CHROM. 4001

FRACTIONATION OF LIPIDS AND PROTEOLIPIDS FROM CAT GREY AND WHITE MATTER BY CHROMATOGRAPHY ON AN ORGANOPHILIC DEXTRAN GEL*

EDUARDO F. SOTO, JUANA M. PASQUINI, RENÉE PLÁCIDO AND JOSÉ L. LA TORRE

Instituto de Anatomía General y Embriología, Facultad de Medicina and Departamento de Química Biológica, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires (Argentina)

(Received February 11th, 1969)

SUMMARY

A method for the fractionation of proteolipids extracted from grey and white matter, involving two successive columns of Sephadex LH-20, is described.

In the first column the total lipid extract (TLE) was eluted with chloroform–methanol (19:1), and in the second, pure chloroform was followed by solvent mixtures of increasing polarity. Ether precipitated TLEs were eluted through the second column.

Two fractions of proteolipids were obtained, one being eluted by a gel filtration mechanism and the other by partition chromatography. Different patterns of elution for grey and white matter extracts were found. The implications of these findings are discussed in relation to the chemical and physicochemical characteristics of the different proteolipids and to the receptor properties shown by some of them in grey matter.

INTRODUCTION

Proteolipids are a group of lipoproteins described by FOLCH-PI AND LEES[1]. They occur in brain and other tissues and have the unusual property of being soluble in chloroform–methanol mixtures but insoluble in water.

Since their discovery, they have been found to be an important component of biological membranes[2]. Myelin isolated from bovine white matter was found to be rich in proteolipids[3], but other subcellular fractions also contain different amounts o these compounds[4,5]. Multilamellar structures (*e.g.* mitochondria) have been found to be especially rich in proteolipids[6]. Their relation to classical neurokeratin has been

* This work was supported by Grant 456-B from the National Multiple Sclerosis Society, U.S.A.

pointed out by LE BARON[7] and their possible presence at the subsynaptic junction has been studied by LAPETINA AND DE ROBERTIS[8].

Recent work at this laboratory suggests that the capacity of the junctional complex to bind dimethyltubocurarine (DMTC) is related to a protein having the solubility properties of proteolipids[9].

Several methods for the isolation and purification of these lipoproteins and studies of their physical-chemical properties have been described in recent years[1,10–16].

The use of Sephadex G-25 columns by WELLS AND DITTMER[17] for the chromatographic separation of lipid components of the nervous tissue led us to use it for the study of proteolipids. Because of the recent availability of a hydroxypropyl derivative of Sephadex G-25, for use especially with organic solvents (Sephadex LH-20) we selected this product for our studies. MOCKRASCH[18] has recently described the use of Sephadex LH-20 for the rapid purification of proteolipids.

The present study was designed to obtain a better concept of proteolipid properties. We will show that a Sephadex LH-20 column gives a consistent fractionation of these unusual lipoproteins, and that the patterns for grey and white matter, respectively, substantially differ from one another.

METHODS AND MATERIALS

Preparation of total lipid extracts (TLE)

Redistilled solvents and analytical reagents were used throughout. Grey and white matter were obtained from cat brain by careful dissection at 4°. The tissue was homogenized in distilled water (10% w/v) in a Porter-Evelhjem type homogenizer fitted with a Teflon plunger, and the homogenate sedimented at 100,000 × *g* in the Spinco L Ultracentrifuge for ½ h to obtain a total particulate (TP).

TP was extracted with chloroform–methanol (2:1) (19 ml of chloroform–methanol per ml of TP) and the extract passed through filter paper.

The crude extract was washed once with water and twice with the "theoretical" upper phase (chloroform–methanol–water, 3:48:47)[19], and the resulting lower phase was cleared by the addition of a few drops of methanol.

Preparation of Sephadex LH-20 columns

Sephadex LH-20 (Pharmacia, Uppsala) was washed prior to column packing to remove soluble material as well as fines. The gel was washed five times with distilled water and five times with acetone through a 2 l fritted glass filter under vacuum. Resuspension of the gel after each washing was done by careful stirring. After the last acetone wash the "gel cake" was left on the filter until most of the acetone had evaporated. It was then spread on a filter paper and left overnight at room temperature. 12–15 g of washed gel were suspended in chloroform–methanol (1:1) in a filtering flask and deaereated for 5 min under vacuum with gentle rotation. The deaereated gel was stored in the same flask for the preparation of other columns. The gel must remain in the chloroform–methanol (1:1) mixture for at least 3 h, to complete swelling.

Column packing

Columns measuring 1.8 × 30 cm, with a porous glass plate were used in all cases. A Teflon stopcock with a 20 gauge needle was fitted to the lower end of the

column and the needle was connected to a fraction collector by means of 2 mm bore Teflon tubing.

The gel slurry was poured into the column and the liquid allowed to drain very slowly. Chloroform–methanol (1:1) was added to the top of the column and passed through the column until there was complete packing. The height of the column was adjusted to 20 cm by aspirating off any excess gel and a 2 cm layer of sand was placed on top. (This was necessary to prevent flotation of the Sephadex during the passage of chloroform or chloroform–methanol mixtures.)

TABLE I

COMPOSITION OF ETHER PRECIPITATES FROM GREY AND WHITE MATTER TLEs

Results are expressed as % of amount present in the original TLE.

	Grey matter	*White matter*
Protein	50–65	95–100
Cholesterol	traces	traces
Lipid phosphorus	12–18	30–40
Cerebrosides	45–55	50–65

Once packed, the column was thoroughly washed with chloroform–methanol (4:1) and finally equilibrated with chloroform–methanol (19:1); or with pure chloroform, depending upon the final use of the column.

Precipitation of TLE with ethyl ether

An aliquot of TLE was placed in a flask in an ice bath and 4 volumes of cold ethyl ether were added slowly. This was vigorously stirred for 20 min. The opalescent mixture was centrifuged for 15 min at 2000 × *g* and the supernatant was decanted. The ether in the precipitate was allowed to evaporate for a few minutes and 1–2 ml of chloroform–methanol (2:1) were added.

Complete dissolution was generally obtained without further treatment. The supernatant remaining after ether precipitation was kept and used for the experiments described below.

The composition of the precipitate obtained from grey and white matter TLEs is shown in Table I.

Chromatography

Chloroform, ½ volume, was added per aliquot of TLE containing approx. 2–4 mg of proteolipid protein. A few drops of methanol were sometimes necessary to clear the extract after the addition of chloroform.

The extract was concentrated at 30° under vacuum to 1–2 ml. The excess solvent above the sand layer on the column was removed completely, the sample was introduced into the column, and elution started.

Two columns were used in succession in our standard procedure for the chromatography of TLE. In the first one (A), the sample was eluted with 80 ml of chloroform–methanol (19:1). The effluent was then evaporated to 2–3 ml and the concentrated sample was placed on the second column (B) and eluted with the following solvent sequence: 80 ml of chloroform; 20 ml of chloroform–methanol (15:1); 20 ml of chloro-

form–methanol (10:1); 20 ml of chloroform–methanol (6:1); and finally, 80 ml of chloroform–methanol (4:1).

Chromatographic separation of the ether precipitated material was carried out using column (B) only.

Aliquots of 2–4 ml were collected with a fraction collector, at 0.5 ml/min, and chemical analysis were performed on the contents of each tube.

Chemical procedures

Protein was determined by the method of LOWRY *et al.*[20], as modified by HESS AND LEWIN[21]. Most of the columns were monitored at 280 mμ, to check the appearance of protein, with a LKB Uvicord model II.

Total cholesterol was determined by SEARCY AND BERGQUIST'S method[22]; total lipid phosphorus according to CHEN *et al.*[23] and cerebrosides-sulfatides (glycolipids) according to FOLCH-PI AND GREANEY[24] with an orcinol–sulfuric acid reagent.

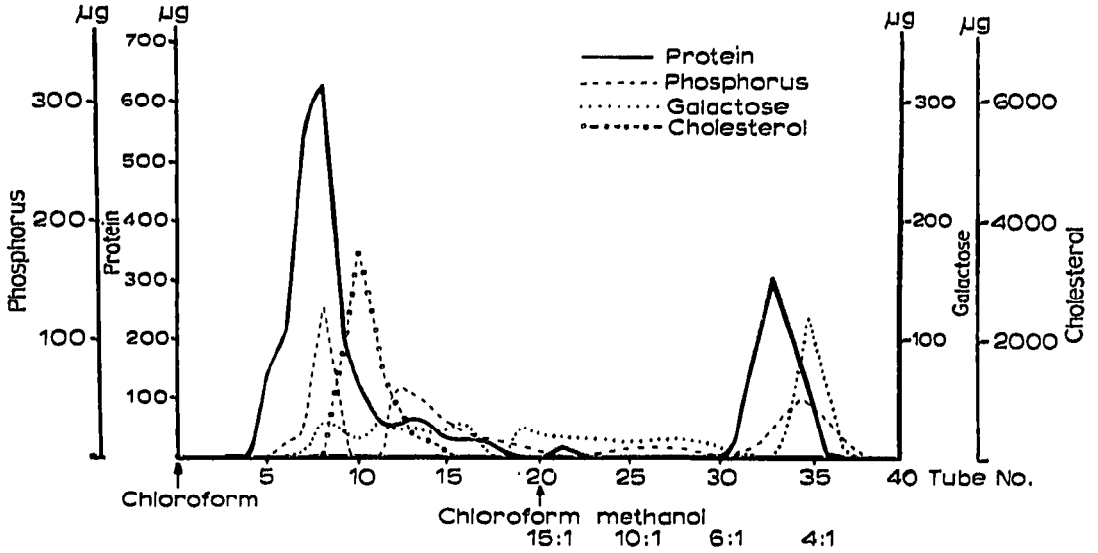

Fig. 1. Elution pattern of a TLE from grey matter passed through column (B). For details see text. Aliquots of 4 ml were collected in each tube.

RESULTS

Chromatography on column (A)

TLEs from grey and white matter passed through column (A) gave a single fraction eluted with the gel void volume, containing 85–90 % of all the constituents present in the original sample.

Chromatography on column (B)

The elution pattern of a TLE from grey matter is shown in Fig. 1. A protein peak which accounts for 60–70 % of the protein recovered is eluted with the gel void volume, together with 20–25 % of the lipid phosphorus. 100 % of the cholesterol and another 50 % of the lipid phosphorus comes out of the column immediately after the protein.

A second protein peak, corresponding to 30–40 % of the protein recovered, is eluted with the more polar solvent mixtures. This peak has no cholesterol but the remaining lipid phosphorus is eluted with it.

The pattern of elution of cerebrosides was not reproducible; in most of the experiments, however, they appeared to coincide with the lipid phosphorus. Recoveries were 80 % or better for all the constituents analyzed.

Fig. 2 shows the pattern given by white matter TLE. A small amount of protein, ranging between 8 and 20 % of the total protein recovered, is eluted with the void volume of the column, accompanied by 10 % of the lipid phosphorus. The cholesterol is eluted as a single fraction immediately after the first protein peak. The remainder of the protein, a complex fraction containing 4 or 5 different peaks, appears soon after the chloroform–methanol mixture is applied to the column. A third of these peaks carries along 80–90 % of the cerebrosides and 40–50 % of the lipid phosphorus. The fraction that contains the remaining 40–50 % of the lipid phosphorus is consistently eluted in two peaks, between tubes 10 and 25. Recoveries were 80 % or better for all the constituents analyzed.

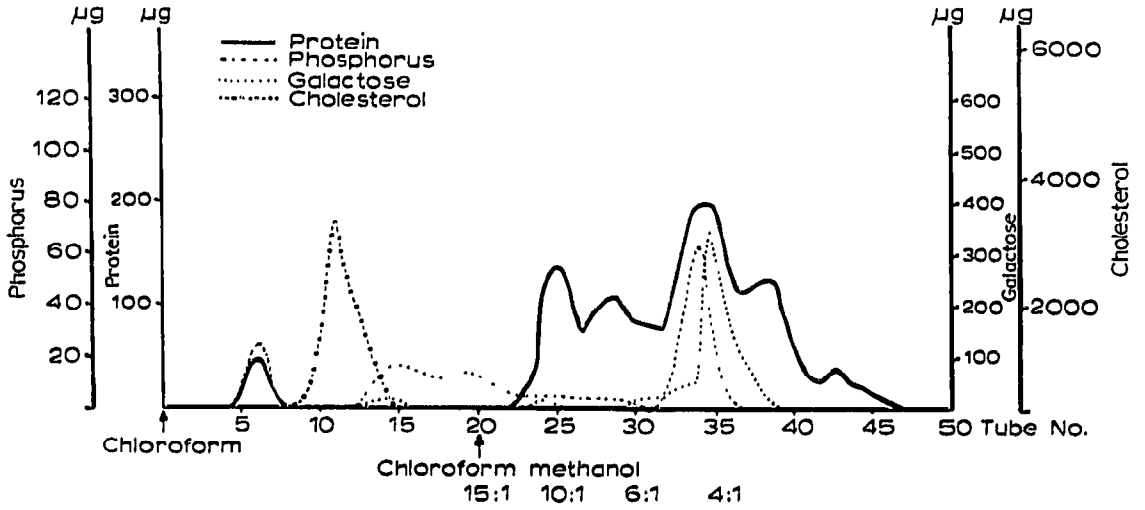

Fig. 2. Elution pattern of a TLE from white matter passed through column (B). For details see text. Aliquots of 4 ml were collected in each tube.

Aliquots of ether precipitated material that were redissolved in 1–2 ml of chloroform–methanol (2:1) were eluted from the standard column (B). The pattern obtained from ether precipitates of grey matter is shown in Fig. 3. Very little protein is eluted with the first three solvent mixtures but as soon as the chloroform–methanol (6:1) enters, a fraction appears which accounts for 30–50 % of the total proteolipid protein present in the original sample placed on the column. This fraction has two or three peaks; the first one contains almost 100 % of the lipid phosphorus and usually represents 18 % of the total protein. As stated above, only 30–50 % of the proteolipid protein applied to the column appears in the effluent, while the recovery for lipid phosphorus is 75 %.

Several experiments were carried out in order to determine the reasons for this retention of protein: an aliquot of the ether precipitate, in chloroform–methanol (2:1), was added to a dry aliquot of the ether supernatant which contained 10 % of the proteolipid protein, 100 % of the cholesterol, 75 % of the lipid phosphorus, and 50 % of the cerebrosides originally in the TLE. The "re-combined TLE" sample was eluted from column (B) and the results are shown in Fig. 4.

The pattern obtained was very similar to that given by a TLE which had not been precipitated with ether, although there was some overlapping of the two peaks, and 100 % of the protein was recovered.

The ether supernatant remaining after the TLE precipitation was also chromatographed. Although it contained a higher amount of cholesterol and lipid phosphorus in relation to the protein present, the pattern obtained was similar to that given by a TLE from grey matter.

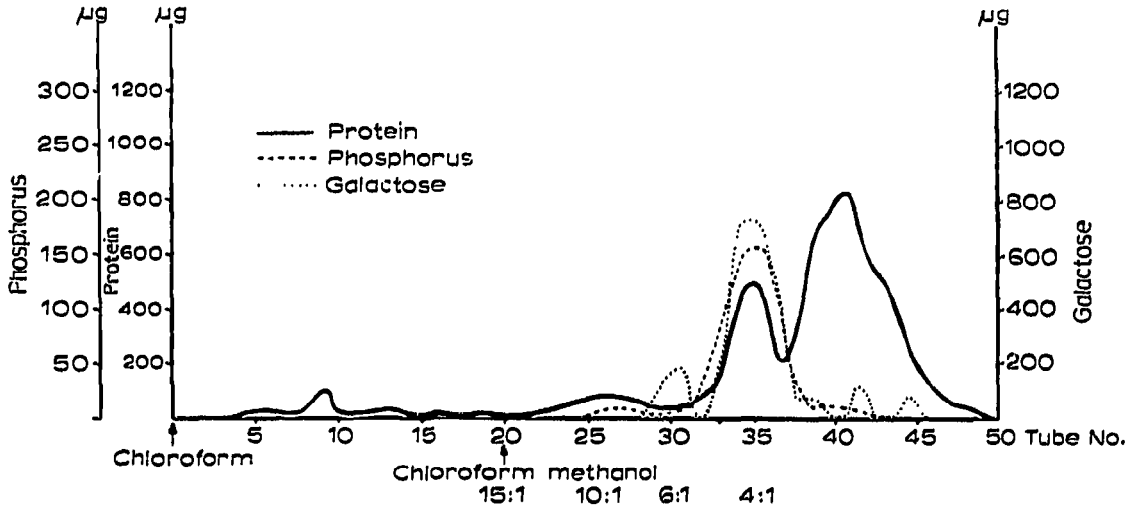

Fig. 3. Pattern of TLE from grey matter precipitated with ether and eluted through column (B). Aliquots of 4 ml were collected in each tube.

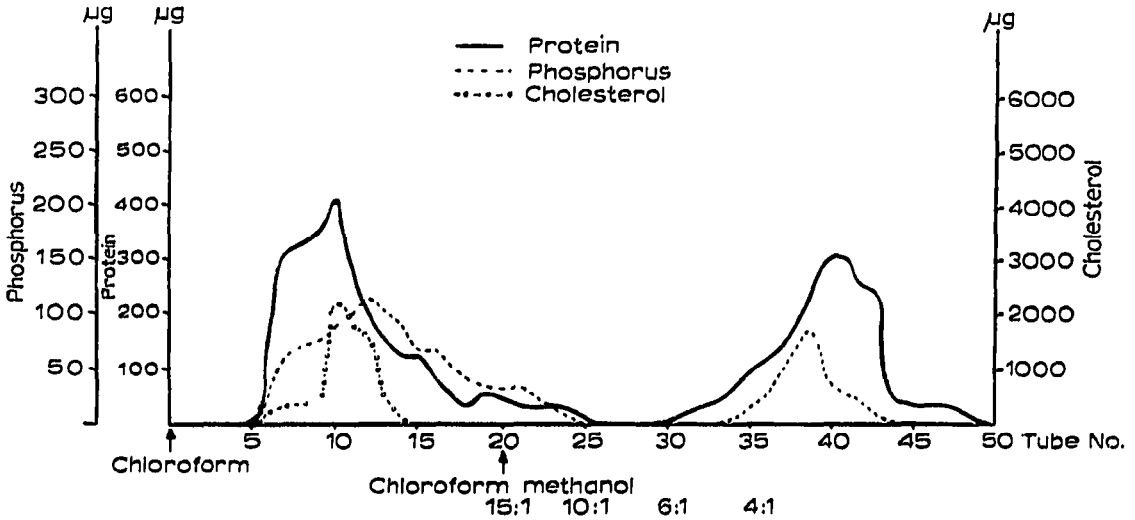

Fig. 4. Elution pattern of a "re-combined" TLE from grey matter. See details in the text. Aliquots of 4 ml were collected in each tube.

The elution pattern from columns containing ether precipitates of TLE from white matter is different from that of grey matter, as shown in Fig. 5.

Two protein fractions are eluted from the column. The first one has 30 % of the protein and coincides with a peak of lipid phosphorus which represents 70 % of the amount recovered. In addition, 70 % of the cerebrosides are eluted by the chloro-

form as a peak appearing immediately after that of the protein. The remaining protein is eluted in three peaks, the first one contains the rest of the cerebrosides plus most of the remaining lipid phosphorus. Recoveries were 75 % or better for all the constituents analyzed.

Re-chromatography

A few re-chromatography experiments were done with grey matter TLE. For this purpose both peak I and peak II from a standard column (B) were passed again, separately, through another column and eluted as usual. Peak II was always eluted in the same position (*i.e.* between tubes 40–50) and as a single fraction. On the other

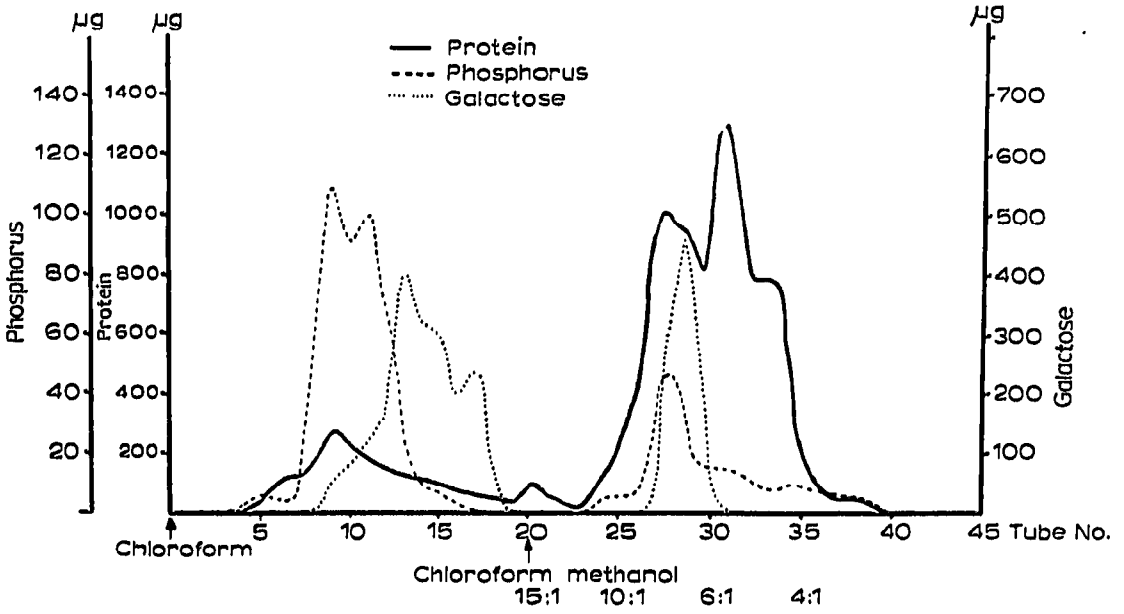

Fig. 5. Pattern of a TLE from white matter precipitated with ether and eluted through column (B). Aliquots of 5 ml were collected in each tube.

hand, peak I, in one experiment gave a single fraction appearing in the original position but in two other experiments it was eluted as two peaks, one appearing in the same position, and one coming out in the position of peak II. In these experiments, which showed further resolution of peak I, the amount of the total protein recovered in the original portion of peak I was 70 %, while 100 % of the cholesterol and 89 % of the total lipid phosphorus was also present in the original position. The remainder of the protein (30 % of the original peak I) appeared much later in the elution pattern.

DISCUSSION

We have shown that nerve ending membranes bind [^{14}C]DMTC and after extraction with chloroform–methanol (2:1) most of the radioactivity is recovered in the washed TLE[9,25]. This binding capacity is due to a protein having the solubility properties of a proteolipid. [^{14}C]DMTC binding is much greater in nerve ending membranes than in myelin. These results suggested that proteolipids from grey and white matter may be different.

There are several methods for the purification and fractionation of proteolipids from white matter which have not yet been used to study grey matter proteolipids [1,10,13,15,16]. The method of MATSUMOTO *et al.*[12] seemed to be adequate for the identification of the components involved in the binding of DMTC but it offered several technical difficulties. The availability of Sephadex LH-20 allowed the development of a new method of column chromatography permitting the separation of proteolipid fractions, using gel filtration procedures. The best results were obtained when the sample was first eluted partially with chloroform, followed by a series of more polar mixtures. Occasionally the sample became turbid after addition of chloroform, and the elution pattern seemed to depend on the presence or absence of turbidity. This phenomenon could be attributed to variable amounts of water remaining in the TLE after evaporation. MOCKRASH[18] has shown that when a TLE is precipitated with ether and then dissolved again in chloroform–methanol for elution, the protein appears as a single peak with the void volume and is followed by the lipids. Elution of our TLE sample using 80 ml of chloroform–methanol (19:1) gave practically the same results. If the effluent was evaporated to 1–2 ml and then passed through a similar column using the solvent sequences described under METHODS AND MATERIALS, no turbidity appeared and reproducible results were obtained.

The results with grey and white matter TLE demonstrate that the column works well for the separation of lipids and proteolipids. In both instances, two well defined fractions of proteolipids were separated; the first appeared with the void volume (approx. 26 ml), while the second fraction was retained by the column during passage of chloroform and was eluted only with more polar solvents.

DETERMANN[26] pointed out that when organophilic gels were used with organic solvents less polar than the substances to be separated, there is a marked affinity between the solute and the gel phase. With Sephadex LH-20, as with other organophilic gels, chloroform retards substances of low molecular weight. However, in solvents containing hydroxyl groups, the elution proceeds in order of decreasing molecular weight. Although retention of a solute in a column may be due to various other types of interaction, in our method the column operates in two different ways: the first proteolipid fraction is eluted by a gel filtration mechanism, while the second is eluted by partition chromatography.

The elution pattern of these last fractions may depend on differences in molecular weight. If this last peak contains low molecular weight material, it might be either a natural proteolipid of small size or a degradation product of the first fraction. The results of the repetitive chromatography experiments, where we attempted to resolve this problem, have been inconclusive and will be the subject of future studies.

The elution pattern of grey and white matter is completely different. In grey matter, the highest percentage of the proteolipid protein (60–70 %) is eluted with the void volume, while in white matter only 8–20 % appears in this volume. In contrast, the bulk of the proteolipid protein is eluted later as a second fraction, usually with four components. Our method does not permit a clear separation of the first proteolipid fraction (eluted with chloroform) from cholesterol, or from one of the phospholipid fractions which are eluted immediately afterwards. These combinations probably results from co-chromatography. However, both proteolipid fractions contain a certain amount of lipid phosphorus, possibly constituents of the proteolipid molecules themselves.

Although the elution patterns of proteolipids from grey and white matter are substantially different, several other arguments support the view that these tissues contain proteolipids that are either chemically or physicochemically different. Of the four or five peaks eluted from white matter TLE by the polar solvent mixtures, only one seems to carry along with it all of the lipid phosphorus and the cerebroside eluted by the chloroform–methanol mixtures. There is also a great difference in the [^{14}C]-DMTC binding capacity of the purified proteolipid from grey matter compared to that of white matter, whether or not the TLEs are precipitated with ether.

Excess lipid not firmly bound to the proteolipids was removed by the ether and gave a starting material for chromatography enriched in proteolipids. Ether was not as effective in precipitating proteolipids when used with grey matter TLE. Moreover, with this procedure, the proteolipid recovery after elution was smaller with grey matter than with white, 50 % *vs.* 80 % respectively.

There were also differences in the elution pattern between the ether precipitates from grey and white matter. The proteolipid retained on the column may be the one eluted with the chloroform in the column (B) procedure, while the retention of this same material (producing a 50 % loss) may be due to the absence of lipids that were previously removed by the ether. These lipids may have mobilized the proteolipid on the LH-20 column (B). The absence of lipids probably increases the retention of proteolipids by the gel of the column to an irreversible point. The results of the recombination experiments support this interpretation.

The last fraction of the ether precipitate from grey matter which was eluted from the column contains no detectable phosphorus. This fraction is completely soluble in chloroform–methanol (2:1), and in pure methanol. It appears to be a highly purified proteolipid fraction which has also the properties of binding [^{14}C]DMTC[25].

The fraction of ether precipitate from white matter eluted with polar solvents has only 2 % phosphorus. This fraction is generally comparable with the purified preparations obtained by emulsion–centrifugation[27] and dialysis[13].

Studies are being carried out on the chemical and physico-chemical characteristics which might explain the different chromatographic behaviors of grey and white matter proteolipids.

It is hoped that such a study will throw further light on the special proteolipid present in nerve ending membranes which has a high affinity for binding DMTC.

ACKNOWLEDGEMENTS

The authors wish to acknowledge the invaluable critical comments and encouragement received from Professors EDUARDO DE ROBERTIS and CARLOS J. GÓMEZ. They also thank Dr. HERMAN DENBER and Dr. DAVID TELLER for revision of the manuscript and discussion of the results and Miss M. T. DE BIASE for technical assistance.

REFERENCES

1 J. FOLCH-PI AND M. LEES, *J. Biol. Chem.*, 191 (1951) 807.
2 J. FOLCH-PI, *Brain Lipids and Lipoproteins and Leucodystrophies*, Elsevier, Amsterdam, 1963, p. 18.
3 E. F. SOTO, L. SEMINARIO DE BOHNER AND M. DEL C. CALVIÑO, *J. Neurochem.*, 13 (1966) 989.
4 M. LEES, *Federation Proc.*, 24, No. 2 (1965).
5 E. G. LAPETINA, E. F. SOTO AND E. DE ROBERTIS, *J. Neurochem.*, 15 (1968) 437.

6 C. D. JOEL, M. L. KARNOVSKY, E. G. BALL AND O. COOPER, *J. Biol. Chem.*, 233 (1958) 1565.
7 F. N. LE BARON, *Brain Lipids and Lipoproteins and Leucodystrophies*, Elsevier, Amsterdam, 1963, p. 31.
8 E. G. LAPETINA AND E. DE ROBERTIS, *Life Sci.*, 7 (1968) 203.
9 E. DE ROBERTIS, S. FISZER AND E. F. SOTO, *Science*, 158 (1967) 928.
10 J. FOLCH-PI, G. R. WEBSTER AND M. LEES, *Federation Proc.*, 18, No. 1 (1959).
11 G. R. WEBSTER AND J. FOLCH-PI, *Biochim. Biophys. Acta*, 49 (1961) 399.
12 M. MATSUMOTO, R. MATSUMOTO AND J. FOLCH-PI, *J. Neurochem.*, 11 (1964) 829.
13 M. LEES, S. CARR AND J. FOLCH-PI, *Biochim. Biophys. Acta*, 84 (1964) 464.
14 M. LEES, *Ann. N.Y. Acad. Sci.*, 122 (1965) 116.
15 D. TENENBAUM AND J. FOLCH-PI, *Biochim. Biophys. Acta*, 115 (1966) 141.
16 R. CAVANNA AND M. M. RAPPORT, *J. Lipid Res.*, 8 (1967) 65.
17 M. A. WELLS AND J. C. DITTMER, *Biochemistry*, 2 (1963) 1259.
18 L. C. MOKRASCH, *Life Sci.*, 6 (1967) 1905.
19 J. FOLCH-PI, M. LEES AND G. H. SLOANE-STANLEY, *J. Biol. Chem.*, 226 (1957) 497.
20 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. S. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
21 H. H. HESS AND E. LEWIN, *J. Neurochem.*, 12 (1965) 205.
22 R. SEARCY AND L. M. BERGQUIST, *Clin. Chim. Acta*, 5 (1960) 192.
23 P. S. CHEN, JR., T. Y. TORIBARA AND H. WARNER, *Anal. Chem.*, 28 (1956) 1756.
24 J. FOLCH-PI AND J. GREANEY, unpublished results.
25 E. DE ROBERTIS, S. FISZER, J. M. PASQUINI AND E. F. SOTO, *J. Neurobiol.*, (1968) in press.
26 H. DETERMANN, *Gel Chromatography*, Springer Verlag, New York, 1968, pp. 79–80 and 134.
27 J. FOLCH-PI, *Expos. Ann. Biochim. Med.*, 21 (1959) 81.