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LIPOPHILIC PROTEINS EXTRACTED FROM CHICK EMBRYO CELL CULTURES BY DIFFERENT METHODS

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SUMMARY : Compounds containing both proteins and phospholipids were extracted by 3 different methods with organic solvents from labeled chick embryo cells cultured in vitro. The extracted material was analyzed by polyacrylamide gel electrophoresis (PAGE) and thin layer silica gel chromatography (TLC). The characteristics of these compounds are :

1) Polypeptides of an apparent molecular weight comprised between 10 and 20 Kdaltons are extracted by each of the 3 methods. Additional polypeptides of a molecular weight ranging from 20 to 70 Kdaltons are extracted with acid chloroform-methanol ; with this method the highest yields are obtained.

2) The phospholipid composition differs from that of the whole cell ; it is enriched in phosphatidylserine and phosphatidylinositol.

3) The compounds are soluble in organic solvents and in aqueous solvents containing SDS.

4) Membrane enriched fractions contain more of these compounds than whole cells.

Compounds containing both proteins and phospholipids have been extracted first from neural tissues (1) and called proteolipids. Subsequently, they have been shown to exist in several other tissues (2). A variety of extraction methods have been described, most of them using chloroform-methanol mixtures as the first extractant (3-5). The majority of the phospholipids can be eliminated from the first extract by prolonged dialysis (1), column chromatography (5), or washing with water, chloroform, ether, ethanol or acetone (2).

Compounds extracted from neural tissue contain a small amount of covalently bound fatty acids(1). The type of bond which maintains the association between the protein moiety and the lipids is thought to be electrostatic and hydrophobic (2, 5, 6), but other types of bonds have been inferred. Negatively charged phospholipids (phosphatidylserine and phosphatidylinositol) are found most frequently though not exclusively associated with the apoprotein (6-8).

The apoproteins, wether extracted from neural or other tissues, are heterogeneous in respect to their molecular weight as determined by

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PAGE. They range from less than 5 Kdaltons to more than 100 Kdaltons (3, 5, 9, 10). Some authors consider the high molecular weight products to be polymers or aggregates of the low molecular weight products (2-4, 9, 10).

Such compounds are present in different animal and plant tissues, but they have not been detected in embryonic tissues.

They have been shown to be membrane components (2).

In the present study we describe the attempt to extract proteolipid-like material from embryonic cultured cells by adapting methods originally designed for the extraction of large amounts of tissue and in particular from brain white matter. We have characterized the material obtained by different extraction methods and compared it to proteolipids described in the litterature. The possibility to extract this putatively membraneous material would permit its study in cells cultured in vitro, and in particular in cell-virus systems.

METHODS: Cells and labeling Chick embryo cells were explanted from 11 days old lymphomatosis free eggs and cultivated in Falcon plastic bottles in Eagle's BHK 21 medium to which 10 % tryptose phosphate broth and 10 calf serum were added. Twenty four hours before labeling, cells were washed with saline and incubated either in phosphate poor medium (45 % Eagle's medium, 45 % Eagle's phosphate free medium, 10 % dialyzed calf serum) or in Eagle's medium lacking valine, containing 2 % calf serum. The same media were used during labeling (10 µCi [^{32}P]/ ml or 5 µCi [^{3}H] valine/ml). Cells were labeled for 48 hours and the medium containing radioactive isotopes was renewed after 24 hours. Cells were grown to semiconfluency. These cells were mostly fibroblasts, but some cells of epithelial origin were also present. The cells were scraped off the surface with a rubber policeman and washed twice with saline before extraction.

Membrane enriched fractions (phase membranes, containing plasma membranes and microsomes) were prepared according to ISRAEL et al. (11). These preparations are enriched in membranes by a factor of 2 as determined by the measure of 5' nucleotidase activity and cholesterol content.

Chemicals Chemicals were purest grade available purchased from either Merck or Carlo Erba. Radioisotopes were obtained from the Commissariat à l'Energie Atomique, Saclay. Autoradiographs were done with a radiofilm Kodak PE 4006. Commercial phospholipids were purchased from General Biochemicals. Radioactive markers for the determination of molecular weight of polypeptides were purchased from New England Nuclear.

Preparation of lipophilic proteins. Three methods of extraction were used :

1) Proteins F were extracted according to a method described by FOLCH et al. (12) as modified by BLIGH and DYER (13). Tissue is extracted by a chloroform-methanol mixture, followed by phase partition and extensive washing of the interphase with chloroform and water.

2) Proteins N were extracted according to a method described by NICOT et al. (5). Again, the first extractant is a chloroform-methanol mixture. The extract is then washed with a water-chloroform-methanol mixture and the proteolipids are precipitated with petroleum ether. In order to obtain a protein concentration high enough to precipitate the lipophilic proteins, we added carrier proteolipids extracted previously by the same method from sheep brain.

TABLE 1

Extraction of lipophilic proteins

A) Preparation of proteins F
1) $5x10^7$ to $2x10^8$ cells suspended in 1 ml H_2O + 4 ml chloroform-methanol (1:2, V/V), 90 min. 20°, intermittent shaking.
2) Centrifugation, 15 min. 8 000 g, 4°C.
3) Supernatant + 1.25 ml H_2O + 1.25 ml chloroform, 1 min. shaking on Vortex.
4) Centrifugation 15 min. 8 000 g, 4°C.
5) Interphase + 1.25 ml H_2O + 1.25 ml chloroform, shaking on Vortex, centrifugation 5 min. 8 000 g 4°C. Repeat 5 times.
Final interphase = Proteins F

B) Preparation of proteins N
1) Pellet of $5x10^7$ to $2x10^8$ cells + 20 ml/g cells chloroform-methanol (2:1, V/V) homogeneization with 35 strokes of Thomas homogenizer.
2) Centrifugation 15 min. 8 000 g, 4°C.
3) Supernatant + 0.2 V H_2O + 1 g glass beads, shaking on Griffith shaker maximum speed, 5 min.
4) Supernatant decanted and centrifuged 20 min., 8 000 g, 4°C.
5) Aqueous phase discarded :organic phase washed with methanol-H_2O-chloroform (97:95:6, V/V/V), methanol added drop by drop to last organic phase and interphase till a one phase system is obtained.
6) 2 mg/ml carrier proteolipid extracted from sheep brain added.
7) + 4 V petroleum ether (40° - 60°) drop by drop, - 15°C.
8) centrifugation 30 min., 23 000 g, - 20°.
Precipitate = Proteins N

C) Preparation of proteins G
1) $5x10^7$ to $2x10^8$ cells suspended in 2 ml NTE buffer (NaCl 100 mM.TRIS 10 mM, EDTA 1 mM, pH 7) + 4 ml chloroform-methanol (2:1, V/V) shaking on Vortex 1 min.
2) Centrifugation 15 min. 8 000 g, 4°C.
3) Aqueous and organic phase discarded. Interphase + 2 ml chloroform-methanol HCl (66:33:1, V/V/V), shaking in Vortex 1 min.
4) Filtration on fiberglass filter Whatman CF/B.
5) Filtrate + 2 V diethylether, 30 min. - 20°C.
6) Centrifugation 15 min. 8 000 g, 4°C.
Precipitate = Proteins G

N.B. Proteins FG are the denatured proteins obtained in A2, extracted by method C. Proteins NG are the denatured proteins obtained by B2, extracted by method C.

3) Proteins G were extracted by a method described by GREGORIADES (14) and TZAGOLOFF (15) : the first extraction is done with chloroform and methanol, the denatured proteins are not discarded as during the extraction of Proteins F and N. They are reextracted with an acid chloroform-methanol mixture and the extract is precipitated with diethyl-ether.

The details of the extraction procedure are described in Table 1.

PAGE AND TLC Thirteen % polyacrylamide gels were prepared according to LAEMMLI (16). For electrophoresis, lipophilic proteins were dissolved in a aqueous solution (TRIS 10 mM, pH 7.2, EDTA 1 mM, SDS 1.5 %, mercaptoethanol 10 %) and heated for 3 min. at 100°. The samples contained 25 % glycerol and a bromophenol blue marker. Electrophoresis was done at 18° and 150 V. After the run, the gels were treated with dimethylsulfoxide and PPO as described by LASKEY et al. (17). A RP X-OMAT film was applied to the dried gels. The readout of the prints was done with a Vernon recording spectrophotometer. Molecular weights were determined by comparison with commercial labeled polypeptides (carbonic anhydrase, ovalbumine, cytochrome C, phosphorylase b, bovine serum albumin.

For TLC, the extracted material was solubilized in a chloroform-methanol mixture (2:1, V/V). Chromatography was done in 2 directions :

1) Chloroform-methanol-water (65:25:4, V/V/V),
2) n-butanol-acetic acid-water (3:1:1, V/V/V),

In order to avoid oxydation, 1 mg of butylated hydroxytoluene was added per ml solvent. After chromatography, a film was applied to the dried plates and the autoradiographs were used to localize the spots which were then scraped off. The radioactivity was counted by Cerenkov radiation. Identification of phospholipids (phosphatidylcholine, phosphatidylserine + phosphatidylinositol, sphingomyelin ,phosphatidylethanolamine, cardiolipin, phosphatidyglycerol, phosphatidic acid) was done by comparing the migration of the labeled samples with that of commercial standards run on the same plates.

Protein content was measured by the method of LOWRY et al. (18).

RESULTS : Yield of extraction Table 2 shows the amount of protein extracted by the different methods, as obtained in a typical experiment, compared to total cellular protein and a fraction enriched in membranes. Proteins F, N, G. FG and NG are defined as the products of different extraction methods, described in Table 1. Proteins N contain unlabeled carrier proteolipids. Therefore only the values of cmp [^{3}H] valine are given.

As also shown in Table 2, proteins F and N constitute less than 1 % of total cellular proteins, whereas proteins G represent 9.3 of all proteins (average yield, 5 - 10 %). If the denatured proteins obtained after the first extraction of proteins F and N are not discarded but re-

TABLE 2

Yield of lipophilic proteins extracted with organic solvents by different methods. Each extraction was done with a different batch of cells.

Sample	µg Protein	cpm[^{3}H]-Valine
Total cellular proteins	17 709 - (100 %)	233 665 - (100 %)
Proteins F	55.4 - (0.31 %)	1 927 - (0.8 %)
Proteins FG	354.1 - (2.3 %)	5.979 - (2.6 %)
Total cellular proteins	-	41 600 000 - (100 %)
Proteins N	-	121 000 - (0.29%)
Proteins NG	-	3 286 400 - (7.9 %)
Total cellular proteins	10 250 - (100 %)	794 555 - (100 %)
Proteins G	950 - (9.3 %)	71 510 (9 %)
Total proteins of membrane enriched fraction	- -	589 000 - (100 %)
Proteins N of this fraction	-	7 500 - (1.27%)
Total proteins of membrane enriched fraction	3 900 - (100 %)	6 010 200 - (100 %)
Proteins G from this fraction	858 - (22 %)	140 710 - (23.4%)

TABLE 3
Yield of phospholipids associated with lipophilic proteins after extraction with organic solvents by different methods.

Sample	cpm[^{32}P]in phospholipids
Total cellular phospholipids	18 211 430 - (100 %)
Proteins F	83 480 - (0.45%)
Proteins FG	163 902 - (0.9 %)
Total cellular phospholipids	6 262 600 - (100 %)
Proteins N	23 100 - (0.38%)
Proteins NG	3 131 - (0.05%)
Total cellular phospholipids	4 001 578 - (100 %)
Proteins G	3 698 - (0.09%)

extracted with acid chloroform-methanol, the quantities of proteins FG and NG extracted amounted to 2.3 - 2.6 and 7.9 % of total cellular proteins respectively.

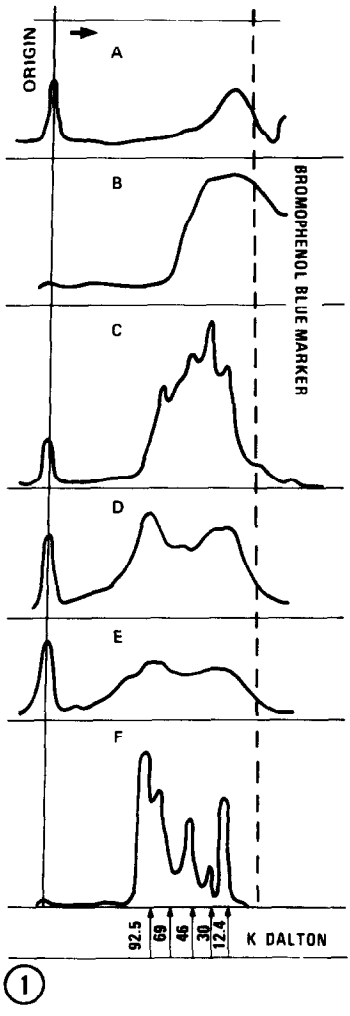
Membrane enriched fractions contained 2-4 times as much proteins N and G as did whole cells ; the enrichement in lipophilic proteins is of the same order as the enrichment in membranes.

Regardless of the extraction method used, phospholipids which represent less than 1 % of total cellular phospholipids remain bound to lipophilic proteins as shown in Table 3.

PAGE Fig. 1 shows the electrophoretic profile of [^{3}H] labeled material, extracted by different methods. The proteins migrate in large bands rather than in neat peaks. Proteins F and N are low molecular weight products, migrating in the region corresponding to a molecular weight of 10-20 Kdaltons. Protein G contains also low molecular weight products, but in addition to these, high molecular weight products ranging from 20 to 70 Kdaltons are present. Proteins FG and NG contain a light and a heavy fraction, both polydisperse.

Some of the phospholipids extracted with the proteins remain associated during electrophoresis, though the majority of these phospholipids migrate rapidly at the same rate as free phospholipids (fig. 2).

Ether precipitation of proteins F tended to increase the amount of polypeptides of apparent high molecular weight, as did prolonged conservation of samples. The phospholipid content of polypeptides of apparent high molecular weight was lower than that of low molecular weight polypeptides (not shown).



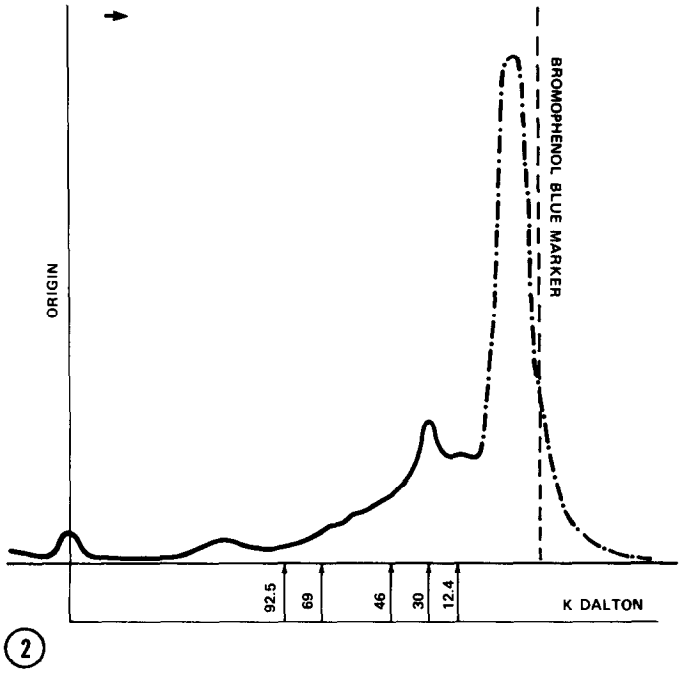


Fig. 1 - Tracing of fluorographs of polyacrylamide gels after electrophoresis of ^{3}H-valine labeled proteins.
A/ - Proteins F
B/ - Proteins N
C/ - Proteins G
D/ - Proteins FG
E/ - Proteins NG
F/ - Commercial standards ; from left to right : phosphorylase b, 92.5 Kdaltons, bovine serumalbumine, 69 Kdaltons, ovalbumine, 46 Kdaltons. carbonic anhydrase, 30 Kdaltons, Cytochrome C, 12.4 Kdaltons.

Fig. 2 - Tracing of fluorograph of polyacrylamide gel after electrophoresis of protein G extracted from ^{32}P labeled cells. Dotted line : free phospholipids, reduced 15 times.

TLC : Fig. 3 shows the autoradiographs of silica gel plates after the migration of [^{32}P] labelled material, and table 4 the relative distribution of the phospholipids. Regardless of the method of extraction used, lipophilic proteins contained a higher proportion of phosphatidylserine

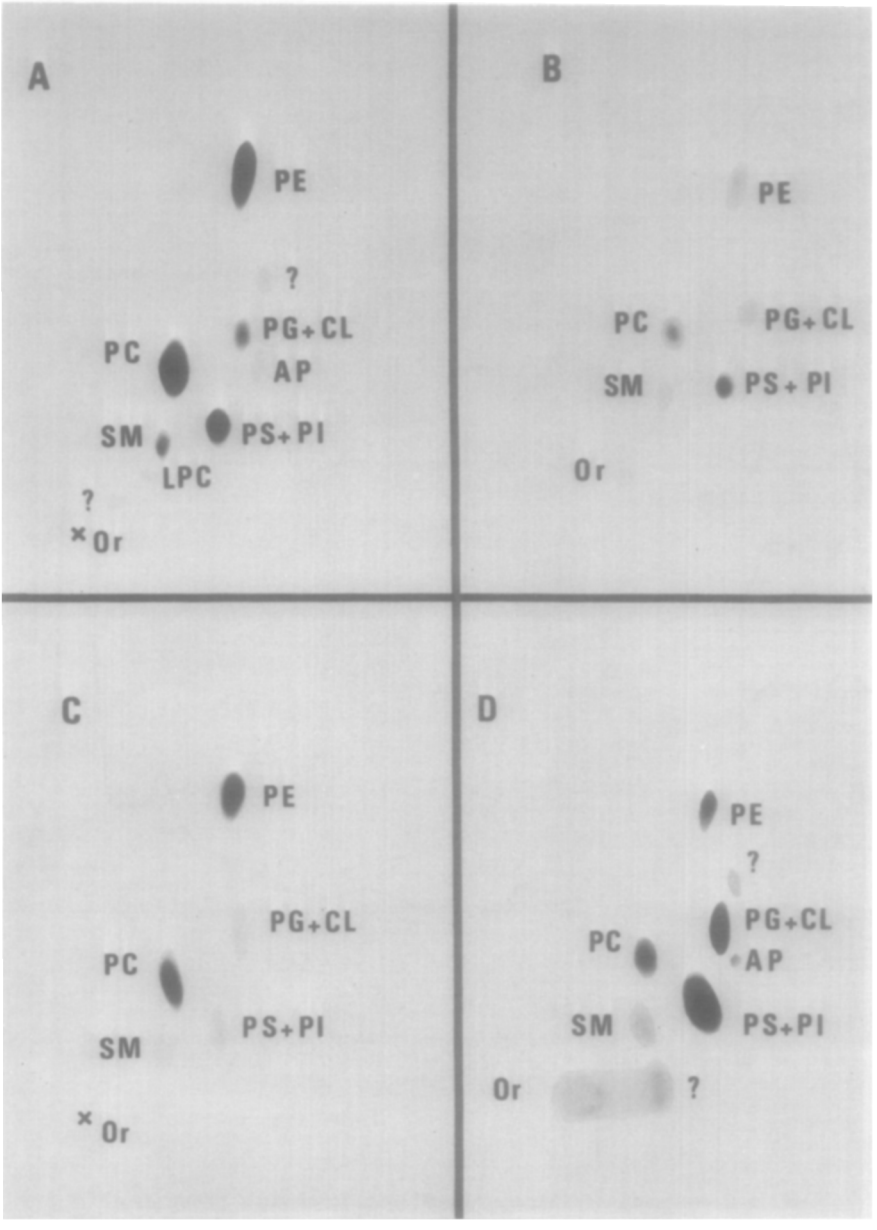


Fig. 3 - Autoradiographs of silica gel thin layer plates after chromatography of lipophilic proteins extracted from ^{32}P labeled cells.
A/ - Total phospholipids (organic phase, Table 1, A4)
B/ - Proteins F
C/ - Total phospholipids (petroleum ether supernatant, Table 1, B8)
D/ - Proteins N
E/ - Total phospholipids (organic phase, Table 1, C3)
F/ - Proteins G

phosphatidylinositol than did whole cells. Minor variations in phospholipid composition could be observed both in whole cell phospholipids and in lipophilic proteins when different extraction methods were used.

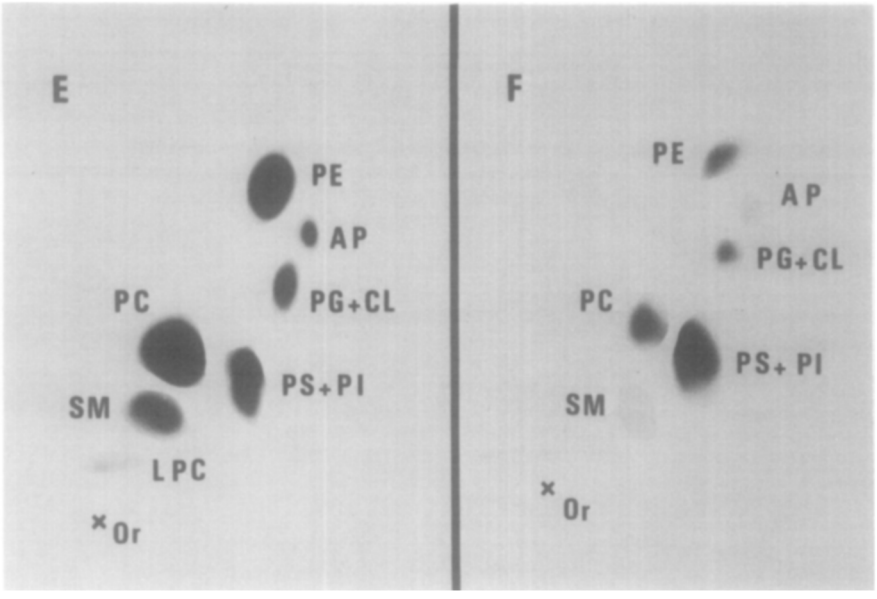


Figure 3 (continued)

TABLE 4

Relative proportions of phospholipids
Whole cells

	Organic phase (Table 1, A4)		Supernatant of petroleum ether (Table 1, B9)		Organic phase (Table 1, C1)	
	cpm	%	cpm	%	cpm	%
SM*	5516	3,6	220	1,9	10565	6,4
PS + PI	24016	15,8	627	5,4	18436	11,2
PC	53431	38,3	5538	47,7	78869	44,1
PE	53253	34,9	4798	41,3	52132	31,5
PG + CL + PA + LPC	11256	7,4	429	3,7	11325	6,8
Non identified	0	0	0	0	0	0

	Proteins F		Proteins N		Proteins G	
	cpm	%	cpm	%	cpm	%
SM*	773	13,5	271	2,2	813	5,3
PS + PI	3035	53,1	7349	59,4	7833	51,4
PC	1033	18,1	992	8	3402	22,3
PE	414	7,2	829	6,7	1945	12,8
PG + CL + PA + LPC	461	8,1	2141	17,3	1243	8,2
Non identified	0	0	788	6,4	0	0

× : SM : sphingomyelin ; PC : Phosphatidylcholine; CL : Cardiolipin ;
PE : Phosphatidylethanolamine; PI : Phosphatidylinositol;
PA : Phosphatidic Acid ; LPC : Lysophosphatidylcholine;
PS : Phosphatidylserine; PG : Phosphatidylglycerol ;

TABLE 5

Proportion of [^{32}P] label migrating in chromatography solvents.

Sample	Total cpm*		^{32}P migrating		^{32}P at origin	
	cpm	%	cpm	%	cpm	%
Cellular phospholipids (organic phase)	152 427	100	152 427	100	0	0
Proteins F	7 473	100	5 716*	76.5	1 757	23.5
The same, ether washed	18 248	100	12 627	69.2	5 621	30.8
Cellular phospholipids (petroleum ether supernatant)	11 612	100	11 612	100	0	0
Proteins N	13 953	100	12 370	91	1 223	9
Cellular phospholipids (organic phase)	165 329	100	165 329	100	0	0
Proteins G	15 632	100	15 632	100	0	0

* 100 cpm can be detected.

Table 5 shows the proportion of [^{32}P] label migrating in the chromatography solvents. It can be seen that most of the label migrates. When lipophilic proteins F were washed with diethylether, the amount of material remaining at the origin increased.

Ninety eight % of the label remained at the origin when [^{3}H] valine labeled extracts were chromatographed. This indicates that proteins which are associated with the phospholipids do not migrate in the chromatography solvents. The remaining portion of the label migrated as phospholipids.

DISCUSSION : Lipophilic proteins can be extracted with organic solvents from embryonic chick cells. These compounds resemble proteolipids in different respects :

1) They are soluble in organic and in aqueous solvents if the latter contain SDS.

2) The proteins and the phospholipids are strongly linked as they remain associated during PAGE and in acidic organic solvents.

3) The majority of the phospholipids associated with the proteins are negatively charged.

4) They are more abundant in membrane enriched fractions than in whole cells.

The presence of proteins in the extract is indicated by the presence of the protein marker [^{3}H] valine and the reaction of the extract with

the protein reagent of LOWRY. However, though most of the label can be attributed to protein, contamination with sugars and lipids cannot be excluded, since the period of labeling is long and both lipids and sugars are labeled after the metabolic breakdown of proteins though to a lesser degree than proteins ; but neither lipids nor sugar migrate on polyacrylamide gels as does the extracted material.

The presence of phospholipids in the extract is proved by the chromatographic characteristics of the material. Less than 1 % of protein G remain at the origin, therefore P containing compounds other than phospholipids such as phosphoproteins are not extracted to any significant amount. When proteins F and N are chromatographed, the material remaining at the origin could be either inositides, phosphoproteins or contaminants which do not migrate or phospholipids bound to aggregated proteins too strongly to be dissolved in the chromatograph solvents. The latter hypothesis is strengthened by the fact that washing with ether which tends to aggregate proteins increases the amount of non migrating labeled material.

A strong association between phospholipids and proteins is proved by the fact that during electrophoresis and after heating to 100° in the presence of detergent phospholipids remain associated with the proteins. Furthermore, the presence of more than 50 % negatively charged phospholipids in proteolipids compared to less than 20 % in whole cells argues that the presence of phospholipids is not simply the results of contamination of proteins with free phospholipids. Owing to the scarcity of material we could not ascertain the presence of covalently bound fatty acids. The presence of covalently bound fatty acids is an essential characteristic of FOLCH-PI proteolipids. However, similar compounds soluble in organic solvents in which the authors could not detect a significant amount of covalently bound fatty acid have been described (15, 19).

The fact that membrane enriched fractions contain more lipophilic proteins than whole cells suggest that these proteins may indeed be membrane components, as has been shown for a protein extracted from mitochondrial membranes (15).

The lipophilic proteins we extracted were heterogeneous in respect to their molecular weight. This may be due to the presence of different apoproteins but also to the formation of polymers, as postulated by some authors (2-4, 9, 10).

More material is extracted with acid chloroform-methanol than with neutral solvents as shown by the yields of proteins G, FC and NG as compared to those of proteins F and N. The molecular weight to the polypeptides differs depending on the extraction method. Extraction with acid solvents

favour either the isolation of high molecular weight products or the formation of polymers. The question wether this difference corresponds to a difference in the apoproteins cannot be answered at the moment. The phospholipid composition suggests that the lipidic material extracted by the different methods is similar.

The present work establishes that lipophilic proteins resembling proteolipids in most respects exist in embryonic cultured cells, though other authors could not show them to exist in embryonic material (14,20). It confirms that in this system lipophilic proteins can be separated from other cellular proteins by extraction with organic solvents. They can be characterized by their phospholipid composition and their solubility in both organic and aqueous solvents. They constitute a useful material in the study of cellular proteins and in particular of membranes of which they seem to be a component.

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