotic shock step in our method to decrease axonal content, and (b) use of discontinuous sucrose gradients in our method rather than continuous iso-osmotic gradients of Ficoll-sucrose. Although the Ficoll-sucrose gradients are theoretically better-suited for isolation of membranes on the basis of density, the presence of axonal membranes in myelin fractions which have not been water-shocked will contribute to the density properties and the lipid composition of the fractions. On the other hand, subjecting myelin membranes to water shock leads to fragmentation of the membrane and perhaps rearrangement of its structure.

Our first objective in the present study was to isolate myelin membranes of differing lipid:protein ratios. We included in our procedure several steps to remove the fraction described by AGRAWAL *et al.* (1970) as a 'myelin-like' or pre-myelin membrane, since this did not morphologically resemble myelin. However, our fraction *D* has many properties in common with the 'myelin-like' fraction; both consist of small vesicular membranes, both have high activities of 2',3'-cyclic nucleotidase, and both have lipid and protein compositions intermediate between those of myelin and microsomes. MORELL *et al.* (1972) have also described a myelin-like fraction isolated on CsCl gradients; it is similar in many ways to that isolated by AGRAWAL *et al.* (1970). Our fraction *D* is morphologically similar to both of these myelin-like fractions. However, unlike these other fractions, fraction *D* has significant amounts of basic protein, some of it probably associated with the occasional myelin fragments we saw in this fraction. The labelling of sulphatide in fraction *D* indicated a rapid turnover of this lipid in comparison to that in the myelin fractions. This rapid turnover could reflect a membrane involved in the transfer of sulphatide from its site of synthesis into myelin. The metabolism of other lipids and especially of basic proteins in this membrane fraction may help clarify its origin and function.

The microsomal fraction we have isolated is a mixture of endoplasmic reticulum and other membranes from various cell types. From previous studies, we know this fraction also contains a small amount of myelin (HERSCHKOWITZ *et al.*, 1968*b*). We would expect that a microsomal fraction from oligodendroglial cells might have an even higher specific activity of cerebroside sulphotransferase than we have reported

in this paper. We have previously reported high specific activities of cerebroside sulphotransferase in 'recycled' microsomal and 'light myelin' fractions from rat brain (FARRELL and MCKHANN, 1971). The low activities of cerebroside sulphotransferase found in the myelin subfractions described in the present paper suggest that the fraction associated with myelin which has high levels of this enzyme is lost during preparation, perhaps during the osmotic shock steps. The identity of the fraction containing high levels of cerebroside sulphotransferase is currently under investigation.

In summary, we have isolated three myelin fractions which differ in density, lipid: protein ratios and sulphatide metabolism. The properties of these fractions are consistent with the hypothesis that each fraction is enriched with membrane fragments from a different region of the myelin sheath. Thus, fraction *A* would be enriched in fragments adjacent to the axon; these fragments might have few binding sites for newly labelled sulphatide, little turnover of sulphatide and high contents of basic proteins and galactolipids. Conversely, fraction *C* might be enriched in fragments adjacent to the oligodendroglial cell; these fragments might have many binding sites for newly labelled sulphatide, some turnover of sulphatide, and lowered contents of basic proteins and galactolipids. Since the fragments of myelin in each fraction are relatively long, we might expect a large degree of overlap; thus in every fraction, there could be some fragments which have newly-forming myelin on one end, and older, more lipid-rich membrane on the other end.

We cannot, of course, rule out the possibility that the myelin fractions we have isolated arise from different regions of brain, or that the apparent metabolic properties of the fractions arise from contamination with a membrane, other than myelin, which also contains sulphatide with a faster turnover than that in myelin. Future experiments to study these possibilities will include examination of myelin from specific areas of brain, and investigation of the synthesis and metabolism of other components highly enriched in myelin, specifically the basic proteins.

Acknowledgements—This research was supported by funds from The John A. Hartford Foundation, Inc., and by USPHS Grant NS08719. We thank Dr. ROBERT HERNDON and Miss LILLIANA DESCALZI for preparing electron micrographs of the myelin fractions.

REFERENCES

ADAMS D. H. and FOX M. E. (1969) *Brain Res.* **40**, 647–661.
AGRAWAL H. C., BANIK N. L., BONE A. H., DAVISON A. N., MITCHELL R. F. and SPOHN M. (1970) *Biochem. J.* **120**, 635–642.
BENJAMINS J. A., HERSCHKOWITZ N., ROBINSON J. and MCKHANN G. M. (1971) *J. Neurochem.* **18**, 729–738.
BRAUN P. E. and BARCHI R. L. (1972) *Brain Res.* **40**, 437–444.
CUZNER M. L. and DAVISON A. N. (1968) *Biochem. J.* **106**, 29–34.
DANIEL A., DAY E. D. and KAUFMAN B. (1972) *Fedn. Proc., Fedn. Am. Socs. exp. Biol.* **31**, 490 Abs.
DAVISON A. N. and GREGSON N. A. (1966) *Biochem. J.* **98**, 915–922.
DEVRIES G. H., NORTON W. T. and RAINE C. S. (1972) *Science, N.Y.* **175**, 1370–1372.
DRUMMOND G., ENG D. and MCINTOSH A. (1971) *Brain Res.* **28**, 153–163.
ENG L. F. and BIGNAMI A. (1972) *Trans. Am Soc. Neurochem.* **3**, 75.
ENG L. F. and NOBLE E. P. (1968) *Lipids* **3**, 157–161.
FARRELL D. and MCKHANN G. (1971) *J. biol. Chem.* **246**, 4694–4702.
FRY, J., LEHRER J. and BORNSTEIN M. (1972) *Science, N.Y.* **175**, 192–193.
GREENFIELD S., NORTON W. and MORELL P. (1971) *J. Neurochem.* **18**, 2119–2128.
HERSCHKOWITZ N., MCKHANN G. M., SAXENA S. and SHOOTER E. M. (1968*a*) *J. Neurochem.* **15**, 1181–1188.

HERSCHKOWITZ N., MCKHANN G. M. and SHOOTER E. M. (1968*b*) *J. Neurochem.* **15**, 161–168.
HIRANO A. (1968) *J. Cell Biol.* **34**, 555–567.
KEAN E. (1968) *J. Lipid Res.* **9**, 319–327.
LIM R. and TADAYYON E. (1970) *Analyt. Biochem.* **34**, 9–15.
LOWRY O. H., ROSEBROUGH N. J., FARR A. L. and RANDALL R. J. (1951) *J. biol. Chem.* **193**, 265–275.
MCKHANN G. M. and HO W. (1967) *J. Neurochem.* **14**, 717–724.
MCMILLAN P. N., WILLIAMS N. I., KAUFMAN B. and DAY E. D. (1972) *J. Neurochem.* **19**, 1839–1848.
MORELL P., GREENFIELD S., CONSTANTINO-CECCARINI E. and WISNIEWSKI H. (1972) *J. Neurochem.* **19**, 2545–2554.
NORTON W. T. (1971) In *Chemistry and Brain Development* (Edited by PAOLETTI R. and DAVISON A. N.) pp. 327–337. Plenum Press, New York.
NORTON W. T. and AUTILIO L. A. (1966) *J. Neurochem.* **13**, 213–222.
OLAFSON R. W., DRUMMOND G. I. and LEE J. F. (1969) *Can. J. Biochem.* **47**, 961–966.
PRITCHARD E. T. (1966) *J. Neurochem.* **13**, 13–21.
RUMSBY M. G., RIEKKINEN P. J. and ARSTILA A. V. (1970) *Brain Res.* **24**, 495–516.
SILBERBERG D., BENJAMINS J. A., HERSCHKOWITZ N. and MCKHANN G. M. (1972) *J. Neurochem.* **19**, 11–18.
SVENNERHOLM L. (1956) *J. Neurochem.* **1**, 42–53.
WAEHNELDT T. V. and MANDEL P. (1972) *Brain Res.* **40**, 419–436.