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RAPID PURIFICATION OF PROTEOLIPIDS FROM RAT BRAIN SUBCELLULAR FRACTIONS BY CHROMATOGRAPHY ON A LIPOPHILIC DEXTRAN GEL

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SUMMARY

Proteolipids from adult rat brain subcellular fractions were purified by a one-step procedure involving chromatography through Sephadex LH-60 eluted with an acidified chloroform—methanol mixture.

The protein peak was eluted with the void volume and was free of adventitious lipids. The degree of purification was similar to that attained with the neutral—acidified chloroform—methanol dialysis method with the advantage that this new procedure can be carried out in only 3 h, with a recovery of proteins of 95—100%. Samples containing different lipid/protein ratios passed through the gel gave similar elution profiles.

When labeled amino acids or palmitic acid were added to myelin total lipid extracts, no radioactivity was eluted with the protein, indicating that the proteolipid apoproteins purified by this method do not adsorb hydrophobic low-molecular-weight compounds.

INTRODUCTION

Proteolipids, first described by Folch and Lees [1], are hydrophobic proteins present in different types of cellular membranes. They are operationally defined as hydrophobic lipoprotein complexes soluble in chloroform—methanol (2 : 1) and insoluble in water. Their apoproteins are also soluble in organic solvent mixtures and can be transferred to aqueous solutions by appropriate procedures.

They are abundant in white matter of the central nervous system, constituting one of the major protein components of the myelin membranes [2]. Peripheral nerve myelin, on the other hand, contains small amounts of proteolipids while grey matter and non-neural tissues contain much smaller amounts than white matter.

Non-myelin proteolipids have been implicated in calcium transport in

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the sarcoplasmic reticulum [3], and in proton transport in bacteria [4] and in mitochondria [5]. Nerve ending proteolipids have been extensively studied by the group of De Robertis [6].

The purification of these hydrophobic proteins, especially the purification of myelin proteolipid, has been the subject of numerous publications describing several procedures such as emulsion-centrifugation [7], precipitation with organic solvents [8], dialysis against neutral chloroform—methanol and acidic chloroform—methanol [9], and chromatographic methods using silicic acid [10], polystyrene gels [11], Sephadex LH-20 [12], Bio-Gel P-10 [13], DEAE-cellulose [14], Dowex resins [15], etc. All these procedures have some drawbacks and only by using neutral and acid dialysis can highly delipidized preparations be obtained. This procedure, however, is time consuming and produces loss of protein material [9]. Effective, rapid and quantitative methods are, up to now, almost absent in the literature.

In this paper, we present a method of purification that involves the use of gel chromatography through Sephadex LH-60, which is very quick (2—3 h), permits almost 100% recovery of the protein material, and furnishes a final product of similar purity to that obtained with the method of Tennenbaum and Folch-Pi [9].

MATERIALS AND METHODS

Preparation of subcellular fractions

Wistar rats, 35—40 days old, of either sex were used throughout. Animals were killed by decapitation and the forebrains rapidly removed and placed in the cold. Subcellular fractions were obtained using the method of De Robertis et al [16] except that the crude mitochondria were subfractionated on a discontinuous gradient of 0.8 *M*, 1.0 *M* and 1.25 *M* sucrose. The layer floating at the 0.32—0.8 *M* interface (fraction A) is myelin; the 0.8—1.0 *M* interface is fraction B; the 1.0—1.25 *M* layer contains synaptosomes (C) and the pellet (D) mitochondria. The layers were carefully removed, diluted with 0.25 *M* sucrose to approximately 0.3—0.4 *M* sucrose and centrifuged at 100,000 *g* for 30 min. The pellet from fraction A (myelin) was further purified according to the procedure of Norton and Poduslo [17]. Microsomes were obtained by centrifugation of the post-mitochondrial supernatant at 100,000 *g* for 60 min, and purified nuclei were prepared from an aliquot of the initial homogenate as described by Krawiec et al. [18]. All the pellets were resuspended in a suitable volume of water for lipid extraction and chemical determinations.

Preparation of proteolipids

Crude total lipid extracts were obtained from an aliquot of the initial homogenate and from the resuspended pellets of the subcellular fractions by the procedure of Folch and Lees [1]. In order to precipitate the basic proteins, 0.05 volume of 0.1 *M* KCl was added to the crude lipid extract, the mixture being left in the cold overnight [19]. After the addition of a few drops of methanol, the precipitate was removed by filtration and the lipid extracts, free of basic proteins, were washed once with water and twice with

the theoretical upper phase (chloroform—methanol—water, 3 : 48 : 47) according to the description of Folch et al. [20]. The washed lower phases (TLE) were concentrated about ten-fold, after the addition of 0.5 volume of chloroform, by evaporating at 30°C under vacuum. To precipitate proteolipids, 5 volumes of acetone were added to the concentrated solutions and the mixture was left at —15°C for 2 h. The precipitate formed (crude proteolipid) was separated by centrifugation in the cold at 4000 *g* for 20 min. The pellets were dried under a stream of nitrogen and dissolved in chloroform—methanol (1 : 1, v/v) containing 5% 0.1 *N* HCl to obtain a protein concentration between 2 and 4 mg/ml. In some experiments acetone precipitation was omitted; instead, the TLEs were carefully concentrated under vacuum and chloroform—methanol (1 : 1, v/v) containing 5% 0.1 *N* HCl (approximately 3 ml per ml of concentrated TLE) was added. The results obtained using either procedure were similar.

Preparation of Sephadex columns

Prior to column packing the Sephadex LH-60 was washed two times with distilled water and two times with acetone through a fritted flass filter under vacuum to remove fines. The gel was then suspended in chloroform—methanol (1 : 1) containing 5% 0.1 *N* HCl and washed three or four times with this mixture. This type of Sephadex must remain in the solvent mixture for at least 3 h at room temperature for complete swelling. Glass columns measuring 40 × 1.8 cm, fitted with a porous glass plate were used in all cases. The gel slurry was carefully poured into the column and the solvent allowed to drain slowly. Four volumes of the solvent mixture were passed through the column, prior to chromatography, in order to eliminate traces of acetone. A similar procedure and column design were used to prepare the Sephadex LH-20 columns.

Chromatography

Crude proteolipid solutions with a volume smaller than 5 ml and containing between 2 and 12 mg of protein were applied to the Sephadex LH-60 or LH-20 column and eluted with chloroform—methanol (1 : 1) containing 5% 0.1 *N* HCl at room temperature (16—22°C) at a flow-rate of 0.3 ml/min; 3-ml fractions were collected, aliquots of which were used for chemical determinations or radioactivity counting.

Chemical procedures and radioactivity counting

Protein was assayed by the method of Lowry et al. [21] as modified by Hess and Lewin [22] using bovine serum albumin as standard. Total cholesterol, lipid phosphorus and galactose were measured by the procedures described by Searcy and Bergquist [23], Chen et al. [24] and Hess and Lewin [22], respectively.

Suitable aliquots from each eluted fraction were evaporated to dryness and counted in a Packard Tri Carb scintillation counter using 10 ml of counting solution containing 5.5% of 2,5-diphenyloxazole and 0.1% of 1,4-bis(5-phenyl-oxazol-2-yl)benzene and Triton—toluene (80 : 20, v/v). Thin-layer chromatography (TLC) of lipids was carried out on 20 × 20 cm silica gel H glass plates [25]. Individual lipids were detected with iodine vapour.

Chemicals and reagents

All the chemicals were reagent grade and the solvents were redistilled before use. Sephadex LH-20 and LH-60 were purchased from Pharmacia (Uppsala, Sweden). L-[4,5-^{3}H]Leucine (55 Ci/mmol), [U-^{14}C]L-phenylalanine (406 mCi/mmol), and [9,10-^{3}H]palmitic acid (17 Ci/mmol) were obtained from New England Nuclear (Boston, MA, U.S.A.).

RESULTS

As shown in Table I, proteolipid proteins are present in all subcellular membranes, except in the soluble fraction. Very small amounts are found in purified nuclei, in agreement with results previously reported for mouse brain by Nussbaum and Mandel [26], while the microsomal fraction contains a higher amount of these proteins. Proteolipid proteins are concentrated in myelin and in fraction B, which together with those present in mitochondria and in the synaptosomal fraction represent 90% of all the proteolipid proteins found in the brain.

TABLE I

SUBCELLULAR DISTRIBUTION OF PROTEOLIPID PROTEINS

Fraction	Morphological constitution	Total protein (mg/g FT)*	Proteolipid proteins		
			mg/g FT*	SC**	RSC***
Total homogenate	—	117.4	3.610	0.031	1
Purified nuclei	—	1.2	0.051	0.042	1.35
A	Myelin fragments	4.6	1.760	0.383	12.35
B	Curved membranes, vesicles and myelin fragments	7.8	0.510	0.065	2.10
C	Nerve endings	7.3	0.410	0.056	1.80
D	Mitochondria	11.5	0.480	0.042	1.35
Microsomes	Curved membranes, vesicles, rough and smooth ribosomes	19.4	0.300	0.016	0.51
Soluble	—	16.9	ND§	ND	ND

*FT = fresh tissue.
**SC (specific concentration) = mg proteolipid protein per mg total protein.
***RSC (relative specific concentration) was estimated as the ratio between the specific concentration in the fraction and that in the total homogenate.
§ND = not detectable.

Fig. 1 shows the chromatographic separation of the protein from the contaminating lipids obtained with a column of Sephadex LH-20. Although an acceptable separation is obtained, traces of lipids are eluted together with the proteins, one of the main lipid contaminants being sphingomyelin, as shown by TLC of fractions 13—14 (Fig. 1B). Similar results were presented by Gagnon et al. [12], who purified human myelin proteolipids by gel chromatography in a similar column using acidified chloroform—methanol. Only

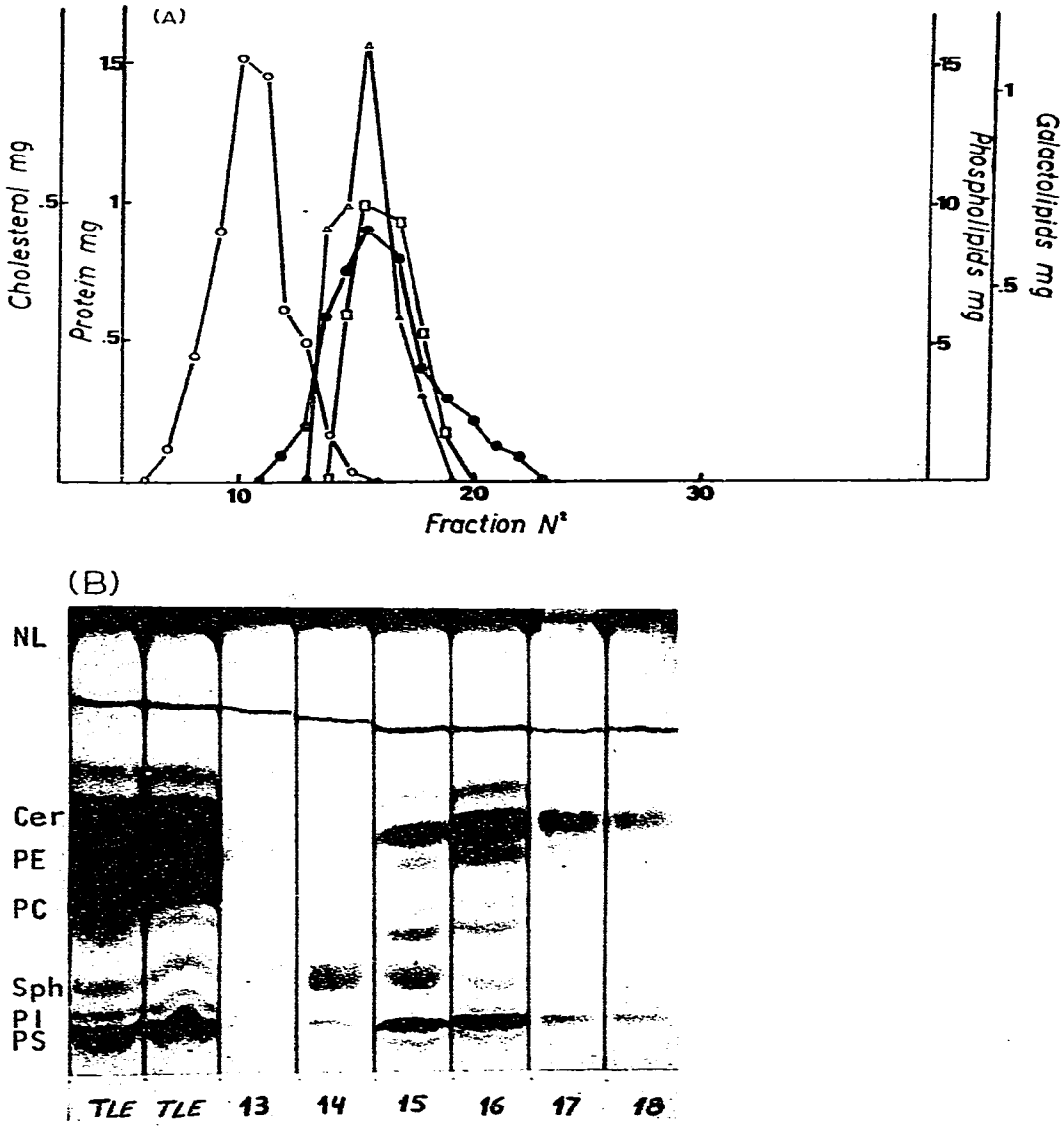
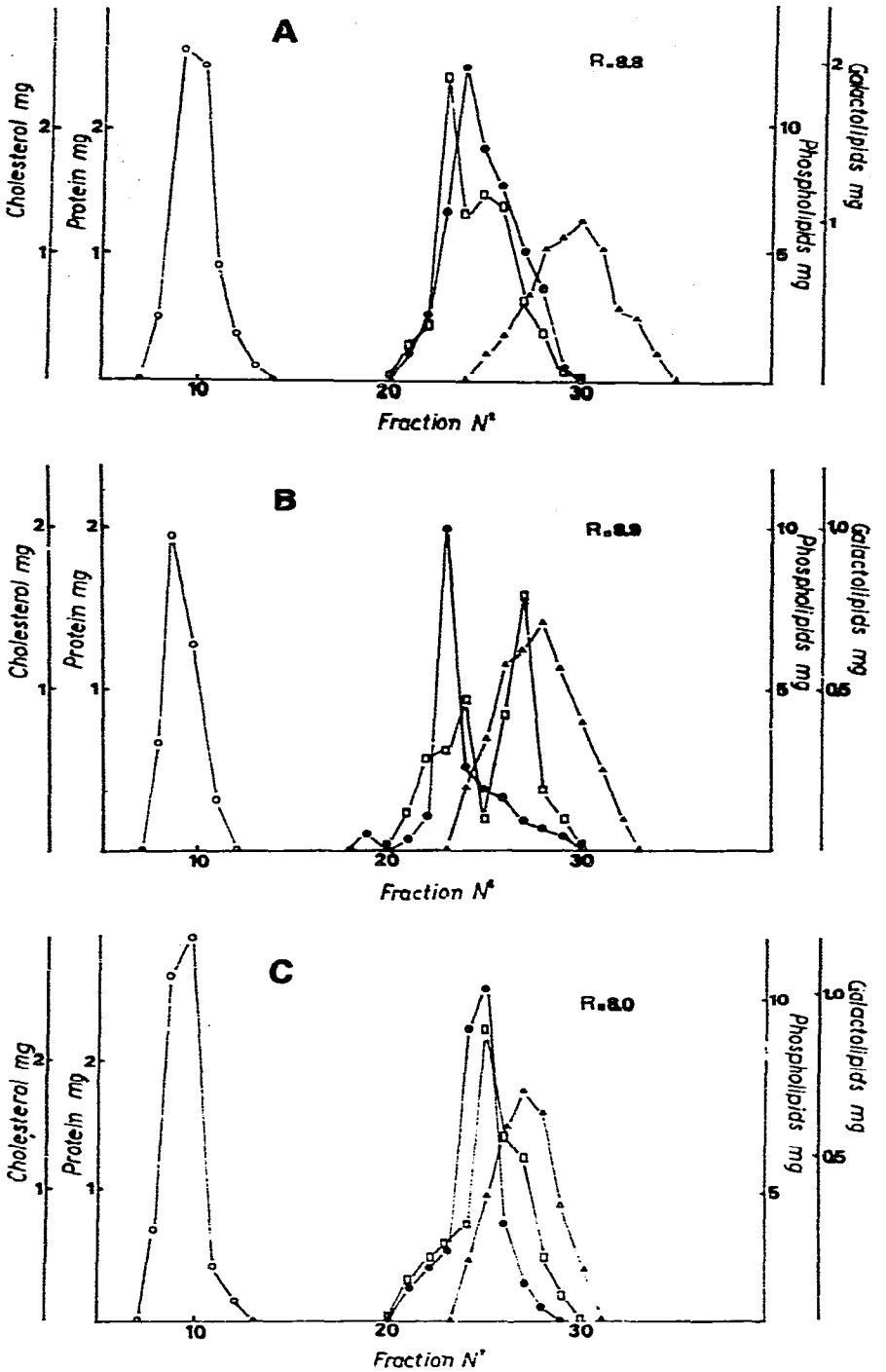


Fig. 1. (A) Purification of total rat brain proteolipids by chromatography on Sephadex LH-20. (○), Protein; (●), phospholipids; (△), cholesterol; (□), galactolipids. (B) Thin-layer chromatograms of the fractions containing lipids. PS = phosphatidyl serine; PI = phosphatidylinositol; Sph = sphingomyelin; PC = phosphatidylcholine; PE = phosphatidylethanolamine; Cer = cerebrosides; NL = neutral lipids.

by repeated chromatography was it possible to obtain a pure material, and protein losses could also occur during this time-consuming procedure. In view of these results, we decided to try a similar methodology but using Sephadex LH-60.

The pattern obtained with rat brain TLE as well as with TLE from different subcellular fractions eluted through Sephadex LH-60 are shown in Fig. 2. The profiles are similar both for lipids and proteins in all membrane frac-


A
R.s.s
Cholesterol mg
Protein mg
Phospholipids mg
Galactolipids mg
Fraction N°
B
R.s.s
Cholesterol mg
Protein mg
Phospholipids mg
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C
R.s.o
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Fig. 2.

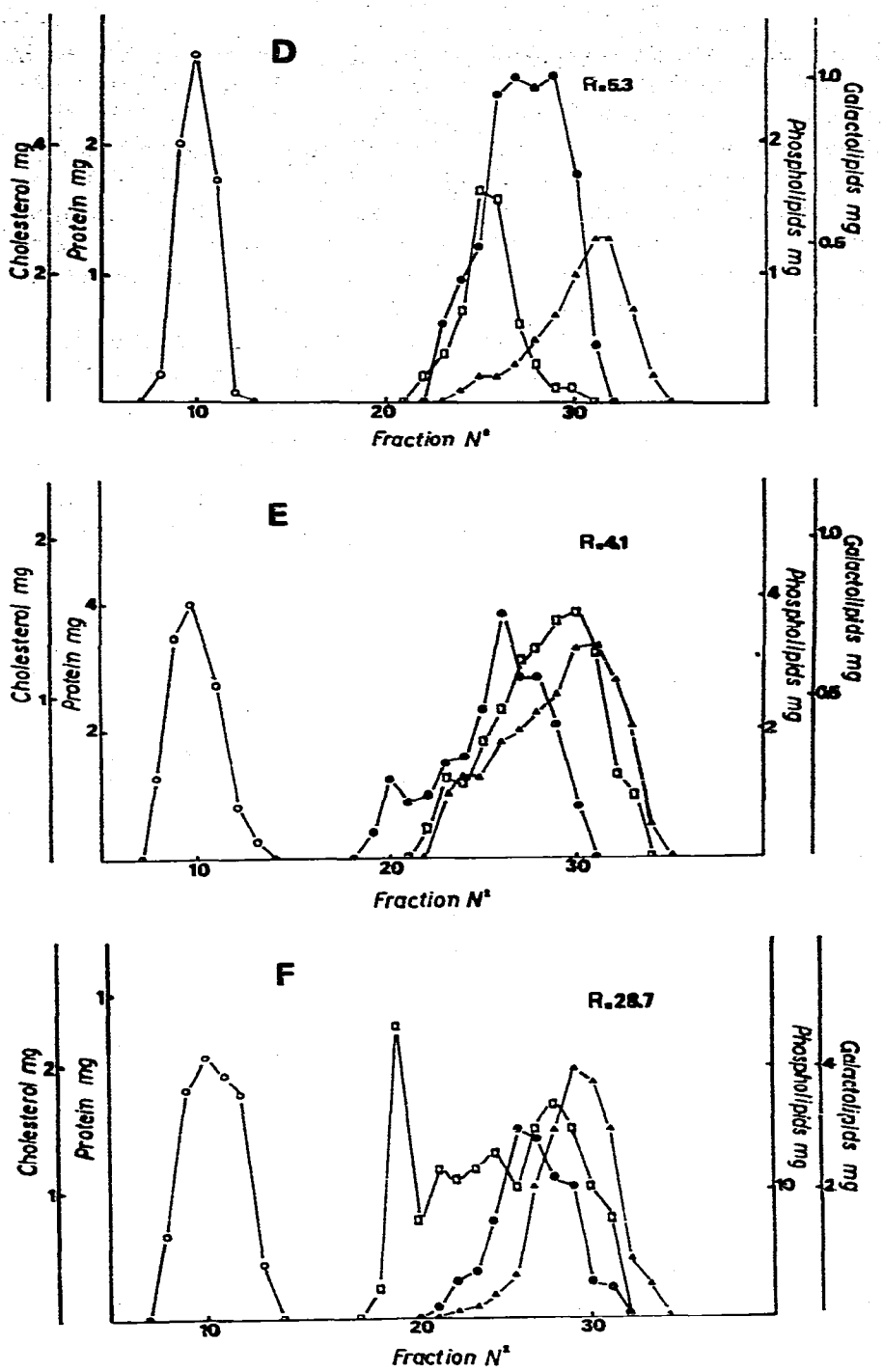

Fig. 2. Purification of proteolipids from rat brain subcellular fractions by chromatography on Sephadex LH-60: (A) whole brain; (B) myelin; (C) fraction B; (D) synaptosomes; (E) mitochondria; (F) microsomes. (○), Protein; (△), cholesterol; (□), galactolipids; (●), phospholipids. R represents the ratio between total lipids and total proteolipid proteins loaded on to the column.

tions. Furthermore they are independent of the lipid/protein ratio present in the samples applied to the column. The protein peak, representing 90—95% of the protein present in the original sample, is eluted within the theoretical void volume and in a very small volume (4—5 tubes). Contrary to what was found using chromatography on LH-20 no lipids co-eluting with the proteins were detected by chemical analysis. Five or six tubes containing no detectable material were collected at the end of the protein peak and before the appearance of the peak containing various lipids, which are eluted in a wide range of tubes, cholesterol being the last to appear.

When the peaks containing the proteins or the lipids were independently re-chromatographed through Sephadex LH-60 using the same solvent mixture, the corresponding materials appeared in their original elution positions. The tubes containing proteins were pooled and concentrated by evaporation under nitrogen in order to perform chemical studies to determine the amount of lipids with reference to protein present in this purified preparation. Lipid phosphorus was found to be 0.035% by weight while galactose and cholesterol were absent. For purposes of comparison, we include in Table II results obtained by other investigators using well-known procedures for the preparation of proteolipid apoproteins. The degree of purification with our method can be compared only with that obtained by extensive dialysis against neutral and acid chloroform—methanol as described by Folch-Pi and Stoffyn [27]. Furthermore, the purified apoproteins can be obtained in only 2—3 h, while the procedure of Folch-Pi and Stoffyn takes at least 14 days. Another important point to be stressed is that there is very little loss of protein material while, as shown by Folch-Pi [28], small peptides are lost during dialysis.

TABLE II

AVERAGE COMPOSITION OF PURIFIED PROTEOLIPID APOPROTEINS OBTAINED FROM BOVINE WHITE MATTER AND MYELIN BY DIFFERENT PROCEDURES

All data are expressed as percentage by weight.

Procedure	Protein	Lipid phosphorus	Galactolipids	cholesterol	$E^{278}_{1\%}$
Emulsion-centrifugation [7]					
Crude proteolipid	30—35	1.0—1.2	5—13	7	6—8
Concentrated	55—60	1.0	3—7	1	9—10
Sephadex LH-20 [15]	75	0.68	10.6	—	13.2
Sephadex LH-20 + Dowex 1-X2 [15]	94	0.074	2	—	16.3
Dialysis against chloroform—methanol [36]	70—85	0.4—1	2.5	0.2	10.5—13.5
Dialysis against chloroform—methanol + acidified chloroform—methanol [27]	99—100	0.04	ND*	ND	16—22
Our preparation (for myelin proteolipid apoprotein)	99—100	0.035	ND	ND	13—14

*ND = not detectable.

It is well known that proteolipid proteins in neutral chloroform—methanol adsorb hydrophobic and aromatic amino acids [29], particularly phenylalanine, and that these amino acids are retained by the protein, even after extensive dialysis. In order to ascertain if free amino acids were co-eluted together with the protein, 20 μCi of [^{3}H]leucine and 5 μCi of [^{14}C]phenylalanine were added to the TLEs, which were subsequently chromatographed as described above. Neither ^{3}H nor ^{14}C was detected in the peak corresponding to protein (Fig. 3A). Recovery of the amino acids was approximately 100%.

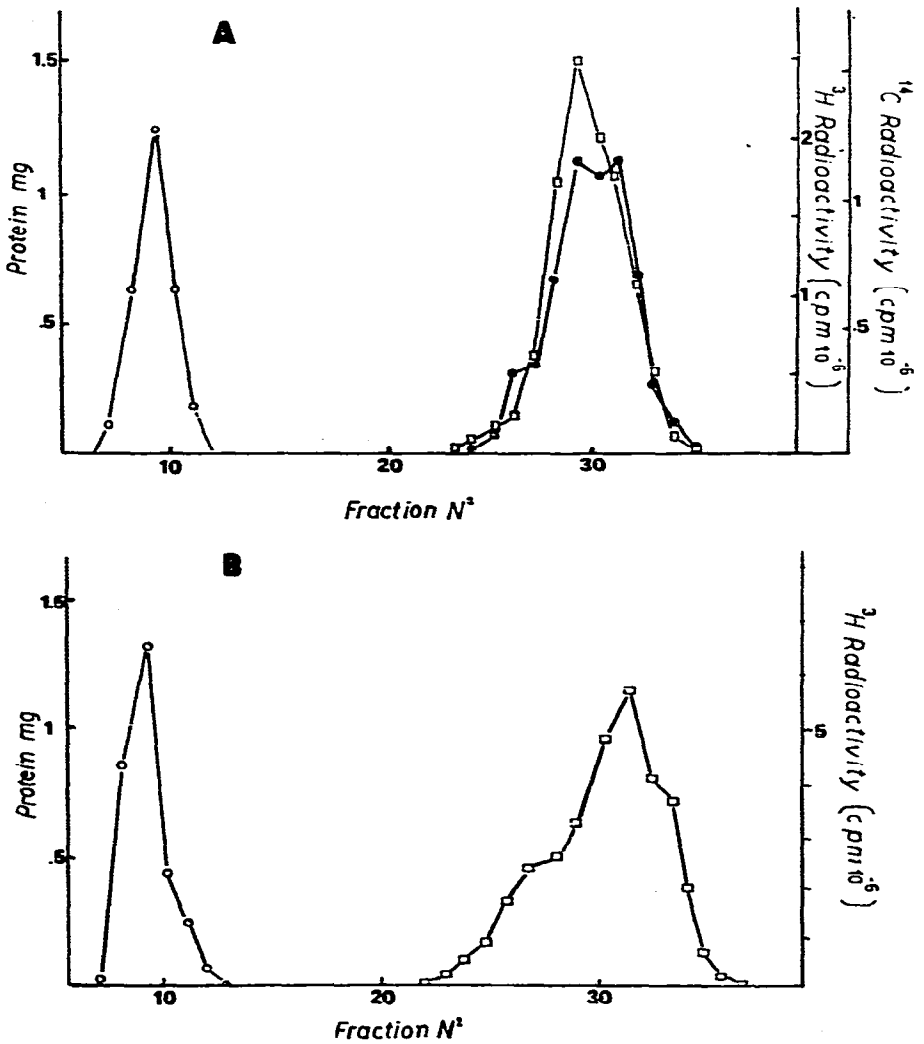

Fig. 3. Column chromatography of myelin TLE on Sephadex LH-60 in the presence of externally added radioactive amino acids or palmitic acid. (A) [^{14}C]Phenylalanine and [^{3}H]leucine were added to TLE containing myelin proteolipids prior to chromatography (see Results). (○), Protein; (□), ^{3}H radioactivity; (●), ^{14}C radioactivity. (B) [^{3}H]Palmitic acid was added to TLE containing myelin proteolipids prior to chromatography (see Results). (○), Protein; (□), ^{3}H radioactivity.

Adsorption of fatty acids to proteolipid apoproteins was also measured as described above, 50 μCi of [^{3}H]palmitic acid being added prior to chromatography (Fig. 3B). The results were similar to those obtained with amino acids.

Using a column of the size mentioned above, the flow-rate can be increased to 0.7 ml/min (if the sample volume and the amount of protein loaded are not changed) without changing the effectivity of the method. If the flow-rate is kept at 0.3 ml/min, the amount of protein loaded can be increased to 30 mg, provided that the sample volume does not exceed 5 ml. This volume, however, can be increased to 8 ml if the flow-rate and amount of protein loaded are maintained within the values mentioned under Materials and Methods.

DISCUSSION

Mokrasch [15] was the first to use hydroxypropyl derivatives of Sephadex G-25 (Sephadex LH-20) for the chromatographic purification of proteolipids. The protein, eluted with the void volume of the column, was re-chromatographed through another column containing Dowex 1-X2, in order to obtain maximal delipidization. Recovery, however, was low and the final product was unstable. In 1969, Soto et al. [30] used a combination of gel chromatography and partition chromatography in Sephadex LH-20 for the fractionation and partial purification of grey and white matter proteolipids. A complete purification of proteolipids from human myelin was obtained by repeated gel chromatography on Sephadex LH-20 eluted with acidified chloroform—methanol [12]. This method has been widely used for the purification of proteolipids by several investigators [31]. Using this solvent, the lipids bound by strong electrostatic interactions are separated from the apoprotein and are eluted according to their molecular weights. If the acid is omitted, the protein eluted from the column is contaminated with acidic lipids while zwitterionic and neutral lipids are separated, the product obtained being very similar to that obtained after extensive neutral dialysis.

If water is omitted from the solvent mixture, or if the chloroform/methanol ratio is increased, recovery of the protein diminishes and partition effects occur.

We thought that Sephadex LH-60 could be a better matrix than Sephadex LH-20 for the separation of the proteins and the lipids and decided to use an acid mixture of organic solvents in order to be sure that gel filtration would occur. Only two problems could invalidate our method: (1) that the matrix of Sephadex LH-60 would adsorb proteolipids, and (2) that the protein could be enclosed within the fractionation range, since Sephadex LH-60, using organic solvents, has an exclusion limit of 15,000. None of these problems occurred since 90—95% of the protein was recovered in a single peak eluted with the void volume.

Fillingame [5] was the first to use hydroxypropyl Sephadex G-50 eluted with chloroform—methanol (2 : 1) for the purification of dicyclohexylcarbodiimide-proteolipid subunits of *Escherichia coli*, obtained a low-molecular-weight protein of 8400 in the void volume. Lees and Macklin [32] in 1972 used Sephadex LH-60 for the separation of the low-molceular-weight hydro-

phobic peptides obtained from bovine white matter proteolipids after digestion with elastase. Lees et al. [33] recently communicated that bovine white matter proteolipids could be fractionated into two peaks by chromatography through Sephadex LH-60 eluted with chloroform—methanol—acetic acid. The protein peak appearing at the void volume contained 25,000 and 20,000 proteolipids, and the peak enclosed within the gel fractionation range 13,000 proteolipid. With our method, and using the solvent mixture proposed, this fractionation does not occur. Gel electrophoresis of the protein peak obtained from a TLE from rat brain shows several bands corresponding to molecular weights ranging between 8000 and 60,000. It is evident that the range of fractionation of Sephadex LH-60, as happens with Sephadex LH-20, is reduced in organic media as compared to aqueous media. Also, bovine white matter proteolipid apoproteins have been shown to be asymmetric [34]. The combination of these two factors is a possible explanation of why proteolipid proteins with relatively low molecular weights (> 8000) are eluted at the void volume.

We have used this procedure for the preparation of the proteolipid apoproteins from different subcellular fractions, which we have analyzed by sodium dodecyl sulphate (SDS) and SDS—urea polyacrylamide gel electrophoresis system, in order to calculate the correct molecular weights using the Ferguson relationship [35], with excellent results.

Since we have demonstrated that the apoproteins obtained by this simple method do not adsorb amino acids, fatty acids or any other lipids, we are currently using the method described above for sample purification of proteolipid proteins from myelin, total homogenate and brain microsomes to study turnover rates of proteolipid apoproteins and of their covalently bound fatty acids.

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