artifacts of isolation must be considered. Our earlier work (Malkin, 1971) indicated that the 22S RNA may be a degradation product of 28S RNA since it is observed in cytoplasmic ribosome preparations only when RNA is prepared by the procedure described in this paper, a procedure which is rather lengthy and which involves an incubation at room temperature under nonprotein denaturing conditions. On the other hand, the 20S RNA was observed as a constant percent component (compared to 18S and 28S RNA) when RNA was prepared by simply dissolving ribosomal pellets in buffer BN containing 1% SDS just prior to sucrose gradient fractionation even though three different ribosome preparations were investigated, each differing in preparation time and lysosome content. These results are similar to those of Attardi and Attardi (1971) who showed that when HeLa cell mitochondria are prepared by a shortened procedure, the 23S peak is no longer seen but the 21S material persists (although reduced in amount relative to the 18S RNA). Experiments are in progress to elucidate these points further.

Acknowledgment

I thank Miss Mary Abell for expert technical assistance.

References

Attardi, B., and Attardi, G. (1969), *Nature (London) 224*, 1079.
Attardi, B., and Attardi, G. (1971), *J. Mol. Biol. 55*, 231.
Bartoov, B., Mitra, R. S., and Freeman, K. B. (1970), *Biochem. J. 120*, 455.
Bishop, D. H. L., Claybrook, J. R., and Spiegelman, S. (1967), *J. Mol. Biol. 26*, 373.
Borst, P., and Grivell, L. A. (1971), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 13*, 73.
de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux R., and Applemans, F. (1955), *Biochem. J. 60*, 604.
Dubin, D. T. (1971), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 30*, 1301.
Dubin, D. T., and Czaplicki, S. M. (1970), *Biochim. Biophys. Acta 224*, 663.
Dubin, D. T., and Montenecourt, B. S. (1970), *J. Mol. Biol. 48*, 279.
Johnson, D., and Lardy, H. (1967), *Methods Enzymol. 10*, 94.
Leskes, A., Siekevitz, P., and Palade, G. E. (1971), *J. Cell Biol. 49*, 264.
Loewenstein, J., Scholte, H. R., and Wit-Peters, E. M. (1970), *Biochim. Biophys. Acta 223*, 432.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Malkin, L. I. (1970), *Proc. Nat. Acad. Sci. U. S. 67*, 1695.
Malkin, L. I. (1971), *Biochem. Biophys. Res. Commun. 43*, 787.
Schnaitman, C., and Greenawalt, J. W. (1968), *J. Cell Biol. 38*, 158.
Scornik, O. A., Hoagland, M. B., Pfefferkorn, L. C., and Bishop, E. A. (1967), *J. Biol. Chem. 242*, 131.
Swanson, R. F. (1971), *Nature (London) 231*, 31.
Swanson, R. F., and Dawid, I. B. (1970), *Proc. Nat. Acad. Sci. U. S. 66*, 117.

Isolation of a Highly Purified Myelin Protein*

J. Gagnon, P. R. Finch, D. D. Wood, and M. A. Moscarello†

ABSTRACT: Myelin was extracted first with mercaptoethanol followed by 0.2 N H_2SO_4. The resulting residue was dissolved in chloroform–methanol (1:1, v/v) containing 5% of 0.1 N HCl and applied to a Sephadex LH-20 column. The major protein fraction was rechromatographed on a similar LH-20 column. The protein was isolated by precipitating with ether and the precipitate was washed with 2-propanol. It migrated as a single component in polyacrylamide gel electrophoresis and appeared to be homogeneous by analytical ultracentrifugation. Amino acid analysis revealed it was relatively high in neutral and low in basic residues. A single N-terminal glycine was found. Of the carbohydrates tested, sialic acid, glucose, mannose, galactose, fucose, and hexosamine, only the latter two were present in appreciable amounts. About 2% of fatty acids was detected, mainly C_{16} and C_{18}. Phosphorus was less than 0.03 μmole/10-mg sample and no glycerol was detected. It was concluded that the fatty acid was not part of triglyceride, phospholipid, sphinogolipid, or galactolipid. Attempts to remove the residual fatty acid by prolonged Soxhlet extraction, treatment with phenol–acetic acid–water containing 2 M urea followed by dialysis, ether extraction of a solution of the protein in 98% formic acid, and charcoal failed to reduce the amount of fatty acid recovered.

At the present time, at least three protein fractions have been isolated from myelin. One of these, a basic protein (also called the encephalitogenic protein), was first isolated by Laatsch *et al.* (1962) and has been subsequently studied in a number of laboratories including our own (Martenson *et al.*, 1969; Lowden *et al.*, 1966; Tomasi and Kornguth, 1967). It has been extensively purified and an amino acid sequence has been reported (Kibler *et al.*, 1969; Eylar, 1970).

The other two protein fractions have been less extensively characterized, largely due to technical difficulties associated with delipidation. One of these protein fractions was first isolated by Folch and Lees (1951) and termed "proteolipid," to denote a mixture of protein and lipid soluble in mixtures of chloroform–methanol. A water-soluble preparation was

* From the Department of Biochemistry, Research Institute, The Hospital for Sick Children, Toronto, Ontario, Canada. *Received March 10, 1971.*

obtained from bovine brain by Sherman and Folch-Pi (1970) which contained 0.02% phosphorus and 2% fatty acids by weight on analysis. The protein was reported not electrophoretically homogeneous.

An acid-soluble proteolipid type was reported by Wolfgram (1966) and Wolfgram and Kotorii (1968). It differed from the previously mentioned fraction in a relatively high content of acidic amino acids. It too was soluble in acidified chloroform–methanol. This fraction has not been delipidated. On disc electrophoresis it too was shown not to be homogeneous.

In the fractionation procedure used in this laboratory, normal human myelin was first extracted with thioethanol followed by acid. The residue was lyophilized and dissolved in acidified chloroform–methanol (1:1, v/v) and applied to a Sephadex LH-20 column. The major protein peak isolated from the column could be transferred into aqueous medium and was electrophoretically homogeneous. A number of studies on the lipid components have shown that no phosphorus, sphingosine or glycerol could be detected in the final material. Carbohydrate analyses revealed little or no galactose was present. However, about 2% of fatty acids was consistently recovered and could not be removed by the different extraction procedures used. Since the fatty acids were not present as phospholipid, sphingolipid, galactolipid, or triglyceride, it was concluded that they are likely associated directly with the protein. This work was reported recently to the American Society for Neurochemistry (Wood *et al.*, 1971).

Materials and Methods

Isolation of Myelin. Myelin was prepared from human white matter by the method previously described (Lowden *et al.*, 1966). A new method using the Beckman L-4 zonal ultracentrifuge which allowed us to make larger quantities of myelin was also used (Murdock *et al.*, 1969).

Preparation of "Residual Material." Lyophilized myelin prepared by either of the above methods was extracted with 0.02 M mercaptoethanol (Lowden *et al.*, 1967) followed by 0.2 N sulfuric acid (Lowden *et al.*, 1966). A flow sheet, showing the preparation of the sample for the LH-20 column, is shown in Figure 1. The pellet remaining after the extraction with sulfuric acid was termed "residual material." The residual material was lyophilized and stored at −20°. Routinely, 300 mg of this material was stirred with 5 ml of acidified chloroform–methanol (1:1, v/v) with 5% v/v of 0.1 N HCl. The suspension was centrifuged yielding an insoluble pellet (50 mg) and a clear, slightly yellow, supernatant. When a large number of columns were to be run in a short time, 5–6 g of lyophilized residual material was homogenized with the appropriate volume of acidified chloroform–methanol (300 mg/5 ml), and the insoluble pellet removed by centrifuging at 2500 rpm for 30 min. The clear supernatant was stored in a glass-stoppered flask, under nitrogen and in the dark. Routinely, 5 ml of this supernatant was fractionated on a single column.

Preparation of Sephadex LH-20 Columns. Approximately 75 g of dry LH-20 was allowed to swell for 3 days in chloroform–methanol (1:1, v/v) acidified with 0.1 N HCl (5% v/v). The glass column used was the Sephadex SR (solvent resistant) column with Teflon adaptors, purchased from Pharmacia (Canada). Column specifications were SR 25/100, diameter 2.5 cm, length 100 cm, and bed volume 340–460 ml.

The bottom adaptor was placed into the column with the outflow left open. Enough LH-20 suspension was added to make a column 90 × 2.5 cm when packed. Solvent was added to the top of the column as needed. When the gel had packed, the top adaptor was put on and downward flow continued for 1–2 hr. The column was then inverted so that the inflow was running upward through the gel. The position of the top adaptor (originally the bottom adaptor) was adjusted. The column was run by upward flow for a further 1–2 hr. A column prepared in this way could be used over and over again provided no cracks in the gel developed. In this laboratory we have used a single column for as many as 15 separate runs.

The column was eluted with chloroform–methanol (1:1, v/v) acidified with HCl as before. The eluting solution was passed from a reservoir through a three-way glass stopcock into the column. The three-way stopcock allowed for the introduction of the sample directly to the column without allowing air bubbles to become trapped in the system. The tubing used throughout was flexible Teflon tubing (i.d. $^1/_{32}$ in.).

The outflow from the column went directly to an ISCO UA-2 scanner, which recorded the A_{280}. From the scanner, the outflow was led to a turntable and 5-ml fractions were collected automatically. In practice it was found that 75 ml of eluent could be collected in bulk before starting the fraction collector. Fractions were collected at the rate of about six tubes per hour. A total of 70 fractions (350 ml) were collected after the application of a 5-ml sample.

At the end of the run, solvent was run through the column for at least 2 hr before another sample was applied. The various peaks, except the one designated N-2, from a number of columns (usually 6) were pooled and precipitated (Mokrasch, 1967) with four volumes of ether at 0°. The precipitate, collected by centrifuging, was washed twice with two volumes of 2-propanol. The largest peak designated N-2 was rechromatographed by pooling only the fractions with the highest A_{280} readings, and applying this material to a similar LH-20 column after precipitation with ether, but omitting the propanol wash because the material was found to be much less soluble after the propanol wash.

Analytical Ultracentrifugation. Ultracentrifugation was carried out in a Beckman Model E analytical ultracentrifuge at 20°. The speed was 59,780 rpm, bar angle 70° using schlieren optics. Two solvents were used for the centrifugation. These were anhydrous (98%) formic acid and phenol–acetic acid–water (2:1:1, v/v) containing 2 M urea. All runs were done using the synthetic boundary cell. A volume of 0.5 ml was placed into the cell and 0.2 ml of solvent in the cup. Photographs were taken at appropriate intervals and the $s_{20,w}$ values calculated.

Equilibrium ultracentrifugation was done in the ANJ rotor at 9000 rpm for 48 hr and 20°. Double-sector Kel F centerpiece was used with uv counterbalance. We are indebted to D. Kels, Department of Biochemistry, University of Toronto, for these runs.

Chemical Methods. Phosphorus was determined on all the fractions by a modified Bartlett (Bartlett, 1959) procedure. Protein was determined by the ninhydrin method as described by Moore and Stein (1954).

Amino acid analyses were done on a Technicon automatic amino acid analyzer. For each analysis 1 mg of protein was hydrolyzed in 1 ml of constant-boiling (5.7 N) HCl, under nitrogen, in a sealed tube, at 100° for 18 hr. In some cases, the protein was hydrolyzed for 48 hr. The HCl was removed using a rotary evaporator, the residue was washed three to four times with water, and evaporated after each washing.

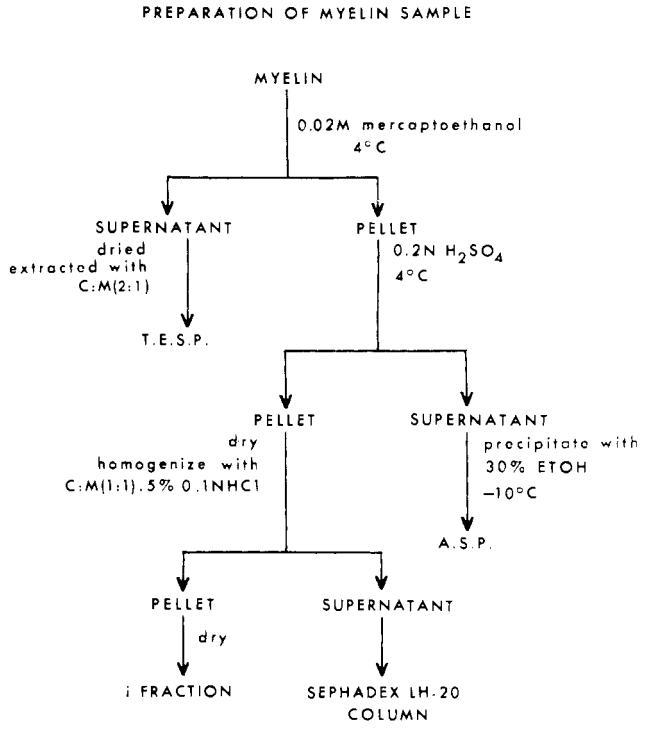

FIGURE 1: Fractionation procedure for normal human myelin. TESP, thioethanol-soluble; ASP, acid-soluble.

The residue was dissolved in acidified sucrose and applied to the column. Tryptophan was determined by the method of Spies and Chambers (1949) using their "Procedure K."

Neutral sugars (glucose, galactose, mannose, and fucose) were determined enzymatically by the method of Finch *et al.* (1969) after hydrolysis of the protein in 2 N HCl at 100° for 4 hr. Glucose was assayed by glucose-6-phosphate dehydrogenase following phosphorylation with hexokinase. Both glucose and mannose are phosphorylated in the hexokinase reaction and the total ADP produced was assayed in the pyruvate kinase and lactic dehydrogenase reactions. The total ADP produced was proportional to the amount of NAD reduced which in turn was shown to be proportional to the amount of glucose plus mannose present. Since glucose was assayed independently in the glucose-6-phosphate dehydrogenase reaction, the amount of mannose present was obtained by difference. Galactose was measured by the galactose dehydrogenase. Fucose was measured by a highly purified fucose dehydrogenase prepared from pork liver by Schachter *et al.* (1969). We are indebted to Dr. H. Schachter of the Department of Biochemistry, University of Toronto, for generous amounts of the enzyme.

Hexosamine was isolated from the protein hydrolysate on a Dowex 50W-X8 column and assayed colorimetrically by the Boas method (Boas, 1953). Hexosamine was routinely removed from the hydrolysate before the neutral sugars were assayed.

Sialic acid was determined by the resorcinal method as modified by Miettinen and Takki-Lukkainen (1959).

Thin-layer chromatography of the lipids was carried out on standard 20 × 20 cm glass plates. The samples were spotted 1 cm from the bottom. The developing solvent chloroform–methanol–water (14:6:1, v/v) was run to 15 cm above the origin. The dried plate was sprayed with a sulfuric acid spray (50 ml of concentrated H_2SO_4 + 50 ml of water + 5 mg of methyl orange) and heated at 100° until the spots had fully developed.

Polyacrylamide gel electrophoresis was carried out according to the method of Takayama *et al.* (1966). Routinely, 7.5% acrylamide gels were prepared, subjected to preelectrophoresis for 2 hr at 2 mA/tube with 10% acetic acid in the tanks and then equilibrated in the sample solvent (phenol–acetic acid–water (3:1:1) + 2 M urea) for at least 2 days before use. After application of the sample electrophoresis was carried out for 2 hr at 2 mA/tube with 10% acetic acid in the tanks. Following electrophoresis, the gels were removed from the tubes and stained in 1% Amido-Schwarz dissolved in 7% acetic acid and then the gels destained in 7% acetic acid; 7.5% gels containing 1% sodium dodecyl sulfate and 0.01 M mercaptoethanol (pH 7.2) were run according to the method of Weber and Osborn (1969).

Glycerol was measured by the method of Chernick (1969) and sphingosine by that of Lauter and Trams (1962). Fatty acids were transmethylated by the method of Hyun *et al.* (1965) and the methyl esters were measured by gas chromatography on a 6-ft column of 10% diethylene glycol succinate on Chromosorb W, H W 60–80 mesh. The temperature was 180° and gas flow 70 cc/min. The fatty acids were detected by flame ionization and the peaks were integrated by an automatic digital electronic integrator (Kent chromalog 2, Kent Instruments Ltd., England). A known amount of C_{19} was used as internal standard which appeared between $C_{18:1}$ and $C_{18:2}$.

Results

Fractionation of Myelin. A flow sheet of the method routinely used to fractionate myelin is shown in Figure 1. The thioethanol-soluble and acid-soluble fractions have been described elsewhere (Lowden *et al.*, 1966, 1967). The residual material was dissolved in acidified chloroform–methanol (1:1, v/v) by homogenization. The slightly cloudy solution was clarified by centrifuging at 2500*g* for 30 min. The clear solution was applied to the Sephadex LH-20 column and eluted with acidified chloroform–methanol (1:1, v/v) as described under Methods.

A typical chromatogram showing the separation of protein from lipid is represented in Figure 2. Protein was measured by A_{280} and lipid phosphorus by the method of Bartlett (1959). Protein in each fraction was also measured by the ninhydrin method (Moore and Stein, 1954) and coincided well with the A_{280} reading except in fractions 46–50 where some of the lipids interfered with the reaction.

As well as obtaining good separation of most of the protein from the lipid, the proteins were fractionated within themselves into one major and five minor peaks. We have termed the major peak N-2.

Thin-layer chromatography of every second fraction showed that the first trace of lipid appeared in fraction 34 (Figure 3). This was identified as sphingomyelin by comparison with a myelin marker.

The material in tubes 26, 27, 28, and 29 was pooled, precipitated with ether, and dissolved in acidified C:M (1:1) and applied to another LH-20 column. Since sphingomyelin was the first lipid detected by thin-layer chromatography, radioactive [^{14}C]sphingomyelin was added to the sample prior to application to the column. As can be seen from Figure 4, the protein was well separated from the sphingomyelin. Thin-layer chromatography of this material has failed to reveal the presence of lipids. However, transmethylation of the rechromato-

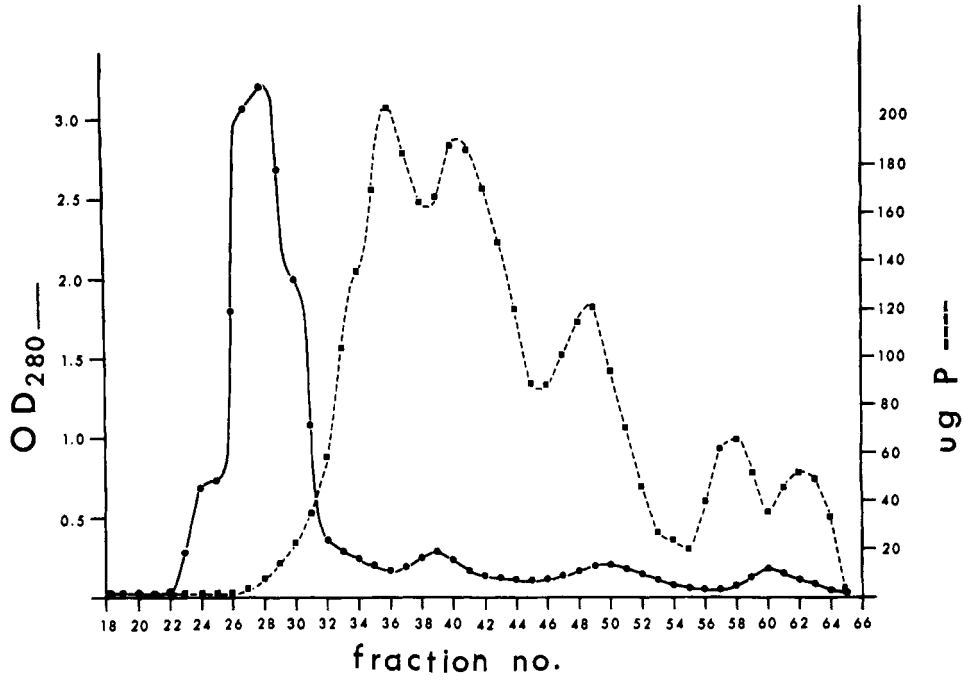

FIGURE 2: Sephadex LH-20 chromatography of residual material from myelin. Residual material (300 mg) was dissolved in 5 ml of chloroform–methanol (1:1, v/v) containing 5% 0.1 N HCl, clarified by centrifuging, and applied to a 90 × 2.5 cm column. Protein was measured by A_{280} and lipid phosphorus by the method of Bartlett.

graphed protein consistently revealed the presence of some fatty acids. A number of studies were then undertaken to elucidate the nature of this residual fatty acid.

The material isolated as N-2 makes up about one-third of the myelin protein. The proportions of the various myelin protein fractions isolated in this laboratory are shown in Table I. The results are expressed as mg dry weight of each protein fraction isolated from 5.85 g dry weight of myelin. Since myelin is generally believed to consist of 20–25% protein, it is clear that N-2 represents one of the major fractions.

Criteria of Homogeneity. Since N-2 is difficult to solubilize, many of the criteria usually used to test for homogeneity could not be applied. Polyacrylamide gel was used in a limited way. The most successful gel was that described by Takayama *et al.* (1966). The protein was dissolved in phenol–acetic acid–2 M urea and 80–100 μg loaded onto each gel. The gels were run as described in Methods. After staining with 1% Amido-Schwarz and destaining with 7% acetic acid, the gels were scanned in a Joyce-Loebl scanner (Transtab type D8, MK2). A typical scan is shown in Figure 5a. It can be seen that the protein has moved a short distance into the gel and migrated as a single component. By contrast, the basic encephalitogenic protein moved further into the gel.

Other gel systems were also used at pH values varying from 2.5 to 8.9. These included the methods of Neville (1967), Martenson and Gaitonde (1969), Reisfeld *et al.* (1962), Weber and Osborn (1969), and Davis (1964). Minor modifications to each system were also made. Gels containing sodium dodecyl sulfate (0.1%) were run using the system of Maizel (1966). In all systems except that of Weber and Osborn, the protein precipitated. A photograph is shown in Figure 5c.

Homogeneity was tested by analytical ultracentrifugation in the Beckman Model E. For this purpose 10 mg of protein was dissolved in the phenol–acetic acid–2 M urea solvent and subjected to a velocity run at 59,780 rpm. A typical schlieren photograph is shown in Figure 6a. A single component was observed throughout the entire run. The s_0 value found in this solvent was 0.95 S. When 98% formic acid was used as solvent, an s_0 of 1.25 S was obtained. In each case the $s_{20,w}$ values at five different concentrations were plotted against concentration of protein to obtain s_0 values.

A water-soluble sample was prepared by dissolving 10 mg of protein in phenol–acetic acid–2 M urea followed by dialysis against decreasing concentrations of acetic acid starting with 25%. For the sedimentation–equilibrium run, a sample giving an OD_{280} of 0.5 was placed in a double-sector Kel F centerpiece and run at 9000 rpm for 48 hr. A plot of the ln of the concentration against $R^2/2$ is shown in Figure 6b. A straight line was obtained indicating homogeneity.

The N-terminal amino acid was determined on 1 ml containing 1 mg of protein by the dansylation reaction as described by Gros and Labouesse (1969). The dansylamino acid was detected on polyamide plates according to the procedure of Hartley (1970). After four solvents, the major spot was found to be glycine. Since no other dansylamino acids were detected, it was concluded that a single N-terminal glycine was present. This supports the above-mentioned data concerning the homogeneity of the protein.

Analysis of Glycerol, Sphingosine, and Fatty Acids. Glycerol was measured enzymatically by the method of Chernick (1969) after hydrolysis of the protein. Samples of 5 mg each were hydrolyzed by three independent methods, 2 N HCl–methanol, 2 N KOH–methanol, and 6 N HCl for 5 and 20 hr in a sealed tube at 100°. After hydrolysis, two volumes of water was added to each tube and the methanol was evaporated from the methanol-containing tubes. The alkali hydrolysates were acidified with HCl. The fatty acids were removed by extraction with one volume of petroleum ether (bp 30–60°) followed by one volume of hexane. The aqueous phase was made to 5 ml with water. For the glycerol analyses, 50–200 μl of each aqueous phase was used. No glycerol was detected in any of the hydrolysates.

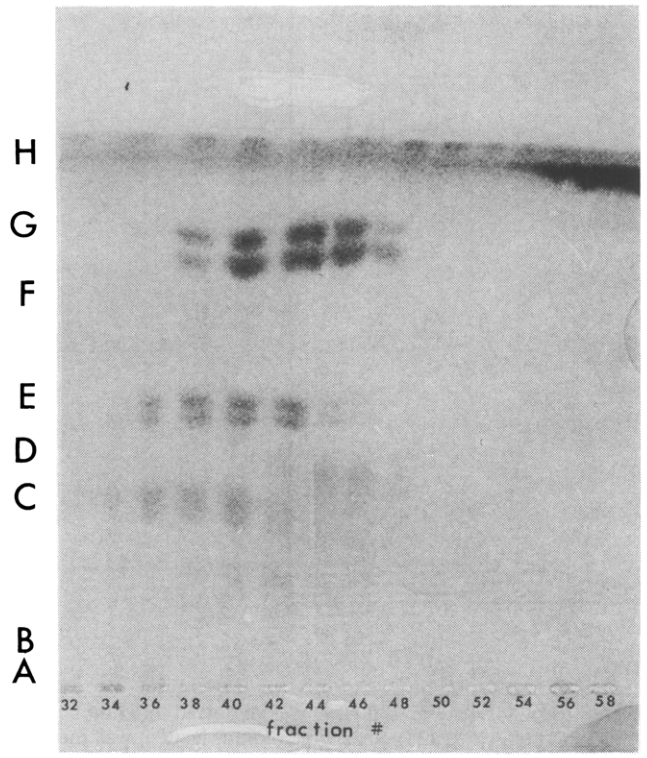

FIGURE 3: Thin-layer chromatogram on silica gel G using standard 20 × 20 cm glass plates of fractions from LH-20 column of normal human myelin. Developing solvent was chloroform–methanol–water (14:6:1, v/v). The dried plate was sprayed with sulfuric acid and heated at 100° until color developed. A myelin marker was included on each plate. (A) Protein, (B) serine glycerophosphatides, (C) sphingomyelin, (D) choline glycerophosphatides, (E) sulfatides, (F) ethanolamine glycerophosphatide, and (H) cholesterol and free fatty acids.

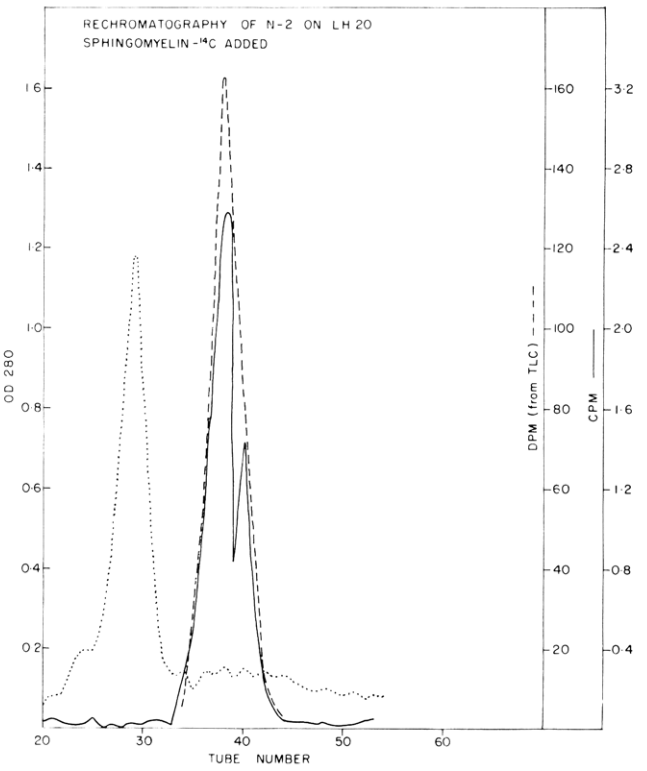

FIGURE 4: Separation of protein from sphingomyelin in a Sephadex LH-20 column. N-2 was mixed with sphingomyelin-^{14}C and applied to the column in the usual way. Counts per minute were measured in each fraction and is represented by the solid line. A portion of each fraction was run on thin-layer chromatography and the radioactivity disintegrations per minute of the sphingomyelin spot was measured. This is represented by the broken line. OD_{280} is shown in the dotted line.

For the measurement of sphingosine and fatty acid, 5- and 10-mg samples were hydrolyzed in 2 N KOH–methanol at 100° for 24 hr. The fatty acid fraction was extracted with petroleum ether and hexane as described above. The fractions were pooled and evaporated to dryness.

TABLE I: Dry Weights of Various Protein Fractions Isolated from Normal Human Myelin.

Material	Weight g	Weight mg
Myelin	5.85	
Acid-soluble fraction		152
Thioethanol soluble		24
Residual material	3.9	
Insoluble residue		672
LH-20 fractions		
N-1		20
N-2		381
N-3		64
N-4		49
N-5		81
Total		1443
Protein recovered (%)		24

The fatty acids were transmethylated by adding 2 ml of BF_3–methanol and 1 ml of benzene (dried over sodium wire) in a sealed vial. Transmethylation was carried out at 65° for 48 hr. After transmethylation, 5 ml of water was added to each vial and extracted with an equal volume of petroleum ether. The extract was evaporated to dryness and the solids were taken up in 1 ml of hexane and applied to a silicic acid column (1.5 × 15 cm). The column was eluted first with hex-

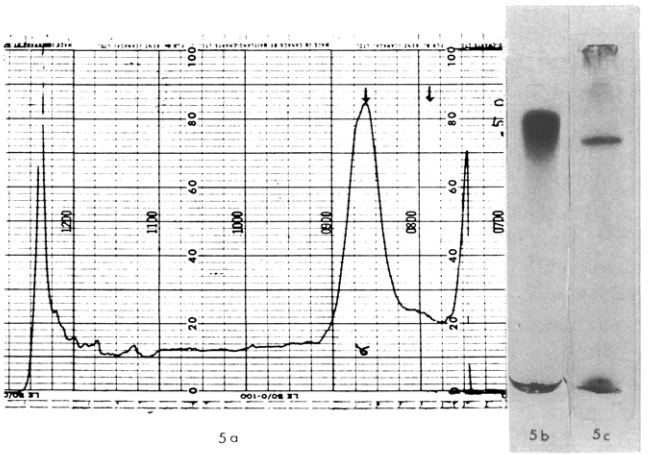

FIGURE 5: Analytical polyacrylamide gel electrophoresis of N-2. (a) Scan of gel run by the method of Takayama *et al.* (1966); (b) photograph of gel used in scan; (c) photograph of gel run by the method of Weber and Osborn (1969).

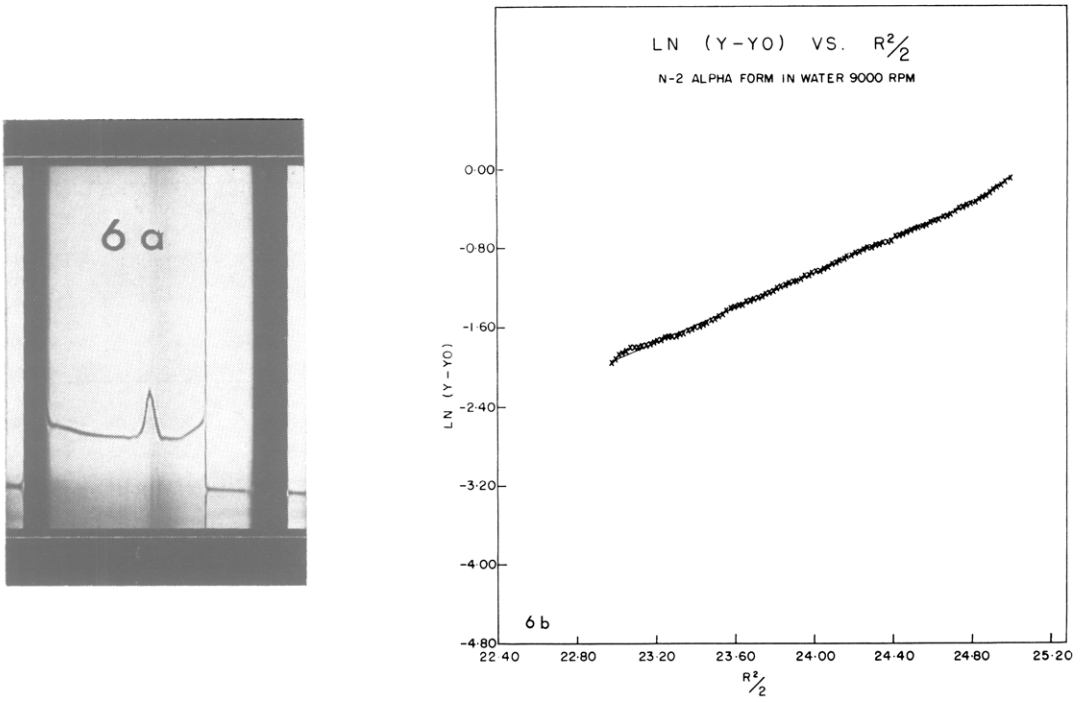

FIGURE 6: (a) Sedimentation–velocity run of N-2 in Beckman Model E analytical ultracentrifuge at 20°, 59,780 rpm, bar angle 70°, schlieren optics. Synthetic boundary cell was used in all runs. (b) Sedimentation–equilibrium ultracentrifugation of N-2 in water at 9000 rpm, ANJ rotor, uv counterbalance, double-sector Kel F centerpiece, 20°, 48 hr.

ane, then hexane containing 5% ether. The fatty acid methyl esters, eluted with hexane +5% ether, were dried down and injected into the gas chromatograph as described under Methods.

The aqueous phase left behind after the petroleum ether, hexane extraction of the hydrolysate, was used for the spingosine assay. It was made slightly alkaline by the addition of KOH and was extracted with ethyl acetate. Sphingosine was assayed colorimetrically as described under Methods. The results for the glycerol, fatty acid, and sphingosine analyses are shown in Table II.

It can be seen that no glycerol was found in any of the hydrolysates, therefore, triglyceride was not present in our material. A small amount of sphingosine was found (0.069 μmole/10-mg sample) and no phosphorus (<0.03 μmole/10-mg sample). Both sphingosine and phosphorus values are so low that they are not considered significant. We are left then with about 0.7 μmole of fatty acid/10-mg sample when the protein was transmethylated directly with BF_3–methanol (65° for 48 hr) and slightly more than 1.0 μmole/10 mg when the protein was hydrolyzed with 2 N KOH–methanol followed by transmethylation of the fatty acids. This difference of 0.3 μmole of fatty acid/10-mg sample which is accounted for by C_{16} and $C_{18:1}$ fatty acids cannot be explained at the moment.

Several methods were used in attempts to remove the fatty acids from the protein. These included: (i) Soxhlet extraction in chloroform–methanol (1:1, v/v) for 48 hr; (ii) prolonged dialysis against acetic acid after dissolving the protein in phenol–acetic acid–water (3:1:1, v/v) + 2 M urea; (iii) 98% formic acid; (iv) the charcoal method described by Chen (1967) for the removal of fatty acids from serum albumin. In all cases the protein was subjected to the above-mentioned treatments and then subjected to transmethylation with BF_3–methanol in the usual way. No loss of fatty acid occurred as a result of any of the treatments.

Amino Acid Analyses. The protein (1 mg) was hydrolyzed in 1 ml of constant-boiling HCl (5.7 N) under nitrogen in a sealed tube at 110° for 18 hr. The HCl was removed on a rotary evaporator and the sample washed several times with water until no HCl remained. The sample was then loaded on

TABLE II: Glycerol, Sphingosine, and Fatty Acid Analyses of N-2 Expressed as μmoles/10-mg Sample.[a]

Compound Assayed	Method of Hydrolysis of N-2 before Transmethylation			N-2 Transmethylated Directly with BF_3–Methanol
	2 N HCl–Methanol	6 N HCl	2 N KOH–Methanol	
Glycerol	0	0	0	
Sphingosine			0.069	
Fatty acids				
16			0.54	0.32
16:1			0.057	
18			0.110	0.16
18:1			0.340	0.21
Total fatty acid			1.047	0.70

[a] For fatty acid and sphinogsine, N-2 was hydrolyzed in 2 N KOH–methanol for 24 hr. In addition, glycerol was also assayed after hydrolysis with 2 N HCl–methanol and 6 N HCl. The values for fatty acids and sphingosine are the means of three determinations. N-2 was also transmethylated directly with BF_3–methanol.

TABLE III: Amino Acid Analysis of N-2 Protein Expressed as Residues per 100 Residues.[a]

Residue	Residues/100 Residues	
	After 18-hr Hydrolysis	After 48-hr Hydrolysis
Aspartic	4.79 ± 0.11	4.2
Threonine	8.41 ± 0.27	7.7
Serine	6.32 ± 0.24	5.1
Glutamic	7.23 ± 1.06	6.3
Proline	2.83 ± 0.25	2.6
Glycine	11.17 ± 0.23	10.0
Alanine	12.03 ± 0.81	11.6
Valine	6.55 ± 0.91	6.6
Cystine	4.17 ± 0.32	3.6
Methionine	1.59 ± 0.23	1.4
Isoleucine	4.38 ± 0.70	4.8
Leucine	11.67 ± 0.53	12.4
Tyrosine	4.89 ± 0.60	4.7
Phenylalanine	8.00 ± 0.78	8.5
Lysine	4.35 ± 0.56	4.6
Histidine	2.34 ± 0.48	2.6
Arginine	1.80 ± 0.62	2.8
Tryptophan	1.33 ± 0.47	

[a] The values represent the means and standard deviations for the 18-hr hydrolysates of eight independent preparations of N-2. The values for the 48-hr hydrolysates represent the means of two determinations.

to a cartridge of a Technicon automatic amino acid analyzer. The amino acid analysis is shown in Table III. The values reported for the 18-hr hydrolysates are the means of eight determinations, each on an independent preparation of the protein. Those for the 48-hr hydrolysates represent the means of two determinations only. Tryptophan was measured colorimetrically by the method of Spies and Chambers (1949).

As can be seen from Table III, this protein is relatively low in basic amino acids but high in neutral and acidic residues. Hydrolysis of the protein for 48 hr resulted in small losses of aspartic, threonine, glycine, and leucine. The largest loss was recorded in the value for serine amounting to about one residue.

Carbohydrate Analyses. Neutral sugars, glucose, galactose, mannose, and fucose were determined enzymatically (Finch *et al.*, 1969); hexosamine, colorimetrically by the Boas method (1953); and sialic acid by the resorcinal method (Miettenen and Takki-Lukkainen, 1959). The data are shown in Table IV. In general the carbohydrate content is low. The low concentration of galactose testifies to absence of galactolipid from this fraction. Appreciable amounts of fucose and hexosamine were found, implying that this protein may be a glycoprotein.

TABLE IV: Carbohydrate Content of N-2 from Normal Human Myelin.

Carbohydrate	μg/mg of Fraction	μmoles/10 mg of Fraction
Sialic acid	0.9	0.03
Glucose	1.1	0.05
Mannose	0.4	0.02
Galactose	0.5	0.02
Fucose	5.2	0.30
Hexosamine	22.7	1.30

Discussion

We have described the isolation of a protein from normal human myelin. It appears to run as a single component in polyacrylamide gel and in analytical ultracentrifugation. It is low in basic amino acid residues but high in neutral and acidic ones. The amino acid composition is not unlike that reported by Folch and Lees (1951) for proteolipid protein and that of Wolfgram and Kotorii (1968) for their trypsin and pepsin resistant fractions. Notable differences are found in the Ser, Glu, Lys, and Val residues. There is little neutral sugar but a small amount of fucose and some hexosamine are present.

Of considerable interest is the presence of about 2% fatty acids which cannot be accounted for as components of phospholipid, galactolipid, sphingolipid, or triglyceride, since our assays of phosphorus, galactose, sphingosine, and glycerol are negative.

Although not a component of the previously mentioned lipids, the fatty acid appears to be rather firmly bound to the protein. Evidence of a strong binding was provided by a number of experiments. Transmethylation with BF_3–methanol liberated 0.7 μmole of fatty acid/10-mg sample. On the other hand, prior hydrolysis with 2 N KOH–methanol followed by transmethylation with BF_3–methanol liberated slightly more than 1.0 μmole. Soxhlet extraction for prolonged periods (48 hr) failed to remove fatty acid. The use of charcoal, a method known to remove fatty acid from albumin, was ineffective with our material. Dissolving the protein in phenol–acetic acid–water + 2 M urea followed by extensive dialysis against decreasing concentrations of acetic acid failed to remove fatty acid. Extraction of a solution of N-2 in 98% formic acid with ether did not decrease the amount of fatty acid associated with the protein. It may be that this residual fatty acid is attached to the protein in an ester-type linkage between fatty acid and protein.

Acknowledgments

We gratefully acknowledge the use of the gas chromatographic facility, kindly supplied by Doctor J. A. Lowden of this institution. The work was supported by a grant from the Multiple Sclerosis Society of Canada. We acknowledge a fellowship from the Multiple Sclerosis Society of Canada to D. D. W. and a studentship to J. G. from the Medical Research Council of Canada.

References

Bartlett, G. R. (1959), *J. Biol. Chem. 234*, 466.
Boas, N. F. (1953), *J. Biol. Chem. 204*, 553.
Chernick, S. S. (1969), *Methods Enzymol. 14*, 627.
Chen, R. F. (1967), *J. Biol. Chem. 242*, 173.
Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 121*, 404.
Eylar, E. H. (1970), *Proc. Nat. Acad. Sci. U. S. 67*, 1425.
Finch, P. R., Yuen, R., Shachter, H., and Moscarello, M. A. (1969), *Anal. Biochem. 31*, 296.
Folch, J., and Lees, M. (1951), *J. Biol. Chem. 191*, 807.
Gros, C., and Labouesse, B. (1969), *Eur. J. Biochem. 7*, 463.

Hartley, B. S. (1970), *Biochem. J. 119*, 805.
Kibler, R. F., Shapira, R., McKneally, S., Jenkins, J., Selden, P., and Chou, F. (1969), *Science 164*, 577.
Hyun, S. A., Vahouny, G. V., and Treadwell, C. R. (1965), *Anal. Biochem. 10*, 193.
Laatsch, R. H., Kies, M. W., Gordon, S., and Alvord, E. C. (1962), *J. Exp. Med. 115*, 777.
Lauter, C. J., and Trams, E. G. (1962), *J. Lipid Res. 3*, 136.
Lowden, J. A., Moscarello, M. A., and Morecki, R. (1966), *Can. J. Biochem. 44*, 567.
Lowden, J. A., Wood, D. D., and Moscarello, M. A. (1967), *Can. J. Biochem. 45*, 148.
Maizel, J. V. (1966), *Science 151*, 988.
Martenson, R. E., Dibler, G. E., Kies, M. W. (1969), *J. Biol. Chem. 244*, 4261.
Martenson, R. E., and Gaitonde, M. K. (1969), *J. Neurochem. 16*, 333.
Miettenen, T., and Takki-Lukkainen, I. I. (1959), *Acta Chem. Scand. 13*, 859.
Mokrasch, L. C. (1967), *Life Sci. 6*, 1905.
Moore, S., and Stein, W. J. (1954), *J. Biol. Chem. 211*, 907.
Murdock, D. D., Katona, E., and Moscarello, M. A. (1969), *Can. J. Biochem. 47*, 818.
Neville, D. M., Jr. (1967), *Biochim. Biophys. Acta 133*, 168.
Reisfeld, R. A., Lewis, U. J., and Williams, D. E. (1962), *Nature (London) 195*, 281.
Shachter, H., Sarney, J., McGuire, E. J., and Roseman, S. (1969), *J. Biol. Chem. 244*, 4785.
Sherman, G., and Folch-Pi, J. (1970), *J. Neurochem. 17*, 597.
Spies, J. R., and Chambers, D. C. (1949), *Anal. Chem. 21*, 1249.
Takayama, K., MacLennan, D. H., Tzagoloff, A., Stoner, C. D. (1966), *Arch. Biochem. Biophys. 114*, 223.
Tomasi, L. G., and Kornguth, S. E. (1967), *J. Biol. Chem. 242*, 4933.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.
Wolfgram, F. (1966), *J. Neurochem. 13*, 461.
Wolfgram, F., and Kotorii, K. (1968), *J. Neurochem. 15*, 1281
Wood, D. D., Gagnon, J., Finch, P. R., and Moscarello M. A. (1971), *Amer. Soc. Neurochem., Transactions 2, 1*, 117

Control of Succinate Dehydrogenase in Mitochondria*

M. Gutman,† Edna B. Kearney, and Thomas P. Singer‡

ABSTRACT: In intact, respiring mitochondria succinate dehydrogenase activity undergoes rapid and extensive changes in response to the metabolic state. Highest succinate dehydrogenase activity is observed in state 4, when coenzyme Q (CoQ) is largely in the reduced state. On transition to state 3 rapid deactivation of the enzyme occurs, in line with data in the literature showing oxidation of CoQ_{10} under these conditions. Lowest activity is observed in state 2 or in the presence of uncouplers, which cause almost complete oxidation of reduced CoQ_{10}. These findings indicate that the activation of the dehydrogenase by reduced CoQ_{10} in membranes also operates in intact mitochondria and is one of the factors governing succinate dehydrogenase activity. The data also help explain reports in the literature of succinate accumulation in state 3 and in the presence of uncouplers and its metabolic removal in state 4. In addition to activation by reduced CoQ_{10} in mitochondria, the enzyme is also activated by succinate and by ATP or a compound in equilibrium with ATP. ATP-induced activation does not seem to involve oxalacetate removal and is not mediated by the energy conservation system, since it is not oligomycin sensitive. In submitochondrial particles and in complex II neither ATP nor GTP seems to activate the enzyme but ITP and IDP do. Activation of succinate dehydrogenase by succinate, substances leading to CoQ_{10} reduction, or ATP occurs more rapidly and with a lower activation energy in mitochondria than in submitochondrial particles or soluble preparations. These observations indicate that the dehydrogenase is under efficient multiple control in intact mitochondria.

It is well established that in soluble or membranal preparations succinate dehydrogenase is converted by substrates and substrate analogs from an unactivated (deactivated) to an activated form (Kearney, 1957) and that it returns to the deactivated state on removal of the activator (Kimura *et al.*, 1967). The possibility that this reversible activation may be of regulatory significance in cell metabolism has been pointed out (Singer, 1968) and became very likely when it was discovered (Gutman *et al.*, 1971a,b) that reduced CoQ_{10}[1] and substances which lead to the reduction of CoQ_{10} in membrane preparations rapidly activate the enzyme, providing thereby a physiologically occurring activator whose concentration changes rapidly and extensively in metabolic transitions (Kröger and Klingenberg, 1966).

It was of interest to examine the extent to which agents

* From the Division of Molecular Biology, Veterans Administration Hospital, San Francisco, California 94121, and from the Department of Biochemistry and Biophysics, University of California, San Francisco, California 94122. *Received August 2, 1971.* This is paper XIX in the series: Studies on Succinate Dehydrogenase. This investigation was supported by grants from the American Cancer Society (P-531 and BC 46A), the National Institutes of Health (HE 10027), and the National Science Foundation (GB 08214).

† On leave of absence from Tel-Aviv University, Israel.
‡ To whom to address correspondence.

[1] Abbreviations used are: CoQ_{10}, coenzyme Q_{10}; ETP and ETP_H, nonphosphorylating and phosphorylating preparations of the inner membrane; PMS, phenazine methosulfate; STM buffer, sucrose–Tris–Mg buffer.