The Maturation of Human White Matter Myelin. Fractionation of the Myelin Membrane Proteins*

L. F. Eng, F.-C. Chao, B. Gerstl, D. Pratt, and M. G. Tavaststjerna

ABSTRACT: Myelin was prepared from bovine and human white matter and bovine sciatic nerve. The human preparations included individuals of age 10 weeks and 4 years, and adults, two of whom had multiple sclerosis. Three myelin protein fractions were obtained by extraction with 0.5% Triton X-100–0.5 M ammonium acetate. A basic protein fraction which accounted for 30% of the total protein in the adult appeared homogeneous on electrophoresis and in the ultracentrifuge. Extraction of the basic protein from mylein with 0.5 M neutral salt suggests that it is bound in the membrane primarily by ionic bonds. A second protein type, which comprised about 50% of total protein, was similar in solubility and amino acid composition to the Folch–Lees protein (Folch, J., and Lees, M. (1951), *J. Biol. Chem. 191*, 807). The remaining 20% of protein had an amino acid composition similar to a protein first described by Wolfgram (Wolfgram, F. (1966), *J. Neurochem. 13*, 461). The proportions of the three proteins from bovine and human white matter were similar; however, sciatic nerve myelin contained a higher proportion of Wolfgram type and lower Folch–Lees type. While total protein and lipid content of myelin at the different ages were similar, the distribution of the various proteins and lipids changed with age. The basic protein increased, the Folch–Lees type decreased, and the Wolfgram type remained constant during maturation. The proportions of galactolipids and cholesterol to phospholipids increased with age. Lecithin content decreased with increasing age.

The major constituents of the myelin membrane are lipids and proteins. The composition of the lipids has been the subject of comprehensive studies recently reviewed by Smith (1967). Three classes of proteins possibly derived from myelin have been demonstrated to be present in white matter. One of these, an encephalitogenic basic protein, was first isolated from guinea pig brain myelin by Laatsch *et al.* (1962). Subsequently, a Folch–Lees (1951) type of proteolipid was identified in bovine brain myelin (Autilio, 1966) comprising 50–65% of the total myelin protein. An acid-soluble proteolipid protein, Wolfgram type (Wolfgram, 1966), is a third protein that might be a constituent of the myelin membrane.

The present report describes a new, simplified procedure for isolating the three classes of CNS[1] myelin proteins, their compositional changes during maturation, and their partial chemical characterization. A discussion of the possible organization of the proteins and lipids in the myelin membrane is included.

* From the Research Laboratory, Veterans Administration Hospital, the Department of Pathology, Stanford University, Palo Alto, California, and Stanford Research Institute, Menlo Park, California. *Received July 12, 1968.* Preliminary reports of parts of these data were presented at the 152nd Meeting of the American Chemical Society, New York, N. Y., 1966, and the 1st International Meeting of the International Society for Neurochemistry, Strasbourg, France, 1967. This investigation was supported by the U. S. Veterans Administration and by U. S. Public Health Service Grant No. B-3821.

[1] CNS, central nervous system; PNS, peripheral nervous system; MS, multiple sclerosis; Triton–salt, 0.5% Triton X-100–0.5 M ammonium acetate; EAE, experimental allergic encephalomyelitis.

Materials and Methods

Human brain tissue was obtained at autopsy. The age of the individuals ranged from 10 weeks to 92 years. Their history suggested, and the gross and histological examination confirmed, the absence of lesions in the CNS in all except two cases with MS. The MS cases had been under clinical observation for many years, and their brains showed typical lesions. Only white matter grossly free of plaques was utilized for study. Bovine brains and sciatic nerve were obtained fresh from a local slaughter house. Wistar rats (16-day old) were used as the source for brain and spinal cord. Triton X-100 (alkylphenoxypolyethoxyethanol) was generously supplied by Rohm and Haas, Philadelphia, Pa. Horse heart cytochrome C was purchased from Calbiochem, Los Angeles, Calif.

Preparation of Myelin. Myelin was prepared from human and bovine white matter by the procedure of Laatsch *et al.* (1962; Gerstl *et al.*, 1967) and further purified by centrifugation in a continuous sucrose gradient (Autilio *et al.*, 1964; Eng and Noble, 1968).

Neutral Salt and Acid Extraction of Myelin. Myelin was first homogenized in the cold with 0.11 M ammonium acetate solution (pH 6.9) for 30 min in a glass tissue homogenizer using a motor-driven Teflon pestle. On centrifugation at an average of 105,000*g* for 1 hr, a myelin pellet and a clear supernatant were obtained. Using this procedure, the myelin was then extracted with 0.5 M ammonium acetate solution (pH 6.9) for 30 min. Water and ammonium acetate in the supernatant were removed by repeated lyophilization (three to five times). Following the final lyophilization, the

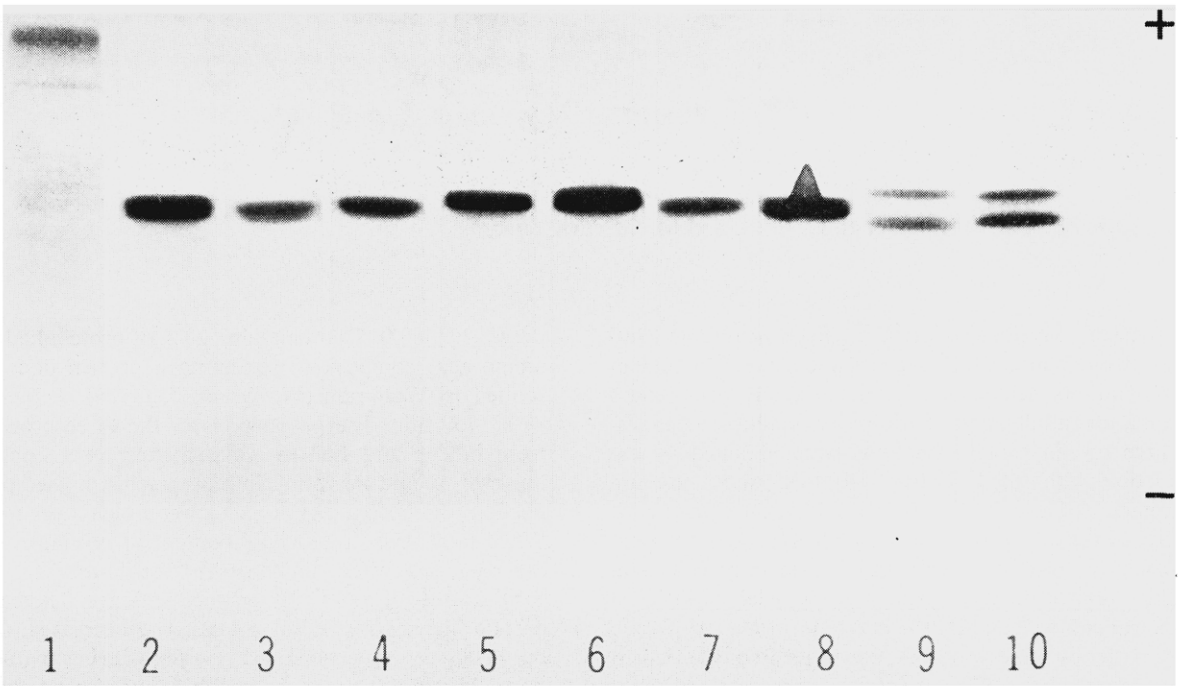

FIGURE 1: Disc electrophoretic analysis in 15% acrylamide gel (pH 4.5) of myelin proteins obtained by salt or acid extraction. Ammonium acetate extraction (0.11 M): (1) 44-yr-old female, 213 μg; ammonium acetate extraction (0.5 M): (2) 44-yr-old female, 58 μg; (3) 57-yr-old male, 36 μg; (4) bovine, 40 μg; 0.05 N HCl extraction: (5) bovine, 50 μg; (6) 41-yr-old male, 60 μg; (7) 47-yr-old male, 30 μg; (8) 10-week male, 55 μg; (9) rat spinal cord, 51 μg; and (10) rat brain, 54 μg.

protein was redissolved in a minimum amount of water and centrifuged at an average of 105,000*g* for 1 hr to remove insoluble material. Further extraction of the myelin pellet with water or 1 M ammonium acetate solution removed only traces of protein, while further extraction with 0.2 N HCl removed a little more.

The myelin pellet remaining after the salt extractions was dissolved in 19 volumes of chloroform–methanol (2:1, v/v) and mixed with 0.20 volume of 0.023 M Tris-HCl in 0.1 M NaCl (pH 8.8) (Webster and Folch, 1961). The protein precipitate at the interphase was washed twice with chloroform–methanol (1:1, v/v) and extracted with 0.05 N HCl. The insoluble proteolipid proteins were removed by centrifugation and the clear supernatant was lyophilized, redissolved in a minimum amount of water, and centrifuged at an average of 105,000*g* for 1 hr to remove insoluble material.

Triton X-100–Neutral Salt Extraction of Myelin. The myelin was first extracted with 0.11 M ammonium acetate solution as described above. The insoluble pellet was next extracted with a Triton–salt solution by homogenization for 30 min in the cold. The resulting clear supernatant, after centrifugation at 105,000*g* for 1 hr, contained a mixture of water-soluble and chloroform–methanol-soluble proteins. The water-soluble protein in the mixture was separated by shaking vigorously with four volumes of diethyl ether and centrifuging at 1000*g* for 10 min. The Triton X-100 and most of the lipid were extracted into the ether phase, the proteolipid protein formed a compact precipitate layer at the interphase, and the water-soluble protein, along with the ammonium acetate, remained in the aqueous phase. Three partitions with ether were made to remove most of the Triton-X-100 and lipids. The water-soluble protein fraction was lyophilized (three to six times), redissolved in a minimum amount of water, and centrifuged at an average of 105,000*g* for 1 hr to remove insoluble material.

The insoluble myelin resulting from this treatment was reextracted with the Triton–salt solution. The resulting soluble extract contained predominantly chloroform–methanol-soluble protein; the insoluble part consisted of insoluble protein, a small amount of chloroform–methanol-soluble protein, and lipids. This pellet was treated with 19 ml of chloroform–methanol (2:1)/g of pellet, and the suspension was centrifuged at 80,000*g* for 15 min in the cold using screw-top polyethylene ultracentrifuge tubes. The resulting clear supernatant contained the bulk of the lipids and proteolipid protein. The pellet consisted of insoluble protein.

Chemical Analyses. Lipids were extracted according to Folch *et al.* (1957), separated by two-dimensional thin-layer chromatography, and the individual lipids were determined by microchemical methods (Eng and Noble, 1968). The proteins were estimated by the procedure of Lowry *et al.* (1951). Hemoglobin was identified by its characteristic ultraviolet and visible absorption spectrum.

Amino Acid Analysis. The various myelin protein fractions were hydrolyzed for 21 hr at 110° with 6 N HCl in vacuum-sealed tubes, and the analysis was performed using a Beckman–Spinco Model 120 automatic amino acid analyzer.

Molecular Weight Measurements. Molecular weight measurements were made by the Archibald technique

TABLE I: Amino Acid Composition of Human Myelin Proteins Obtained by Extraction with 0.5 M Ammonium Acetate or 0.05 N HCl.

Amino Acid	Approximate Integral Molar Ratios			
	Salt Extraction		Acid Extraction	
	47-Year Control	43-Year MS	47-Year Control	43-Year MS
Lysine	7	7	6	6
Histidine	6	5	5	5
Arginine	9	9	9	8
Aspartic acid	6	6	5	5
Threonine	4	4	4	4
Serine	8	8	8	8
Glutamic acid	5	5	5	5
Proline	6	6	7	7
Glycine	12	12	11	12
Alanine	6	6	6	6
Valine	2	2	2	2
Half-cystine	0	0	0	0
Methionine	1	1	1	1
Isoleucine	2	2	2	2
Leucine	4	4	4	4
Tyrosine	2	2	2	2
Phenylalanine	4	4	4	4
Number of residues in smallest possible unit	82	81	81	81
Molecular weight (minimum)	9,218	9,080	8,835	8,742
Molecular weight (Archibald)	8,900	9,500	12,000	17,000

as modified by Trautman (1956), in a Beckman–Spinco Model E analytical ultracentrifuge with a rotor temperature of 20° at a rotor speed of 35,600 rpm. The proteins were dissolved in 0.1 M sodium acetate buffer (pH 4.5) containing 0.1 M NaCl, to give final concentrations of 5–8 mg/ml. A partial specific volume of 0.725 for the protein was calculated according to McMeekin and Marshall (1952).

Tlc separation of the myelin proteins (Andrews, 1964) on Sephadex G-75 and G-200 was accomplished on 20 × 20 cm glass plates descending at an angle of 25° to the horizontal, with 0.5 M NaCl or 0.5% Triton X-100 in 0.5 M NaCl as developing solvents. The developed plate was covered with a sheet of filter paper to absorb the separated proteins. The filter paper was dried, the proteins were stained with 0.1% Amido Black in 5% acetic acid, the paper was redried, and excess dye was removed with 7% acetic acid.

Disc Electrophoresis. Electrophoresis on polyacrylamide gel at pH 4.5 was carried out according to Reisfield *et al.* (1962) at 6–8 mA/tube for 1 hr. Electrophoresis at pH 8.9 was performed according to Ornstein (1964) and Davis (1964).

Biological Assay. The myelin basic protein preparations emulsified with complete Freund's adjuvant were tested for encephalitogenic activity in guinea pigs by intradermal injection above the anterior sternum (H. Rauch, personal communication).

Results

Extraction of Basic Myelin Protein with Neutral Salt and Dilute Acid Solutions. The extraction of purified myelin with 0.11 M ammonium acetate solution (pH 6.9) removed 2–4% of the total myelin protein, and a following extraction with 0.5 M ammonium acetate solution solubilized 10–15% of the total protein. While further extraction with 1 M ammonium acetate solution or 0.2 N HCl yielded 2–3% additional protein, removal of the lipids with chloroform–methanol and treatment of the resulting protein precipitate with 0.05 N HCl solubilized 10–15% more of the myelin protein.

The fraction obtained with 0.11 M ammonium acetate solution contained hemoglobin and both basic and acidic proteins, and thus was heterogeneous (Figure 1, tube 1). The protein fractions obtained with 0.5 M ammonium acetate solution (Figure 1, tubes 2–4) or 0.05 N HCl (tubes 5–8) migrated toward the cathode as one main band both at pH 4.3 and 8.9, and thus may be designated as homogeneous as far as net charge is concerned. At pH 4.3, their migration toward the cathode was slower than that of horse heart cytochrome C. The corresponding fraction obtained from rat brain and spinal cord migrated as two main components (Figure 1, tubes 9 and 10).

Amino Acid Composition and Molecular Weight of Myelin Basic Proteins. The amino acid composition

TABLE II: The Extraction of Myelin Proteins with Solutions Containing 0.5 M Ammonium Acetate and Varying Amounts of Triton X-100.[a]

	% Total Protein					
	Basic		Folch–Lees		Wolfgram	
No. of Extractions	1	3[b]	1	3[b]	1	3[b]
Extracting solvent						
A. 0.5 M ammonium acetate	17	28	2	51		21
B. 0.5 M ammonium acetate–0.05% Triton-X-100 (n = 2)	25	29	5	51		20
C. 0.5 M ammonium acetate–0.1% Triton X-100	28	30	15	53		17
D. 0.5 M ammonium acetate–0.5% Triton X-100	24	25	46	58		17
A–D. (av n = 4)		28		53		19

[a] Aliquots of myelin prepared from the brain of a 41-year-old male were used. The protein concentration of the homogenates were 2.0–2.5 mg/ml. [b] Summation of the initial and two subsequent extractions with 0.5% Triton X-100–0.5 M ammonium acetate.

of the basic proteins extracted with 0.5 M ammonium acetate solution and with 0.05 N acid from two adult human myelin preparations (Table I) showed high proportions of basic amino acids and glycine and absence of cysteine. There was little variation between the amino acid composition of the four samples. The minimum molecular weight calculated by taking approximate integral ratios of the amino acids was approximately 9000 for each of the four samples. The Archibald technique yielded similar values for the ammonium acetate solution extracted proteins but molecular weight of 12,000 and 17,000 for the 0.05 N HCl-extracted proteins (Table I).

TABLE III: Proportions of the Three Classes of Protein in Mylein.

Myelin Sample	% Dry Wt of Total Myelin Protein	% Total Protein		
		Basic	Folch–Lees	Wolfgram
Human brain				
10-week male	24	16	68	16
4-year male (av n = 2)[a]	24	21	60	19
41-year male (av n = 5)	24	28	53	19
43-year female (MS)	23	27	53	21
45-year male	24	27	52	21
63-year male (MS)	22	27	54	19
73-year male	23	28	51	21
92-year male (av n = 3)	23	29	51	19
41–92 yr (av n = 6)	23	28	52	20
Bovine brain (av n = 2)	22	29	54	17
Bovine sciatic nerve	19	21	23	55

[a] n, number of aliquots of myelin subjected to the Triton–salt extraction procedure.

The Separation of Myelin Proteins with 0.5% Triton X-100–0.5 M Ammonium Acetate. Attempts to obtain the total basic protein fraction from myelin without exposure to acidic solvents led to the use of a nonionic detergent, Triton X-100, which in conjunction with ammonium acetate solution was as effective as an ionic detergent. Unlike the ionic detergent, sodium lauryl sulfate, used by Hulcher (1963), which has a hydrophobic side chain attached covalently to a hydrophilic group, the hydrophobic Triton X-100 could be removed easily from the aqueous ionic medium by partition with ether. Using a Triton–salt solution, it was possible to separate the myelin proteins into three general classes: one which remained soluble in aqueous solution after removal of the Triton X-100 (basic protein); the second which was soluble in Triton–salt solution or chloroform–methanol–water (12:6.3:1, v/v) but insoluble in ammonium acetate solution after removal of the Triton X-100 (Folch–Lees-type protein); and a third which was insoluble in water, Triton–salt solution, and chloroform–methanol–water (Wolfgram-type protein).

The solubility of the myelin proteins in mixtures of 0.5 M ammonium acetate solution and increasing concentrations of Triton X-100 are presented in Table II. It is evident that 0.5 M ammonium acetate solution

TABLE IV: The Amino Acid Composition of the Myelin Proteins (Mole %).

	Triton–Salt Soluble							Triton–Salt Insoluble Wolfgram (C)				Total Myelin Protein			
	Basic (A)			Folch–Lees (B)											
	Human	Bovine		Human		Bovine		Human		Bovine		0.28A + 0.52B + + 0.20C Human		0.29A + 0.54B 0.17C Bovine	
Amino Acid[a]	43–47 yr *c*	*d*	*e*	41 yr *f*	45 yr *d*	*d*	*g*	41 yr *f*	45 yr *d*	*d*	*h*	*i*			*j*
Lysine	7.7	8.1	8.2	4.7	4.9	4.8	4.3	7.8	7.5	7.7	6.4	6.2	5.9	6.3	6.0
Histidine	6.3	5.8	5.9	2.5	2.6	2.3	1.9	2.3	2.4	2.2	2.1	3.6	3.1	3.3	2.4
Arginine	10.4	9.8	11.2	3.2	3.2	3.0	2.6	5.0	5.5	5.5	5.4	5.6	4.6	5.4	4.0
Aspartic	6.9	7.0	5.8	4.7	4.5	4.4	4.2	6.5	8.1	7.6	9.1	5.8	6.2	5.7	6.3
Threonine	5.0	4.4	4.3	7.8	7.7	8.6	8.5	6.1	6.4	6.2	4.8	6.4	7.4	7.0	6.5
Serine	9.7	9.9	9.6	5.2	5.8	6.7	5.4	5.9	6.6	7.2	5.4	6.8	6.1	7.7	10.2
Glutamic	6.0	6.6	6.4	7.4	6.5	6.6	6.0	10.8	11.4	11.9	12.0	7.5	7.8	7.5	7.4
Proline	7.7	7.3	6.8	2.8	3.8	2.3	2.9	4.3	5.7	5.3	4.3	4.9	4.6	4.3	4.0
Glycine	14.6	15.0	15.6	11.2	10.2	11.0	10.3	9.0	8.4	8.7	7.4	11.4	10.8	11.8	9.8
Alanine	7.2	8.3	8.6	10.8	10.7	11.8	12.5	8.5	8.6	7.9	7.8	9.4	9.5	10.1	9.1
Valine	2.5	1.8	0.8	6.3	6.8	7.0	6.9	6.1	6.8	6.4	5.4	5.4	6.0	5.4	6.0
Half-cystine[b]	0.0	0.0	Trace	4.2	3.1	2.4	4.2	2.5	0.0	0.0	0.9	1.9	1.7	1.3	3.8
Methionine	0.9	1.1	1.2	1.4	1.6	0.5	1.7	1.4	0.2	1.5	2.0	1.2	1.5	0.8	1.2
Isoleucine	2.5	1.9	1.2	4.3	5.0	5.3	4.9	4.7	5.0	4.9	4.0	4.0	4.2	4.3	4.2
Leucine	5.1	6.0	6.0	10.8	10.8	11.1	11.1	9.7	10.1	9.3	8.9	9.0	9.2	7.8	9.0
Tyrosine	2.2	2.3	2.6	4.9	4.6	4.0	4.7	4.0	2.2	2.6	2.7	3.7	3.3	3.3	3.4
Phenylalanine	5.4	4.9	4.9	7.8	8.2	8.0	7.8	5.4	5.2	5.2	3.9	6.7	6.2	6.6	5.4

[a] Tryptophan was not determined. [b] Uncorrected for degradation caused by acid hydrolysis. [c] Average of 4; analyses were kindly done by Dr. John Peters of Stanford Research Institute, Menlo Park, Calif. [d] Analyses were kindly done by Dr. F. Wolfgram. [e] Nakao *et al.* (1966). [f] Analyses were performed by the Amino Acid Analysis Service, Beckman Instruments Inc., Palo Alto, Calif. [g] Tenenbaum and Folch-Pi (1966). [h] Wolfgram (1966). [i] Gerstl *et al.* (1967). [j] Hulcher (1963).

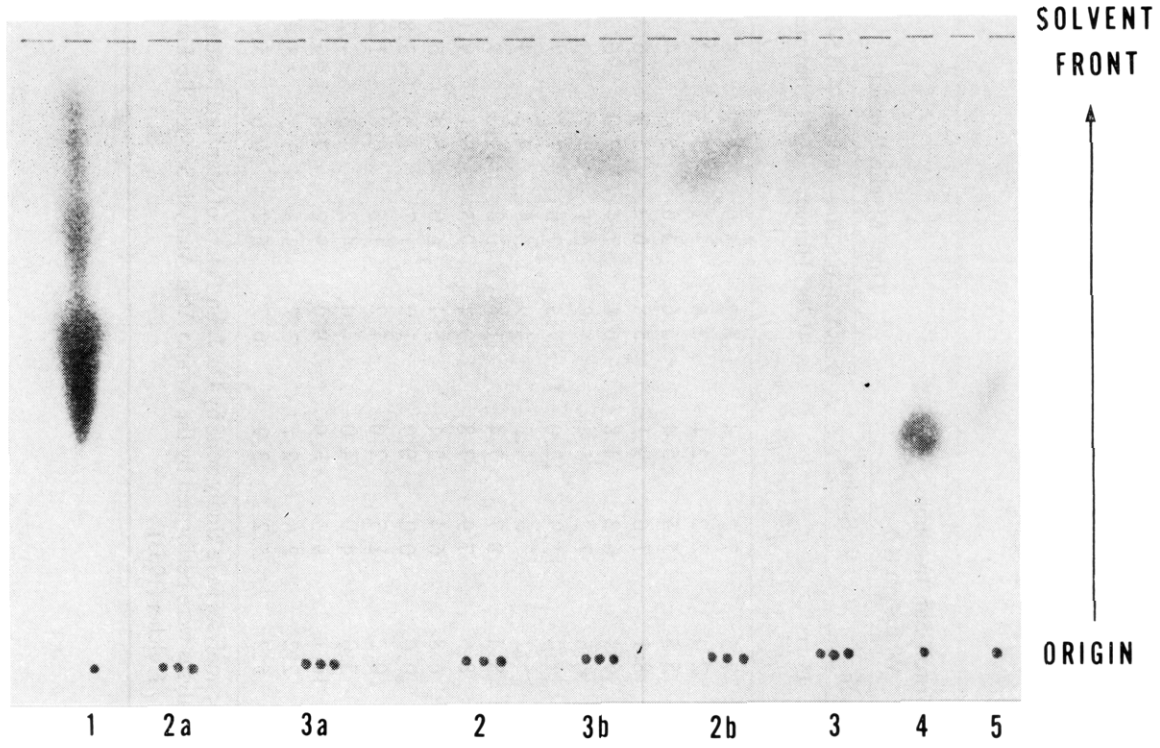

FIGURE 2: Thin-layer chromatogram of 0.5% Triton-X-100–0.5 M ammonium acetate soluble myelin proteins on Sephadex G-200. The plate was developed with a solution of 0.5% Triton X-100–0.5 M NaCl descending at an angle of 25° from the horizontal for 4 hr: (1) human serum; (2) 43-yr-old female, MS; (3) 4-yr-old male; (4) horse heart cytochrome C; and (5) bovine ribonuclease. 2–3 represent the first Triton–salt extract and each contains basic and Folch-Lees proteins; 2a–3a represent the basic protein after precipitation of the Folch-Lees protein; 2b–3b represent the second Triton–salt extract of the myelin and contain primarily the Folch-Lees protein.

alone extracts about 50% of the total basic protein from mylein and that addition of Triton X-100 in concentrations of 0.05–0.5% equally extracted over 80% of the total basic protein. Increasing the concentration of Triton X-100, however, increased the amount of Folch–Lees-type protein that became soluble. Two additional extractions of each aliquot (A through D, Table II) with Triton–salt solution yielded 10–20% more of the total basic protein and varying amounts of Folch–Lees-type protein. The average values of proportions on the four aliquots of myelin were: basic protein, 28%; Folch–Lees, 53%; and Wolfgram, 19%.

The solubility of the Folch–Lees-type protein in Triton–salt was also dependent upon the total protein concentration in the homogenate. Increasing the homogenate concentration from 2.5 to 10 mg of protein per ml did not result in proportionate increases in soluble Folch–Lees-type protein. Homogenates, with protein concentrations of 5, 7, 8, and 10 mg per ml, yielded on initial extraction over 90% of the total basic protein but only 20, 24, 25, and 28% of the Folch–Lees-type protein, respectively. Additional extraction with Triton–salt solubilized the remaining Folch–Lees-type protein.

Solutions of 0.5% Triton X-100 or 0.5% Triton X-100 in 0.11 M ammonium acetate solution extracted not more than 5% of the total myelin protein. Apparently the high salt concentration was an important factor in releasing the basic protein from the myelin membrane, and the Triton–salt was necessary for solubilizing the Folch–Lees-type protein. The Folch–Lees-type protein extracted with Triton–salt remained soluble when mixed with 19 volumes of chloroform–methanol (2:1, v/v).

Proportions and Properties of the Myelin Proteins. Table III summarizes the proportions of the three classes of myelin proteins in eight human and one bovine brain and one bovine sciatic nerve myelin preparations. Although the ratio of total protein:total lipid varied little over the age span of 10 weeks to 92 years, the proportion of basic protein appeared to increase and that of Folch–Lees-type protein to decrease, during maturation. Adult myelin of four control brains contained 25–30% basic, 50–55% Folch–Lees-type, and 15–20% Wolfgram-type proteins. The proportions of these proteins in the myelin of two cases of MS were within the range of the normal adults. Bovine brain myelin showed a protein composition similar to that of adult human myelin. Sciatic nerve myelin could also be fractionated by Triton–salt extraction but the proportions of the three classes were at variance with those found in brain myelin (Table III). The amino acid compositions of the three classes of myelin proteins from two human myelin preparations were similar to the corresponding fractions from one bovine sample (Table IV).

An indication of the relative molecular size and homogeneity of the basic and Folch–Lees-type proteins

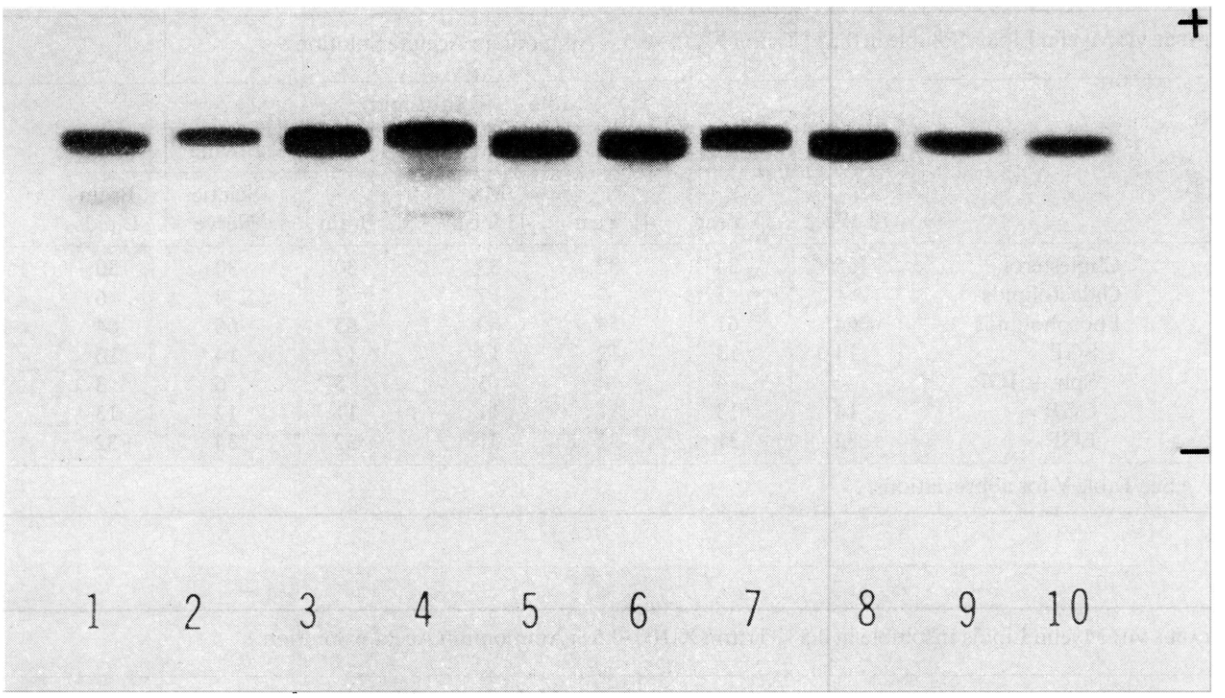

FIGURE 3: Disc electrophoretic analysis in 15% acrylamide gel (pH 4.5) of brain myelin basic proteins obtained by extraction with 0.5% Triton X-100–0.5 M ammonium acetate: (1) bovine, 80 μg; (2) 57-yr-old male, 25 μg; (3) 44-yr-old female, 90 μg; (4) 92-yr-old male, 125 μg; (5) 41-yr-old male, 100 μg; (6) 47-yr-old male, 100 μg; (7) 26-yr-old male, 43 μg; (8) 26-yr-old male, 84 μg; (9) 43-yr-old female, MS 72 μg; and (10) 4-yr-old male, 58 μg.

TABLE V: Composition of Myelin Lipids.[a]

	Mole % of the Major Lipids							
	Human						Bovine	
	10 Week	4 Year	41 Year	45 Year	MS 43 Year	MS 63 Year	Brain	Sciatic Nerve
Cholesterol	40	42	42	42	42	41	41	38
Galactolipids	15	17	20	20	18	20	22	16
Phospholipids	45	41	38	37	40	39	37	46
SGP	9	8	8	8	10	9	9	9
Sph + IGP	4	8	7	7	7	7	6	14
CGP	15	11	9	9	9	10	9	9
EGP	17	14	14	13	14	13	14	14
Galactolipid + Sph + EGP/ cholesterol	0.90	0.93	0.98	0.95	0.93	0.98	1.02	1.16

[a] Abbreviations: SGP, serine glycerophosphatide; Sph, sphingomyelin; IGP, inositol glycerophosphatide; CGP, choline glycerophosphatide; EGP, ethanolamine glycerophosphatide.

was obtained by Sephadex G-75 and G-200 thin-layer chromatography. Samples of basic protein isolated by ammonium acetate solution or HCl extraction migrated at a rate faster than ribonuclease and cytochrome C and slower than trypsin, chymotrypsin, and serum albumin on Sephadex G-75 thus corresponding to a molecular weight of 20,000. Folch–Lees-type protein, basic protein, and a mixture of both were chromatographed on a Sephadex G-200 plate (Figure 2). The Folch–Lees-type protein migrated at the rate of the γ-globulins suggesting a molecular weight of at least 150,000.

The electrophoretic pattern of Triton–salt-extracted basic myelin proteins from human and bovine brain are shown in Figure 3. Preparations 2–3 and 7–10 were

TABLE VI: Myelin Lipids Soluble in 0.5% Triton X-100–0.5 M Ammonium Acetate Solution.[a]

	Mole % Total Lipid						
	Human				Bovine		
	10 Week	4 Year	41 Year	MS 43 Year	Brain	Sciatic Nerve	Brain Lipids
Cholesterol	35	34	32	33	30	30	30
Galactolipids	1	5	9	7	5	4	6
Phospholipids	64	61	59	60	65	66	64
SGP	14	13	12	13	17	14	16
Sph + IGP	4	4	4	5	5	6	3
CGP	14	13	11	11	11	12	13
EGP	32	31	32	31	32	34	32

[a] See Table V for abbreviations.

TABLE VII: Myelin Lipids Insoluble in 0.5% Triton X-100–0.5 M Ammonium Acetate Solution.[a]

	Mole % Total Lipid					
	Human				Bovine	
	10 Week	4 Year	41 Year	MS 43 Year	Brain	Sciatic Nerve
Cholesterol	49	46	46	47	45	45
Galactolipids	19	24	27	23	30	17
Phospholipids	32	30	27	30	25	38
SGP	6	6	6	7	6	7
Sph + IGP	5	7	7	7	7	16
CGP	12	9	7	8	6	7
EGP	9	8	7	8	6	8

[a] See Table V for abbreviations.

nearly homogeneous while 1 and 4–6 showed minor components estimated at less than 5% of the total.

Composition of the Myelin Lipids. The lipid composition of the various myelin preparations is given in Table V. It appears that during maturation of the human brain, there is a decrease in the proportion of phospholipids and an increase in proportion of galactolipids. Lecithin content decreased with increasing age. The lipids of bovine brain myelin contained a higher proportion of cholesterol and galactolipid than bovine sciatic nerve myelin. The lower content of galactosphingolipid in sciatic nerve myelin is compensated for by a higher proportion of sphingomyelin.

The basic protein fraction extracted with 0.5 M ammonium acetate solution from myelin was essentially free of lipids. The Triton–salt extracts, however, contained several lipids, with the total amount of lipid being independent of the amount of myelin originally present in the homogenate (1–6 mg of myelin/ml). The maximum amount extracted was about 1 μmole of phospholipid/ml of homogenate. There was no direct correlation between the amount of Folch–Lees type or basic protein extracted and the amount of lipid extracted.

The proportions of the lipids that were soluble in Triton–salt solution (Table VI) differed markedly from those in total myelin, that of phospholipids being higher, and galactolipids much lower than that of cholesterol. Ethanolamine glycerophosphatide was the most soluble phospholipid. When a mixture of protein-free bovine lipids was homogenized with the Triton–salt solution, the quantity and proportions of the extracted lipids were identical with those obtained from bovine myelin (Table VI). This suggests that solubility in the solvent, rather than any specific lipid–protein binding, determines the type and quantity of lipids extracted by this procedure.

The composition of the lipids associated with the Wolfgram-type protein after complete removal of the Triton–salt-soluble proteins reflected that of the original total myelin lipids (Table VII).

Encephalitogenic Activity of the Basic Myelin Protein.

TABLE VIII: The Amino Acid Composition of Bovine Spinal Root Myelin Proteins (Mole %).

Amino Acid	Triton–Salt Soluble: Basic A[a]	Triton–Salt Soluble: Folch–Lees B[a]	Triton–Salt-Insoluble Wolfgram C[b]	Total Myelin Protein 0.21A + 0.23 B + 0.54 C	*a*
Lysine	10.7	2.3	6.3	6.2	7.7
Histidine	1.8	1.8	2.7	2.3	2.4
Arginine	4.9	3.4	5.1	4.6	4.9
Aspartic	8.9	4.2	6.9	6.6	7.3
Threonine	8.2	4.6	5.7	5.9	5.6
Serine	7.6	7.8	7.4	7.5	12.5
Glutamic	8.8	5.5	8.9	8.0	8.8
Proline	3.5	2.1	3.2	3.0	3.7
Glycine	9.4	9.0	9.3	9.2	8.2
Alanine	7.1	7.1	7.3	7.2	6.8
Valine	7.6	12.0	10.3	10.0	8.5
Half-cysteine	0.0	1.6	0.0	0.4	0.8
Methionine	0.8	1.8	1.0	1.1	1.4
Isoleucine	4.8	6.8	5.9	5.8	4.7
Leucine	8.4	15.9	7.4	9.5	9.3
Tyrosine	1.5	4.4	3.3	3.2	2.3
Phenylalanine	4.8	6.9	5.0	5.4	4.2
Tryptophan	1.2	2.9		0.9	2.2

[a] Wolfgram and Kotorii (1968). [b] F. Wolfgram, personal communication.

Basic myelin proteins at doses between 15 and 20 μg of protein per animal, prepared either by 0.5 M ammonium acetate solution, Triton–salt, or 0.05 M HCl extraction, produced lethal EAE in over 90% of 100 guinea pigs tested. Preliminary results indicate that basic protein obtained by 0.5 M ammonium acetate solution extraction is active at a lower dose than is the fraction extracted with Triton–salt following the ammonium acetate solution extraction. Basic protein extracted with ammonium acetate solution or Triton–salt, in a total dose of 6–8 mg of protein, injected into rabbits in small portions over a period of 1 month, elicited the production of precipitating humoral antibodies to the basic protein fraction while basic protein obtained by HCl extraction failed to do so. A detailed report of these findings is to be published.

Discussion

The data presented demonstrate that after a preliminary purification by extraction with 0.11 M ammonium acetate solution, the myelin proteins can be separated into three fractions by selective solvent treatments. These, the basic fraction, Folch–Lees type, and Wolfgram type, had been isolated by others using different procedures (Einstein *et al.*, 1962; Kies, 1965; Caspary and Field, 1965; Wolfgram, 1965; Folch and Lees, 1951; Wolfgram, 1966), and their amino acid composition (Nakao *et al.*, 1966; Tenenbaum and Folch-Pi, 1966; Wolfgram, 1966) correspond to the preparations described in the present study (Table IV). When the amino acid composition of total myelin protein is calculated by combining the appropriate proportions of the various amino acids found in the three classes, the sum agrees with the data reported for human and bovine myelin (Gerstl *et al.*, 1967; Hulcher, 1963) (Table IV), thus supporting the data given in Tables II and III.

The relative proportions of the three classes of myelin proteins in sciatic nerve differed from those of CNS myelin (Table III). When the data on the amino acid composition of the three myelin proteins of spinal roots (Wolfgram and Kotorii, 1968) were applied to the proportions of the corresponding proteins of sciatic nerve myelin, the quantitative amino acid composition of total spinal root myelin was in general agreement with that found for sciatic nerve myelin (Table VIII).

The proportions of the three classes of myelin protein obtained from white matter of two MS brains, as well as the amino acid composition of the basic protein from one case, were similar to those in the controls. This supports an earlier report that the amino acid composition of total myelin protein in MS is similar to that in controls (Gerstl *et al.*, 1967).

Some information on the orientation and binding of the proteins in the myelin membrane can be deduced from their composition, physical properties, and relative solubilities in various solvents. The basic myelin protein, by virtue of its high content of basic amino acids and its extraction from myelin with neutral salt or dilute acid, suggests that it is probably bound in the membrane, primarily by ionic bonds, with acidic lipids or proteins. This is in accord with the binding of other

basic proteins to acidic lipids (Das *et al.*, 1965), gangliosides (Booth, 1962), nucleic acid (Davidson, 1967), hydrophobic protein (Edwards and Criddle, 1966), and serum proteins (Truffert *et al.*, 1958).

The absence of disulfide linkages and the relatively high proline content of the basic protein suggest that its configuration is that of an extended, strandlike molecule. Molecular weight estimations by ultracentrifugation and calculations from amino acid composition yield values lower than those obtained by gel filtration. Nakao *et al.* (1966) and Tomasi and Kornguth (1967) reported similar differences using the ultracentrifuge and gel filtration procedures. This would be explicable on the assumption of a rodlike, rather than globular, molecule, but it could also be due to partial dimer formation which becomes more apparent on gel diffusion than on centrifugation (Kakiuchi *et al.*, 1964). The histidine content of this protein (Table IV) and the firm binding of zinc to the myelin proteins, reported earlier (Gerstl *et al.*, 1967), would support this suggestion. Most of the basic protein preparations behaved as homogeneous species in both acrylamide gel electrophoresis and on ultracentrifugation. Several others showed additional minor electrophoretic components that may have resulted from decomposition or proteinase action (Einstein *et al.*, 1968). The cause of the difference in the molecular weight of the basic protein obtained by salt extraction from that by acid, as determined by ultracentrifugation, is not apparent and requires further study.

The Folch–Lees type of protein, by virtue of its solubility in chloroform–methanol, high proportion of aliphatic amino acids and sulfhydryl groups, and tendency to aggregate upon removal of the lipids, chiefly suggest hydrophobic bonding, although the possibility of ionic interaction is not excluded. The molecular weight of this protein was estimated by gel filtration to be at least 150,000.

Little, other than its amino acid composition showing a high proportion of acidic residues, is known about the third class of myelin proteins, the Wolfgram type. Since it is soluble in acidified chloroform–methanol, it has been classified as a proteolipid. These properties suggest that it may bind equally well by ionic or hydrophobic bonds in the myelin membrane.

The lipid changes occurring in human brain myelin during maturation are in line with those found previously (Gerstl *et al.*, 1966; Horrocks, 1968; Cuzner and Davison, 1968; Eng and Noble, 1968). The proportions of cholesterol and galactolipids increased, and that of lecithin decreased, during development. The present lipid data (Table V) also agree with the concept of a 1:1 molar relationship for the sum of the ethanolamine glycerophosphatide and sphingolipids to cholesterol (Eng and Smith, 1966). Reports (O'Brien *et al.*, 1967; Horrocks, 1967) that the lipid composition of brain myelin is different from peripheral nerve myelin were corroborated by the present study.

The major contribution of lipids to the stability of the myelin membrane was recently reviewed by Vandenheuvel (1963) and O'Brien (1967). However, the importance of the protein moiety in preservation of the membrane structures of peripheral nerve myelin and mitochondrial membrane was demonstrated by electron microscopy (Napolitano *et al.*, 1967; Fleischer *et al.*, 1967).

Properties other than structural and encephalitogenic have not yet been attributed to the myelin basic protein (Laatsch *et al.*, 1962). This is in contrast to other basic proteins which exhibit enzyme activity (cytochrome C, ribonuclease, and lysozyme) and stimulate transport of ions into mitochondria (Johnson *et al.*, 1967) and of proteins into tumor cells (Ryser, 1968).

The amounts of basic protein and sphingolipids in human myelin increase from infancy to adulthood. Similar lipid changes have been observed in the rat (Eng and Noble, 1968). Since lipids in a variety of mixtures can form bimolecular layers of varying stability, it is tempting to speculate that a template is necessary to orient the myelin lipids in a compact and stable conformation. It is proposed that the basic protein fraction in myelin is bound mainly by ionic bonds with the acidic lipids or proteins and serves primarily as a framework around which the remaining lipids and proteins can condense to form the stable myelin membrane.

The Wolfgram and Folch–Lees proteins, as found for CNS and PNS myelin, show qualitative and quantitative similarity of amino acid composition, which is not the case for the basic protein fractions from the two sources. Since proteolipid proteins isolated from other membranes have similar amino acid composition and serve structural purposes (Wolfgram, 1967), it is suggested that the proteolipid proteins of myelin have a similar function.

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