An improved preparation of bovine brain proteolipid

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SUMMARY An improved preparation of proteolipid from bovine brain white matter is described. The product obtained by repeated acetone precipitation is completely soluble in chloroform–methanol and has a fairly constant composition: 35% protein, 40% galactocerebroside, and about 25% phospholipid.

KEY WORDS bovine brain · white matter · proteolipid · galactocerebroside · lipid–protein interaction · lipid antigens

The isolation of proteolipid, a tissue lipoprotein that is soluble in organic solvents, was first described by Folch and Lees (1). Three proteolipid fractions were obtained from bovine white matter, and these (designated A, B, and C) differed in the quantity and type of lipid mixture associated with the protein. The solubility of proteolipid preparations in chloroform–methanol 2:1 was dependent on the presence of critical amounts of water. The lipid in these preparations is bound to protein with varying degrees of firmness, and in subsequent reports, Folch and coworkers (2–4) described other methods for preparing proteolipid with much higher protein content (up to 65%). In the preparations most recently described little glycolipid was present, and thus, the remaining lipids were mainly phosphatides.

Our interest in proteolipids was stimulated by two observations made in this laboratory. The first was the demonstration that galactocerebroside was immunologically active and would react with some anti-brain sera in amounts less than 5 nanograms in the presence of suitable quantities of lecithin and cholesterol (5). The second observation was that reactions of some antisera with pure CNS myelin could occur solely through the availability in myelin of galactocerebroside determinants (6).

It was therefore of considerable interest to study a substance containing both galactocerebroside and protein, but one less complex than myelin. Proteolipid B (1) was the most suitable choice, since it was reported to be crystalline and to contain 20% cerebroside and 50% protein (and 30% phosphatide) and was thought to have some antigenic activity in experimental encephalomyelitis (7). In attempts to prepare this substance, we were unable to obtain preparations with consistent lipid composition. Small changes in operational variables (temperature, concentration, type of agitation, shape of

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vessels) appreciably affected the final results. We have reinvestigated the preparation of bovine brain proteolipid, and by introducing several modifications, we have obtained a preparation with reasonably constant composition. This preparation contains the bulk of the organic solvent-soluble protein present in white matter, and the method avoids the original fragmentation into proteolipids A, B, and C which subsequent studies indicate to be solely an operational artifact (8). Proteolipid prepared in this way is immunologically active and reactions with anti-cerebroside antibody can detect less than 1 μg (9).

Analytical Methods. Glycolipid analysis by the anthrone method was carried out according to Radin, Lavin, and Brown (10), with pure bovine cord galactocerebroside as a standard. Phosphorus was determined by a method based on that of Beveridge and Johnson (11), after perchloric acid digestion. For analysis of protein, the method of Lowry, Rosebrough, Farr, and Randall (12) was used, except that the sample of proteolipid (100 μg) was digested with 0.5 ml of 1 N NaOH at 25°C for 20 hr with gentle magnetic stirring. Bovine serum albumin was used as the standard. Plasmalogens were determined by the *p*-nitrophenylhydrazone method (13).

Preparation of Crude Proteolipid. All procedures were carried out at 4°C unless specified otherwise. White matter was dissected from fresh adult bovine brain, and scraped free from gray matter (about 40 g of tissue was taken from each brain). Batches of 25 g of white matter were extracted in a Waring Blendor with 20 volumes of cold chloroform–methanol 2:1 for 2 min at low speed. The suspension was filtered by gravity (through Schleicher and Schüll sharkskin paper). The clear filtrate was transferred to a 600 ml beaker containing a magnetic stirring bar 1.5 × 0.38 inches. The beaker was positioned in the center of a 6 liter cylindrical container resting on a magnetic stirrer. The beaker was held firmly in position, gentle magnetic stirring was started, and the outer vessel was slowly filled with water to a level 2 inches above the top of the beaker. With 400–500 ml of solution in the beaker and slow stirring, the turbulence produced was slight. After 6 hr, stirring was discontinued and the system was allowed to stand overnight. The beaker contained a firm gel embedded in the organic phase and a clear aqueous phase which could easily be removed by aspiration. The beaker was placed at −10°C for 24 hr, and the frozen gel was gravity-filtered at −10°C through sharkskin paper moistened with chloroform. The clear filtrate containing free lipids showed no absorption at 280 mμ. The solid was washed twice (on the filter) with chloroform at −10°C, transferred to 50-ml stainless steel centrifuge tubes, and allowed to thaw at 4°C.

After centrifugation at 40,000 *g* for 30 min at 0°C, three phases were obtained: an upper aqueous layer, a tightly packed interfacial gel, and a bottom phase of chloroform. The stiff gel was transferred to a 500 ml round-bottom flask, shaken into a homogeneous suspension with an equal volume of water, and evaporated under reduced pressure to about two-thirds the original volume in order to remove organic solvents completely. The residual suspension was lyophilized. The crude proteolipid, a cream-white powder, was obtained in a yield of 2.2 g. It was readily soluble in chloroform–methanol 2:1, and had the following characteristics (average of 10 preparations): $A_{1\,cm}^{1\%}$ (280 mμ, chloroform–methanol 2:1), 3.0 ± 0.1; glycolipid (anthrone analysis, calculated as galactocerebroside equivalent), 44 ± 4%; phosphorus, 1.42 ± 0.2%; and cholesterol, <3%. The preparations were stored in a desiccator at −10°C.

Preparation of Final Product. The crude proteolipid was precipitated repeatedly from chloroform–methanol solution with acetone at 4°C as follows. A sample of material, 0.8 g, was dissolved in cold chloroform–methanol 2:1 in a tared 250 ml glass centrifuge bottle to make a clear 2% solution. With vigorous magnetic stirring, 4 volumes of cold acetone were added rapidly, and the mixture was stirred vigorously for 15 min. The

TABLE 1 Composition of Individual Proteolipid Preparations

Preparation No.	Protein*	Glycolipid†	Total Phosphorus
	%	%	%
From individual brains			
1‡	37.3	40.3	0.94
2‡	34.4	37.6	0.96
3	37.3	41.1	1.04
4	39.0	37.4	1.10
5	37.5	40.2	1.20
6	35.8	42.7	1.21
7	35.8	39.1	1.22
8	33.4	38.5	1.22
Mean ± SD	36.3 ± 1.8	39.6 ± 1.8	
From single pool			
9a	34.8	40.0	1.18
9b	35.5	40.3	1.22
9c	30.8	40.9	1.23
9d	33.0	42.0	1.26
9e	35.4	40.4	1.20
9f	36.9	38.3	1.24
9g	35.5	38.6	1.19
9h	35.2	40.0	1.19
9i	33.6	38.0	1.23
9j	33.3	40.0	1.22
Mean ± SD	34.4 ± 1.7	39.9 ± 1.2	1.22 ± 0.03

* As bovine serum albumin equivalents (Lowry).
† As galactocerebroside equivalents (anthrone).
‡ Six acetone precipitations.

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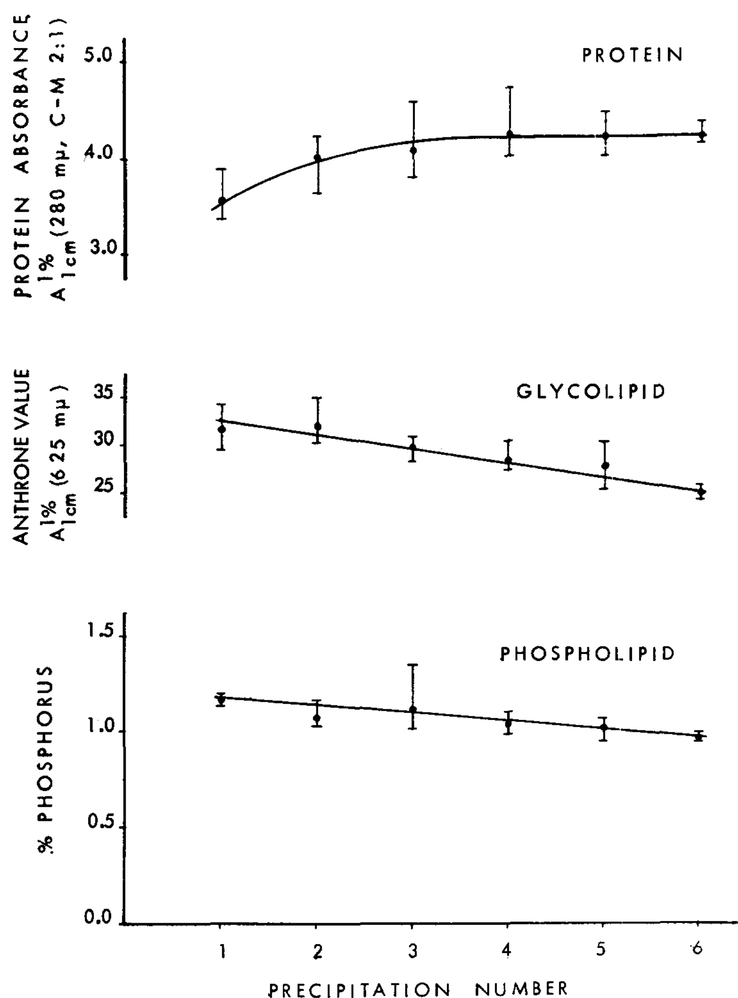

FIG. 1. Composition of proteolipid preparations obtained by repeated acetone precipitation. Points represent average of four preparations and vertical lines indicate range of values. C, chloroform; M, methanol.

flocculent colorless precipitate was collected by centrifugation for 10 min (2000 rpm, International Refrigerated Centrifuge), and the clear supernatant solution was decanted. The precipitate was washed twice by resuspension in 1 volume of acetone (based on the original chloroform–methanol solution) and centrifugation. The final precipitate was carefully dried by spreading it evenly on the walls of the centrifuge bottle, and subjecting it in sequence to (*a*) a stream of nitrogen, (*b*) respreading and evacuation until acetone was removed and (*c*) crushing and desiccation over P_2O_5 in vacuo. The granular, grayish, pale yellow powder was weighed, stirred at room temperature with sufficient chloroform–methanol 2:1 to make a 2% solution, and from the clear solution (obtained in 30–60 min), a small sample was removed for analysis. The solution was then chilled to 4°C, and the precipitation with acetone was repeated. After the final reprecipitation (the fourth in the procedure finally adopted), the moist precipitate was suspended in water, the acetone was removed under reduced pressure, and the aqueous suspension was lyophilized. The yield was 440–576 mg (55–72% of the weight of the crude proteolipid). Eight preparations obtained from different animals and 10 preparations obtained from the same pool of white matter showed reproducible composition (Table 1) that consisted almost entirely of protein, glycolipid, and phospholipid. Cholesterol was absent. The molar ratio of plasmalogen to phosphorus was 0.05. These preparations were soluble in chloroform–methanol 2:1 without addition of water, and 15 of the 18 gave optically clear solutions. The specific absorbance ($A^{1\%}_{1\,cm}$) at 280 mμ had an average value of 4.25.

Effect of Reprecipitation with Acetone. The procedure described was selected after trying precipitation with 1, 2, 3, 4, 5, and 10 volumes of acetone. It was found that 4 volumes of acetone would permit recovery of all the proteolipid protein while its association with the largest amount of glycolipid was maintained.

Analysis after successive precipitations (Fig. 1) showed that changes were minor after the fourth, al-

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though the phosphorus and glycolipid contents apparently continued to decrease slowly at a constant rate.

Discussion. The purpose of this study was to establish conditions for obtaining a reproducible preparation of brain proteolipid suitable for immunological study. The preparation described satisfies the requirement of reproducibility reasonably well, since noncovalent binding of lipid to protein in this complex does not permit the application of the criterion of constancy of composition normally associated with chemical compounds and more specific complexes. Variation in the final products obtained from different animals exceeds that of final products obtained from the same pool of tissue, which suggests that at least some of the variation is attributable to the biological state of the tissue. The rest is due to the manipulation of the complex during isolation, particularly the reprecipitation from chloroform–methanol solution with acetone. The conditions described appear to be sufficiently gentle to reduce to a minimum the changes that occur during this step. Analysis of the lipid components by thin-layer chromatography on Silica Gel H showed that successive precipitations produced a considerable increase in the ratio of phrenosine to cerasine and the loss of a considerable portion of the sulphatide. Ethanolamine glycerophosphatides progressively decreased, while phosphatidyl choline, sphingomyelin, and minor phospholipids were relatively constant. It is clear that the lipids in proteolipid are bound with varying degrees of firmness. The preparations described here correspond more closely to the proteolipid B described by Folch and Lees (1) than to those previously designated A and C. They have a somewhat higher concentration of lipid, and this is mainly glycolipid. Variations in phosphorus content exceed those in glycolipid content (preparations 1–4), but the reason for this is not clear. Extraction methods indicate that some phosphorus that is tightly bound may be covalently linked to the amino acid residues of the protein or be present as so-called phosphatidopeptide.

We believe that the optical clarity of solutions of these proteolipids in chloroform–methanol (without addition of water) is an important criterion in judging their quality. In this respect they represent, in our experience, a considerable improvement over preparations of proteolipid previously described. Despite the clarity of these solutions, attempts to evaluate protein content by optical absorption at 280 mμ proved to be less satisfactory than the Lowry method. The absorption values at 280 mμ with a given preparation on different occasions showed variations that possibly reflect differences in degree of exposure of the chromophores.

Studies of the immunological properties of proteolipid are in progress.

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