Interactions of Lipids with a Membrane Structural Protein from Myelin*

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ABSTRACT: A water-soluble, delipidated membrane protein from bovine brain myelin combines with anionic lipids to form insoluble complexes. The minimal amount of phosphatidylserine, phosphatidylinositol, cerebroside sulfate, and oleic acid required to completely precipitate the protein corresponds to 39 mole % of the total basic amino acid groups in the protein. Complexes precipitate optimally near neutrality and are stabilized by divalent cations. Nonionic lipids (cholesterol and cerebroside) and lecithin form nonprecipitating complexes with the protein which can be demonstrated by centrifugation in sucrose density gradients. These lipids also bind to protein–anionic lipid complexes. Succinylation of the protein greatly reduces interaction with lipids and abolishes the capacity to form insoluble complexes. These observations are discussed with respect to the structural role which different kinds of lipids might assume in myelin.

The nature of the weak interactions between the protein and lipid components of cellular membranes is a question central to considerations of supramolecular cell architecture which has not been satisfactorily answered. Various recent reviews of membrane structure have dealt with the importance of lipophilic versus ionic associations of lipid and protein and have attempted to evaluate their contribution to the architectural arrangement and stability of membranes (Finean, 1966; Maddy, 1966; Korn, 1966; Green and Tzagoloff, 1966; Rosenberg, 1967; Rothfield and Finkelstein, 1968). Despite the paucity of experimental supporting data, current views of membrane structure minimize the significance of charge–charge interactions and emphasize the domination of lipophilic bonding between lipid and protein. Several models of cellular membranes based on this supposition have recently been described (Benson, 1966; Lenard and Singer, 1966; Wallach and Gordon, 1968).

Myelin is a membranous structure of particular interest because of its critical function in the nervous system and because of its relatively simple protein composition. There appear to be only three major proteins in central nervous system myelin, two of which can be obtained in a lipid-free and water-soluble form (Lowden *et al.*, 1966; Tenenbaum and Folch-Pi, 1966; Eng *et al.*, 1968). We describe here experiments which demonstrate *in vitro* the association between the major structural protein of myelin with several anionic, zwitterionic, and nonionic lipids. Our data support the premise that all the lipids tested interact lipophilically with the protein but that the anionic lipids play a special role in rendering the complex insoluble.

Materials and Methods

Myelin. White matter was carefully dissected from fresh beef brains and stored frozen. Large-scale myelin isolations were carried out with the Beckman B-14 zonal rotor according to the procedure of Autilio *et al.* (1964). The final preparation, following osmotic rupture and numerous water washes to remove axonal fragments, consisted of a dense slurry pellet.

Proteolipid Protein. A modification of the procedure described by Tenenbaum and Folch (1966) provided the myelin protein used in all the present studies. Freshly prepared myelin was dissolved in 19 volumes of chloroform–methanol (2:1, v/v). The clear solution was partitioned with water according to Folch *et al.* (1957) and the lower phase, containing all the soluble proteolipid, was transferred to chloroform–methanol extracted dialysis tubing. Following dialysis for 7 days in chloroform–methanol the dialysate was acidified to 0.04 N with concentrated HCl. The precipitate which appeared at this point was removed by centrifugation (20,000*g*, 15 min). The clear supernatant fluid was returned to fresh tubing and dialyzed against chloroform–methanol–HCl. The transfer of the delipidated protein to aqueous solution was performed by successive dialysis in solutions of increasing methanol and water and finally in water alone. A small amount of suspended material was removed by centrifugation at 100,000*g* for 60 min. The pH of the final, clear aqueous solution of protein was close to neutrality. Protein solutions were concentrated to 1–10 mg/ml by either pervaporation or precipitation (pH 9–10) with dilute NH_4OH followed by resolution in very dilute acetic acid and dialysis. Since proteolipid protein is denatured by lyophilization or by freezing and thawing (Tenenbaum and Folch, 1966), protein solutions were stored at 4° with a few drops of chloroform added as a preservative.

The chloroform–methanol–HCl-insoluble protein was redissolved in chloroform–methanol. Transfer of this protein to aqueous solution was accomplished by the above procedure. Considerable losses of this protein occurred, as it is dialyzable. This is most probably the low molecular weight basic myelin protein isolated previously by other procedures (Lowden *et al.*, 1966; Roboz Einstein *et al.*, 1968; Eng *et al.*, 1968).

Myelin slurry (436 g; 16.1 g of myelin, dry weight) yielded 1.5 g of water-soluble proteolipid protein and 0.8 g of basic protein. A third protein fraction remained at the interface during sol-

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vent partition of myelin. Preliminary attempts to solubilize this material were unsuccessful.

Succinylation of Protein. Proteolipid protein was succinylated by the procedure of Klotz (1967). Following dialysis against water the protein solution was acidified to pH 4 with acetic acid. The precipitated protein was redissolved in water with a few drops of dilute NH_4OH, dialyzed, and stored as a 7-mg/ml solution at 4°.

Analysis of the Proteolipid Protein. The lipid content of the preparation was estimated by determining the phosphorus content after wet ashing (Bartlett, 1959) and by quantitative gas–liquid partition chromatography of the fatty acids released by acidic methanolysis (Kishimoto and Radin, 1965). An internal standard was added prior to methanolysis. Analysis for cholesterol by the method of Lowry (1968) was performed on 1 mg of dry protein on a layer of silica gel G.

Since HCl in anhydrous methanol is the commonly employed medium for esterifying proteins (Wilcox, 1967), we examined the possibility of methyl ester formation during dialysis of the proteolipid protein in chloroform–methanol–HCl. A 1-mg sample of the protein was hydrolyzed in 30 μl of 2 M KOH for 1 hr in a sealed tube at 50°. Sublimation of the reaction mixture yielded the aqueous methanol solution which was injected in 3-μl portions into a gas chromatograph fitted with a flame ionization detector and an Apiezon L column. Concentrations of methanol as low as 70 ng/μl could be determined readily by comparison with standard solutions.

The amino acid composition of the protein was kindly determined by Dr. Robert Zand with a Beckman automatic amino acid analyzer. The number of free carboxyl groups (as opposed to total glutamine, glutamate, asparagine, and aspartate) was determined by titration of the protein (5 mg in 1 ml) with 0.01 N HCl under nitrogen at room temperature. The titration curve was corrected by substracting a titration curve for water. The optical density of a 1% solution of the protein was found to be 15.3 at 280 nm.

Acrylamide Gel Electrophoresis. The method of Davis (1964) was used to prepare 7.5% polyacrylamide gels. The gels contained 5 M urea and 0.5% Triton X-100. The buffer was either Tris-glycine (pH 8.1) (Davis, 1964) or sodium formate (50 mM) (pH 3). Electrolyte buffers also contained Triton X-100 (0.5%). Samples of protein (100–200 μg) in 40% sucrose and 0.5% Triton X-100 were applied directly to the top of the gel, immobilized with a small amount of Sephadex G-25, and overlaid with electrolyte. Current was applied for 90 min at 5 mA/gel. Gels were stained with Amido Black and destained by diffusion.

Reagents. Pure PC,[1] PE, PS, oleic acid, and cholesterol were obtained from Applied Science, Inc., or Supelco, Inc. PI was prepared as the NH_4^+ salt by the method of Colacicco and Rapport (1967). Cerebroside, [^{3}H]cerebroside, and cerebroside sulfate were pure compounds available in this laboratory. A mixture of brain mono-, di-, and triphosphoinositides was a gift of Dr. B. Agranoff. Anionic lipids were converted into the Na^+ salt with the aid of a Chelex column (Carter and Weber, 1966). ^{14}C-Labeled PC, PS, and cholesterol were purchased from Tracerlabs and New England Nuclear Corp.

The chemical and radiochemical purity of all lipids was confirmed by thin-layer chromatography and autoradiography. All lipids were stored in chloroform, chloroform–methanol, or benzene in the cold. The nonionic detergents Triton X-100 and Myrj 59 (supplied by Atlas Chemicals) were used without purification. Crystalline bovine serum albumin was supplied by Pentex, Inc.

Dispersal of Lipids in Water. Optically transparent aqueous dispersions of all lipids could be obtained by ultrasonic radiation. Detergents were required in some cases. Appropriate solutions of lipid and detergent were coevaporated in glass scintillation vials at 30° under a stream of N_2. Water was added and the mixture was sonically irradiated with the probe of a Branson Sonifier until a clear solution was obtained (30–60 sec). Lipid solutions were stored at 4° and discarded after 10 days. Concentrations of various lipids and detergents which provided clear, stable solutions are listed in Table I.

TABLE I: Concentrations of Lipid and Detergent Used to Obtain Clear Aqueous Dispersions.

Lipid–Detergent	Concn (μg/ml)
Phosphatidylserine	400
Phosphatidylinositol	400
Phosphatidylcholine	400
Cerebroside–Myrj 59	100:300
Cerebroside–PC	100:200
Cerebroside–PC–PE	100:200:100
Cerebroside sulfate–PC	200:400
Cholesterol–PC	100:300
Cholesterol–Myrj 59	100:100

The Na^+ salt of the phosphoinositide mixture is readily dispersed in water without sonication and is an excellent solubilizing agent for a variety of less readily dispersed lipids such as cerebroside and cholesterol.

Formation and Determination of Protein–Lipid Complexes. Aqueous dispersions of lipids were added to solutions of proteolipid protein in water and incubated at room temperature for 15 min on a rotary shaker. Insoluble protein–lipid complexes were designated to be those which were completely sedimented as a well-packed pellet in 20 min at 40,000*g*. Residual protein in the supernatant solution was estimated by the method of Lowry *et al.* (1951) using proteolipid protein as a standard, and residual lipid by radioactivity determination in the scintillation counter or by phosphorus analysis according to Bartlett (1959).

Protein–lipid mixtures were also applied to sucrose gradients as follows. After dissolving 250 mg of sucrose in 0.4 ml of the mixture, the sample was layered over a 0.5-ml cushion of 60% sucrose in a 5-ml tube. A 4-ml linear sucrose gradient (20–30%) was then applied above the sample. Centrifugation for 4.5×10^6 g-hr was done in the Beckman SW65 rotor and 0.3-ml fractions were collected by tube puncture. After dilution to 1 ml with water, the protein was estimated at 280 nm. Lipid was determined by radioactivity measurement of a 0.5-ml aliquot of the diluted fractions in 10 ml of a standard 2,5-

[1] Abbreviations used are: PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; Ce, cerebroside.

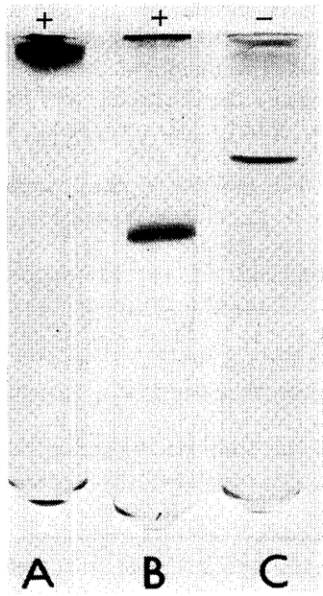

FIGURE 1: Electrophoresis of myelin proteins on acrylamide gels. A = proteolipid protein, pH 3; B = basic protein, pH 3; C = succinylated proteolipid protein, pH 8.1. In each case migration was downward.

diphenyloxazole–1,4-bis[2-(4-methyl-5-phenyloxazolyl)]–toluene solution containing 9% Bio-Solv BBS-3 (Beckman solubilizer). The sucrose did not affect counting efficiency.

Results

Characteristics of the Proteolipid Protein. The phosphorus content of the protein was found to be 7.7 nmoles/mg, a value lower than that reported by Tenenbaum and Folch (1966). The fatty acids (primarily stearate, palmitate, and oleate), which are associated with the protein in either the free form or as components of complex lipids, amounted to about 70 nmoles/mg. It would appear that much of the lipid present is not phospholipid. The test for cholesterol, which detects less than 2 μg, proved to be negative.

The methanol obtained by saponification of the protein was found to be about 62 nmoles/mg, presumably arising during the dialysis in chloroform–methanol–HCl. The physical entrapment of methanol by the protein cannot be entirely ruled out but seems improbable in view of the prolonged aqueous dialysis to which the protein is subjected. The amino acid analysis of our protein, in good agreement with that of Tenenbaum and Folch (1966), indicates that the total acidic amino acids (including their amides) comprise 10.2 mole %, or 0.95 μmole/mg. Thus, 6.5% of the acidic amino acid residues might occur as their methyl esters in our preparation.

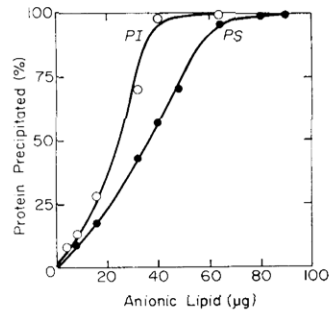

FIGURE 2: Precipitation of protein–anionic lipid complexes as a function of PI and PS concentration. Each tube contained 290 μg of proteolipid protein (0.4 ml), 0.5 mM $CaCl_2$, and aqueous dispersions of the lipid, in a final volume of 1 ml. Identical curves were obtained when the mixtures contained, in addition to the anionic lipid, an amount of PC dispersion equal to that of the anionic lipid.

TABLE II: Lipids Which Were Tested for Their Ability to Form Insoluble Complexes with Myelin Proteolipid Protein.

Lipid	Amt of Lipid Added to Protein[a] μg	Amt of Lipid Added to Protein[a] nmoles[b]	Amt of Protein Pptd (%)
Phosphatidylserine	80	95	100
Phosphatidylinositol $(NH_4)^+$	65	90	100
Cerebroside sulfate (Na^+)	85	95	98
Oleate (NH_4^+)	28	95	100
Phosphatidylcholine	10–100		0
Phosphatidylethanolamine	10–100		0
Cerebroside (in PC or Myrj 59)	10–50		0
Cholesterol (in PC or Myrj 59)	10–50		0

[a] 290 μg of proteolipid protein; 238 nmoles of total Lys, Arg, and His. [b] Calculated from approximate formula weights of the lipids; the minimal amount of lipid required for complete precipitation of the protein.

Titration of the protein between pH 6 and 3 required 2.5 μmoles of HCl for 5 mg, indicating that only 53% of the acidic residues are titratable in this region. The above two values are in agreement with preliminary ammonia determinations (as part of the amino acid analysis) showing that 40–50% of the total acidic residues is in the amide form. Since the lysine, histidine, and arginine contents are about 4.3, 1.9, and 2.6 mole %, respectively (8.7 total), and the free carboxyl content is about 5.4 mole %, it would appear that the protein bears a net positive charge in neutral solution.

Figure 1A illustrates the electrophoretic behavior of the proteolipid protein in acrylamide gel. No faster migrating proteins were detectable at either shorter or longer times. A single band which penetrates the gel only with difficulty was observed at several pH values (pH 2.5, 4, and 11.5 in formate, acetate, and phosphate buffers, respectively) and in gels of two different concentrations (5 and 7.5%). This observation is compatible with viscosity determinations showing the protein to be a large random coil, and with ultracentrifugal analysis which illustrates a marked tendency of the protein to aggregate (work in progress). Aggregation of the protein seems to be minimized during electrophoresis when 0.5% Triton X-100 is present. The electrophoretic mobility of the smaller, highly basic myelin protein which precipitated from chloroform–methanol–HCl is much greater than that of the proteolipid protein (Figure 1B) and is not affected by detergent. A small amount of contaminating proteolipid protein is also observed in the gel. This basic protein migrates toward the cathode even

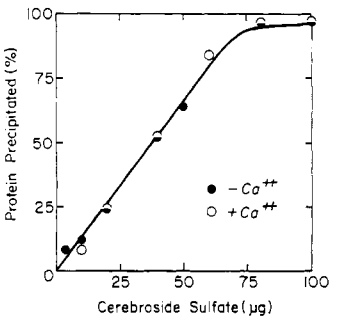

FIGURE 3: Precipitation of a protein–cerebroside sulfate complex as a function of lipid concentration. The cerebroside sulfate–PC dispersion (1:2) was added to 290 μg of aqueous proteolipid protein in a final volume of 1 ml. When present in the mixture, the $CaCl_2$ concentration was 2.5 mM.

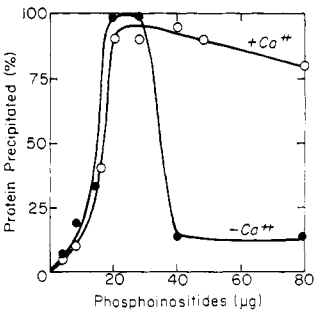

FIGURE 4: Precipitation of a protein–phosphoinositide complex as a function of lipid concentration, with and without Ca^{2+} (2.5 mM). The mixture of brain phosphoinositides was added to 260 μg of proteolipid protein and the volume was adjusted to 1 ml with water.

at pH 11.5. Succinylated proteolipid protein migrates as a discrete band and appears to be homogeneous (Figure 1C).

Complexes of Protein with Anionic Lipids. All negatively charged lipids tested form insoluble complexes with proteolipid protein in water. These complexes appear on visual inspection as opalescent dispersions, most of which form aggregates that settle out on standing, especially when low concentrations (0.5 mM) of $CaCl_2$, $MgCl_2$, $MnCl_2$, or higher concentrations (15 mM) of NaCl are present. The nonacidic lipids which were tested (Table II) do not form these sedimentable complexes. The minimal molar amounts of anionic lipids (regardless of structure) required to form insoluble aggregates with all of the protein (Table II) are equivalent to about 39% of the total basic amino acid residues in the protein.

Figures 2 and 3 illustrate the correlation between the amount of anionic lipid added and the percentage of added protein which sediments centrifugally as a complex with lipid. This precipitation phenomenon exhibits a sigmoidal curve with PS and PI (Figure 2), whereas a simpler relationship is observed with cerebroside sulfate (Figure 3). Since PC was used as a dispersing agent for cerebroside sulfate, it was of interest to compare the effect of PC on complex formation in response to PS. The curve obtained when equal amounts of PC and PS were added to the protein was identical with that obtained with PS alone (Figure 2).

Addition to protein of anionic lipid in large excess over that amount required to just precipitate all the protein results in partial resolubilization of the complex. This effect is most pronounced with the mixture of phosphoinositides (Figure 4) and may be accounted for by the excellent detergent properties of this lipid mixture (see Materials and Methods). The insoluble protein–lipid complexes are, however, nearly completely resistant to this type of resolubilization when Ca^{2+} is present (Figure 4).

In the presence of Ca^{2+} (0.5 mM) sufficient PI can be added to the protein to reach a saturation level (Figure 5). Assuming that all basic residues (119 nmoles in 145 μg of protein) are available for interaction with anionic lipid, then about 88 μg of PI (approximate mol wt 740) may associate electrostatically with the protein; the remaining lipid (37 μg of PI) must therefore interact lipophilically with the protein. The optical

TABLE III: Comparison of the Chloroform–Methanol Extractability of Anionic and Nonionic Lipids from Protein–Lipid Complexes.

Lipid Distribution	Experiment[a] A, [^{14}C]PS (μg)	B, [^{3}H]Ce (μg)
Labeled lipid in protein–lipid complex	980	200
Labeled lipid in upper layer	20	2
Labeled lipid in lower layer	660	198
Labeled lipid bound to interfacial protein	300	None

[a] Both reaction systems contained protein (1.45 mg), $CaCl_2$ (2.5 μmoles), and lipid (A, 1 mg of [^{14}C]PS (5 × 10^5 cpm); B, 400 μg of unlabeled PS and 600 μg of [^{3}H]Ce/Myrj 59, 1:2 (1.23 × 10^5 cpm)), and water in a final volume of 5.0 ml. Lipid–protein complexes were centrifuged as described in Methods. The supernatant fluid was decanted from the pellet. To the pellet was added 1.2 ml (estimated to be 19 volumes) of chloroform–methanol (2:1), and the suspension was partitioned with 0.24 ml of water according to Folch *et al.* (1957). Centrifugation yielded a thin, tightly packed pad at the interface. The clear layers and the interfacial pad (washed once with chloroform–methanol) were analyzed by liquid scintillation counting.

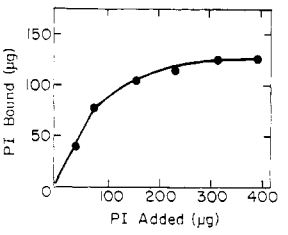

FIGURE 5: Binding of PI to proteolipid protein as a function of PI added. The aqueous PI dispersion and 0.5 μmole of $CaCl_2$ were added to 145 μg of protein (0.2 ml). The final volume was 1 ml. The uncomplexed PI was determined by phosphorus analysis of the supernatant fluid (see Methods).

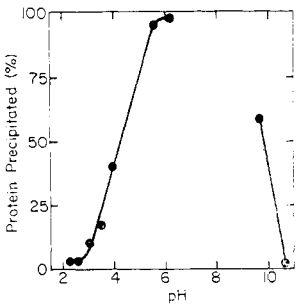

FIGURE 6: Precipitation of the protein–PS complex as a function of pH. Each tube contained 290 μg of protein and 80 μg of PS. The pH was adjusted with dilute solutions of HCl or NaOH.

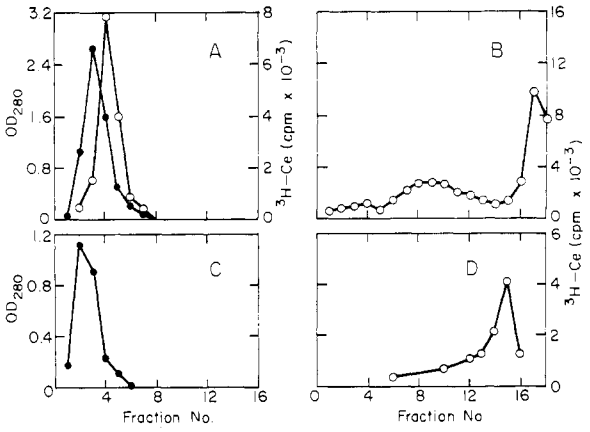

FIGURE 8: Protein–cerebroside interaction determined by centrifugation in sucrose gradients. (A) 2 mg of proteolipid protein and 40 μg of [^{3}H]Ce in 120 μg of Myrj 59; (B) 15 μg of protein and 40 μg of Ce in 120 μg of Myrj 59; (C) 1 mg of protein; (D) 10 μg of [^{3}H]Ce in 30 μg of Myrj 59. (●) Protein (OD_{280}); (O) lipid (counts per minute). In all cases the top of the gradient is on the right.

transparency of the phosphoinositide solution remained unaffected by this concentration of Ca^{2+}.

The ionic nature of protein–phosphatidylserine interaction is further attested to by the resistance of the bound PS to extraction with chloroform–methanol (Table III, expt A). The addition of PS to protein in excess of that required to completely bind to all possible basic residues results in a complex from which 70% of the total PS can be removed by chloroform–methanol. The remaining 30% (360 nmoles) is tightly bound to the protein and corresponds to about 30% of the total basic groups (1190 nmoles) in the protein, assuming a one-to-one binding relationship. By comparison, approximately 39% of the basic groups must interact ionically with lipid before all of the protein becomes insoluble.

Formation of insoluble complexes between myelin proteolipid protein and an anionic lipid such as PS is pH dependent (Figure 6). A similar dependence upon pH is observed with protein–phosphoinositide complexes. This effect could not be studied in the range of 7–9.5, the region in which the protein itself is insoluble. Above pH 9.7 and below pH 5 the protein–lipid precipitation phenomenon is markedly reduced. No changes in the optically clear phosphatidylserine solution could be detected over this pH range.

If charge–charge interactions between anionic lipids and the basic amino acid residues of the protein are indeed the determinant of insoluble complex formation, then reduction of the number of positively charged sites on the protein by succinylation of amino groups should reduce or eliminate this interaction. In experiments parallel to those described in Figures 2–4, succinylated myelin structural protein does not form any sedimentable complexes; the solution of protein and anionic lipid remains optically transparent. Similar results are obtained when bovine serum albumin is used instead of myelin protein. Serum albumin is well known for its ability to bind various lipids. Under the conditions described here no insoluble complexes are observed with this protein and any of the lipids tested.

Interaction between Primary Protein–Lipid Complexes and Nonionic or Zwitterionic Lipids. Once they are formed, the binary complexes of structural protein and anionic lipids are capable of binding substantial amounts of phosphatidylcholine, cerebroside, and cholesterol, alone or in various combinations. This binding occurs equally well when the protein is added to the aqueous mixture of both kinds of lipid. The amount of cholesterol–PC required to saturate the protein–PI complex is shown in Figure 7. Saturation is reached in this instance when the total lipid to protein ratio is 1.4. Attempts to saturate the binary complex with phosphatidylcholine or cerebroside–Myrj 59 were unsuccessful since the protein complex became partially resolubilized as a consequence of the detergency of these lipid preparations.

In contrast to the tightly bound acidic lipid, cerebroside is easily extracted from the protein (Table III). The complete extraction of cerebroside from a ternary complex by chloroform–

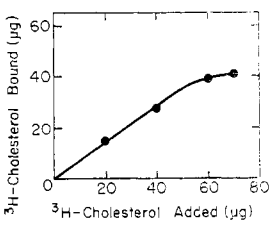

FIGURE 7: Incorporation of cholesterol into insoluble protein–PI complexes as a function of cholesterol added. Each tube contained 145 μg of protein, 40 μg of PI, 0.5 μmole of $CaCl_2$, and appropriate amounts of [^{3}H]cholesterol–PC (1:3). Unbound cholesterol was determined by liquid scintillation counting.

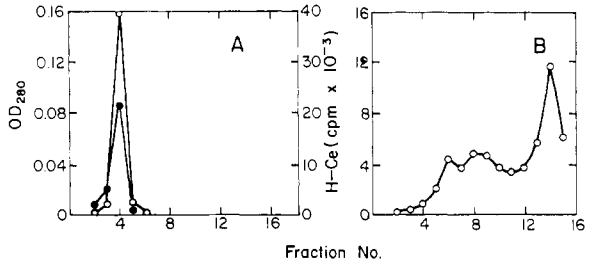

FIGURE 9: Interaction of protein with cerebroside dispersed in PC–PE. (A) 100 μg of proteolipid protein and 40 μg of [^{3}H]Ce in 120 μg of PC–PE (2:1); (B) 30 μg of proteolipid protein and 40 μg of [^{3}H]Ce in 120 μg of PC–PE (2:1). (●) Protein; (O) lipid.

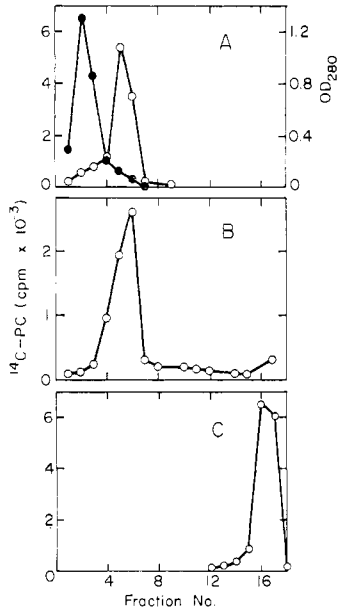

FIGURE 10: Interaction of protein with phosphatidylcholine. (A) 2 mg of proteolipid protein and 80 μg of [^{14}C]PC; (B) 50 μg of protein and 160 μg of [^{14}C]PC; (C) 80 μg of [^{14}C]PC. (●) Protein; (○) lipid.

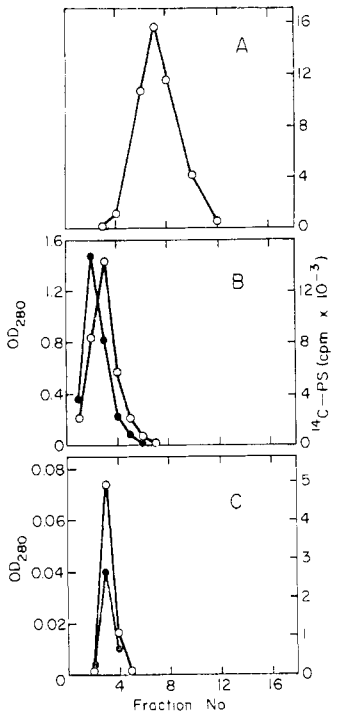

FIGURE 11: Density gradient centrifugation of the protein–PS complex. (A) 160 μg of [^{14}C]PS; (B) 2 mg of proteolipid protein and 80 μg of [^{14}C]PS; (C) 50 μg of proteolipid protein and 160 μg of [^{14}C]PS. (●) Protein; (○) lipid.

methanol emphasizes the weaker interactions between nonionic lipids and the membrane protein.

Interaction of Lipids with Proteolipid Protein. It is apparent from Figure 7 and related experiments described above that nonacidic lipids such as cholesterol, PC, and cerebroside interact with myelin protein by a mode different from that of the anionic lipids. The interaction of nonionic and zwitterionic lipids with protein can be observed by centrifugation in linear sucrose density gradients (Figures 8–10). It is evident from Figure 8A that all of the cerebroside is associated with the protein when the lipid:protein ratio is low (the Myrj 59 is considered to be a component of the total lipid); however, not all of the protein is associated with the lipid in the lipoprotein peak at this ratio. The density of the lipoprotein under these conditions is only slightly lower than that of the protein alone (Figure 8C). Proteolipid protein alone in amounts ranging from 50 μg to 3 mg displayed a profile identical with that shown in Figure 8C in all centrifugation experiments. Flotation at the top of the gradient (Figure 8D) was observed for all lipids, except PS, over a range of 10–240 μg. When the lipid:protein ratio is high (Figure 8B) the radioactivity profile broadens (fractions 5–14), presumably as a consequence of the formation of lipoprotein particles with a wide range of densities. The sharper band at the top of the gradient is likely uncomplexed lipid (*cf.* Figure 8D). The limited amount of lipid (160 μg) which could be suitably dispersed in the appropriate sample volume necessitated the use of very small amounts of protein when high lipid:protein ratios were desirable. In Figure 8B the 15 μg of protein could not be readily monitored in the gradient. The cerebroside profile obtained at densities greater than the control lipid (Figure 8D) is taken as an indication of interaction with the protein.

Similar centrifugation profiles are observed when cerebroside is codispersed in a mixture of PE and PC instead of in Myrj 59 (Figure 9). In this experiment, an increase in the ratio of total added lipid to protein from 3.2 (Figure 9A) to 5.3 (Figure 9B) is again seen to effect a large alteration in the density of the lipoprotein peak; however, the ratio of bound lipid to protein, calculated from total lipid and protein in fractions 4–12, remains constant (2.9–3.1). Because of the small amount of protein used (Figure 9B) a protein profile could not be obtained. All of the protein could, however, be accounted for by pooling fractions 4–12, precipitating the protein with trichloroacetic acid, washing the centrifuged pellet free of sucrose, and estimating the protein colorimetrically by the method of Lowry *et al.* (1951).

Figure 10A shows that the binding of a small amount of phosphatidylcholine to protein is similar to the binding of small amounts of cerebroside (Figure 8A). The bulk of the PC interacts with only a fraction of the total protein to form a lipoprotein band which has a density slightly lower than that of the protein alone. Again, a shift of the radioactivity (lipoprotein) profile to lower density occurs in response to a slight excess of lipid (Figure 10B). Although protein was not monitored in Figure 10B, this peak is presumed to consist of lipoprotein, since it is considerably denser than the lipid alone (Figure 10C) but less dense than the protein control which bands around fraction 4 (profile not shown). In all these experiments, when lipid:protein was close to 3:1, the lipoprotein region of the gradient after centrifugation was visually discernible as an opalescent band approximately 1 mm thick. Lipid and protein regions were not visually apparent.

Additional support for the bimodal association of anionic lipid with proteolipid protein, demonstrated in Figure 5 and Table III, is derived from Figure 11. The protein–PS behavior in a sucrose gradient is similar to that of the protein–cerebro-

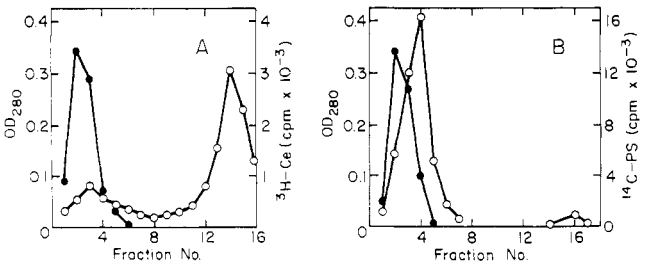

FIGURE 12: Interaction of serum albumin with cerebroside and phosphatidylserine. (A) 2 mg of albumin and 20 μg of [^{3}H]Ce in 60 μg of Myrj 59; (B) 2 mg of albumin and 80 μg of [^{14}C]PS. (●) Protein; (○) lipid.

side and protein–PC complexes (Figures 8–10), with the exception that a larger proportion of the protein molecules are associated with PS (Figure 11B). Whereas insufficient PS (160 μg) was added to react with all basic groups of the protein (2 mg) in expt B, the high PS:protein ratio (3:1) in Figure 11C illustrates the lipophilic capacity of the myelin protein. Here, as in Figure 9A, a relatively high lipid:protein ratio results in greater coincidence of the lipid and protein profiles than is observed with low lipid:protein ratios. It appears, therefore, that not all protein molecules are associated with equal amounts of lipid when subsaturating levels of lipid are present. PS micelles (Figure 11A) also possess a greater buoyant density than cerebroside or PC (Figures 8 and 10).

Although the lipid-binding ability of serum albumin is well known (Goodman, 1958; Reynolds *et al.*, 1968), this protein has a very low capacity for cerebroside and PS (Figure 12) compared with myelin protein. The proteolipid protein is seen (Figures 8 and 11) to interact with 300 times more nonionic lipid, and 40 times more anionic lipid than does serum albumin.

Lipid Interaction with Succinylated Protein. Succinylation of the proteolipid protein drastically reduces its affinity for lipids (Figure 13). The total amount of PS which can interact with the succinylated protein by ionic or nonionic forces is 0.2% of that which interacts with the unmodified protein. The conversion of the lysine amino groups into acidic functions also diminishes greatly the ability of the protein to associate with cerebroside and PC (Figure 13C, B). The affinity for these two lipids was reduced by factors of 500 and 800, respectively.

Discussion

A specific role for anionic lipids in membrane structure is indicated by the experiments reported above. This is shown by the ability of anionic lipids, such as those found in myelin, to form insoluble, aggregated complexes with a myelin protein. Lipids which are not negatively charged also interact with the protein but the resultant complexes remain in a "soluble" or dispersed form. Additional evidence for specificity of binding is the observation (Figures 2 and 3) that the precipitation phenomenon is unaffected by the presence and binding of nonacidic lipids. A third type of evidence for two different binding modes is derived from the finding that the anionic lipid, phosphatidylserine, is only partially removed from the ternary complex. The resistance of phosphoinositides in myelin to organic solvent extraction has previously been cited as evidence

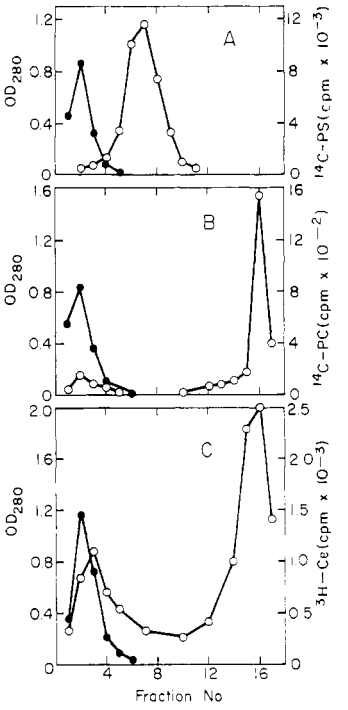

FIGURE 13: Density gradient centrifugation of succinylated protein-lipid mixtures. (A) 1.3 mg of succinylated proteolipid protein and 80 μg of [^{14}C]PS; (B) 1.3 mg of succinylated protein and 40 μg of [^{14}C]PC; (C) 2 mg of succinylated protein and 10 μg of [^{3}H]Ce in 30 μg of Myrj 59. (●) Protein; (○) lipid.

for electrostatic binding of anionic lipids in membranes (LeBaron, 1963; Folch-Pi, 1966).

Our inability to demonstrate protein–anionic lipid aggregates at pH extremes is consistent with the requirement for negatively and positively charged groups on lipids and protein, respectively, before insoluble complexes can occur. This effect may, however, be a result of enhanced solubility of the protein or the complex at low and high pH values.

Divalent cations prevent, to a large extent, the dispersion or disaggregation of the protein–anionic lipid complexes by detergent-like lipids (Figure 4). These ions (Ca^{2+}, Mg^{2+}, and Mn^{2+}), at a concentration of 0.5 mM, probably stabilize the complex by inter- or intrachain cross-linking through protein carboxyl or lipid phosphate groups. Similarly, monovalent cations (Na^+ and K^+) at a much higher concentration (15 mM) may reduce the hydrophilicity of the protein–lipid complexes, thereby stabilizing their aggregated arrangements. Our observations with respect to cation stabilization of membrane complexes are in agreement with many similar observations by other investigators working with intact membranes.

The binary complexes of protein and anionic lipid, despite their aggregated form, bind substantial amounts of nonacidic lipids (Figure 7). That this interaction is not due to the ionically bound lipid alone is seen from density gradient centrifugation profiles of lipoprotein complexes which are obtained in the absence of acidic lipid (Figures 8–10). In a similar centrifugation experiment, in which phosphatidylserine was added to a protein–cerebroside mixture, the ratio of nonionically bound cerebroside to protein (approximately 3:1) was not affected by the anionic ligand. This observation emphasizes the independent binding of these two different lipids and the

simultaneous accessibility of both cationic and apolar regions of the protein.

It is evident from these data that there are different kinds of binding sites for the two types of lipids. Anionic lipids are most likely bound to the cationic groups of the protein whereas the neutral lipids are probably associated with lipoidal regions of the protein. A small amount of neutral lipid may, however, be associated with cationic sites by prior complexing with anionic lipids, as in the complexing behavior observed between cerebroside sulfate and lecithin (Abramson and Katzman, 1968). The high content of apolar amino acid side chains, characteristic of myelin proteolipid protein (Folch-Pi, 1966), does not, however, appear to account entirely for the large amount of lipid which can associate with the protein. The conformational arrangement of the protein likely provides extensive hydrophobic domains capable of accommodating many molecules of lipid. This view is supported by our finding that succinylation of the lysine amino groups almost completely abolishes the capacity of the protein to associate with lipids; this effect is likely a consequence of drastic alterations in the three-dimensional structure of the protein which are induced by the negatively charged succinyl groups.

Specific differences exist in the nature of the protein–lipid interaction within each category of lipids. The sigmoidal shape of the curve showing aggregation *vs.* anionic ligand (Figure 2) suggests that protein–phosphatidylserine and protein–phosphatidylinositol complexes may interact in a cooperative fashion, perhaps to form elementary particles or subunits reminiscent of the cooperative phenomena in biological membranes which have been proposed by Changeux *et al.* (1967). The precipitation of the protein–cerebroside sulfate complex, on the other hand, exhibits a simpler relationship to the amount present of this anionic lipid.

Differences in the interaction of nonacidic lipids with the protein are shown in centrifugation studies. Phosphatidylcholine, at subsaturating levels, is associated with only a small fraction of the protein (Figure 10A) whereas cerebroside and PS, at a similar level, are bound to a somewhat larger fraction of the protein (Figures 8A and 11B). This phenomenon may be indicative of cooperative interaction between the protein and lipid, the initial interaction resulting in an enhanced affinity of the lipoprotein for additional lipid.

The remarkable agreement between our lipid:protein ratios at "saturation" and the lipid:protein ratio of natural myelin (Norton and Autilio, 1966) may be fortuitous, however, since the proteolipid protein comprises only about 55% of the total protein; the remaining 45% is apparently accounted for by two other proteins in roughly equal amounts (Eng *et al.*, 1968). Preliminary experiments have shown that our preparation of basic protein also is able to form complexes with lipids and thereby may contribute to the high lipid content of myelin. A paper that appeared when this study was completed (Palmer and Dawson, 1969) has indeed shown that the basic protein can combine with triphosphoinositide together with large amounts of PC, and that this protein has many of the lipid-binding properties of myelin structural protein.

Our observation of a sudden shift of the lipoprotein peak in the sucrose density gradient to a region of lower density when the lipid:protein ratio exceeds 3:1 (Figures 8 and 9) may indicate a structural rearrangement of the lipoprotein particles with the exclusion of water; the more hydrophobic particles would tend to be less dense. Of interest in this regard is the observation by Autilio *et al.* (1964), that the "heavy" band of myelin in a sucrose density gradient had a slightly lower lipid:protein ratio than the "light" myelin band.

We conjecture that the fundamental structural units of myelin are lipoprotein complexes composed of a variety of lipids and several proteins, and that these units are assembled into a two-dimensional array, with the assistance of anionic lipids and divalent cations. This alignment of elementary particles may be a cooperative phenomenon dependent upon ligands such as anionic lipids. The assembly process could provide a protomyelin lattice of repeating units whose ultimate arrangement is contingent on the incorporation of additional lipids and proteins. Studies by Dobiasova and Radin (1968) on the incorporation *in vitro* of lipids into isolated and partially delipidated myelin preparations support the idea of self-assembly processes in the biogenesis of myelin.

Regardless of what the biological assembly mechanism might be this concept emphasizes an essential feature of all membranes, namely, their insolubility in an aqueous environment. Proteolipid protein is capable of association with large amounts of nonacidic lipid, but these complexes only assume a "tight" aggregated form when relatively small amounts of anionic lipid are present. Although this view has been derived from a study of myelin components, it may have more general applicability for other membrane systems. A compilation by Wallach and Zahler (1968) of amino acid compositions of several membranes illustrates that they all contain basic proteins, a feature shared by myelin. Moreover, there is ample evidence that all membranes also contain anionic lipids. At this point it may be useful to emphasize that recent studies on myelin cast some doubt on the frequently cited atypical nature of this membrane. The well-established metabolic turnover of both lipids and proteins of myelin (Smith, 1968) and the intimate association with this structure of two enzymes, a peptidase (Adams and Davison, 1965) and a highly active cyclic nucleotide hydrolase (Kurihara and Tsukada, 1967; Olafson and Drummond, 1969), attest to the similarity between myelin and other membranes. The high lipid content of myelin likely accounts for the natural role of this structure as neural insulator but it does not rule out the possibility of additional functions in the nervous system.

We predict that the bioassembly of myelin involves conformational alterations in the structural proteins when they interact with lipids. Experiments are in progress to examine these transitions by optical rotatory dispersion and circular dichroism, and to elucidate further the relationship between the components of myelin.

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Effect of Calcium Ion on S-100, a Protein of the Nervous System*

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ABSTRACT: A specific protein of the nervous system, S-100, appears to exist in multiple forms when subjected to acrylamide gel electrophoresis in the presence of either Ca^{2+} or 8 M urea, but only in a single form in their absence. Calcium ion causes a limited conformational change in S-100 leading to a more unfolded structure in a region probably containing its single tryptophan, several of its tyrosine and phenylalanine, and two of its three cysteine residues. These effects are not seen with Mg^{2+}. Monovalent cations antagonize the effect of Ca^{2+}, K^+ being more effective than Na^+. At physiological levels of K^+ and Na^+ the effects of Ca^{2+} occur also in its physiological range.

A protein, called S-100 since it was soluble in saturated $(NH_4)_2SO_4$ at pH 7, has been shown to be specific to the nervous system and to be present in an immunologically similar form in the nervous systems of all vertebrates (Moore, 1965; Levine and Moore, 1965; Kessler *et al.*, 1968; Moore *et al.*, 1968). It was characterized by its high mobility during starch or acrylamide gel electrophoresis as a consequence of its high content of glutamic and aspartic acid residues and its small size.

Hyden and McEwen (1966), Vincendon *et al.* (1967), and Gombos *et al.* (1966) found that S-100 showed immunologically similar, multiple forms when electrophoresed, either in pure form or from brain extracts, on acrylamide gel. We attempted to answer the question: Do the multiple forms of S-100 represent similar proteins differing in primary structure? Although this question has not been answered unequivocally, the results of the first experiments led to the bulk of the work in this paper which demonstrates that S-100 shows interesting specific interactions with calcium ion which might give a clue to the function of S-100 in the nervous system.

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