CHEMICAL STRUCTURE OF SOLUBLE LIPOPROTEINS OF BRAIN

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Dedicated to Professor F. Šantavý on the occasion of his 60th birthday.

The chemical composition of soluble lipoproteins of brain was studied. The ratio of lipids to proteins in the supernatant fraction of brain is 0·03. The quantity of phospholipids and cholesterol in the lipid moiety of supernatant lipoproteins was determined; these two types of lipids accounted for 35% of total supernatant lipids. The presence of mono-, di-, and triglycerides, of free fatty acids and cholesterol esters in the supernatant fraction was established chromatographically; the concentration of these substances is considerably higher than in the lipid extract of the whole brain tissue. Phosphatidylcholines and phosphatidylserines are most abundant among the phospholipids. The presence of residual blood in the tissue was without effect on the results of analyses of proteins, total lipids, and phospholipids yet it partly increased the value of cholesterol in the supernatant fraction of brain.

Eichberg and coworkers[1] and Seminário and coworkers[2] were the first to demonstrate the presence of lipids in the soluble (supernatant) fraction of brain. These authors voiced the opinion that these lipids are not present in free state in the supernatant but that they are bound in complexes to proteins. The lipoproteins of brain supernatant are very poor in lipids compared to membrane lipoproteins. A very low lipid to protein content in the supernatant brain fraction was observed also by Lapetina and coworkers[3] and Mézešová[4]. Herschkowitz and coworkers[5] were the first to study the structure of lipoproteins from the supernatant fraction in more detail; they examined the distribution of phospholipids, glycolipids, and cholesterol in lipoproteins from the supernatant of brown rat brain and compared the analyses of this fraction with those obtained by the analysis of the whole brain tissue, microsomal fraction, myelin, and blood serum. The authors conclude from their studies that even if possibly contaminated by lipids from other fractions, the lipoproteins of the brain supernatant represent a distinctive group of lipid-protein complexes whose structure markedly differs from that of membrane and blood plasma lipoproteins.

Recorded data on the composition of the supernatant brain fraction are scarce and very little information on the chemical structure of soluble brain lipoproteins is available at present. Continuing our preceding studies[4,6], we investigated the

chemical composition of the supernatant brain fraction with special attention to the structure of the lipid moiety of the lipoprotein complexes investigated.

EXPERIMENTAL

Material. Brains of male guinea pigs (weight 350—450 g) and bovine brains were used in our experiments. Guinea pig brains washed free of blood with 0·9% sodium chloride were also used. The brain was washed in ether narcosis as follows: 100 ml of 0·9% sodium chloride was introduced intracardially and blood together with the washing solution was allowed to drop from opened femoral veins. The guinea pigs were sacrificed by decapitation, the brain without the cerebellum was taken out, washed immediately with cool 0·9% sodium chloride, and freed completely of the meninges and surface blood vessels. Bovine brain was stored over ice and 110 g portions of its frontal part were taken for the experiments. The subsequent treatment was the same as that of guinea pig brains.

Methods. The brain tissue was homogenized always with a ten-fold volume of 0·1M-KCl in a glass homogenizer equipped with a teflon piston. The supernatant brain fraction was separated by ultracentrifugation at 105000 *g* (centrifuge VAC 60) for 90 min and its protein content was determined[7]. Proteins and lipoproteins in the supernatant were precipitated by 20% trichloroacetic acid at 8—12°C and lipids were extracted from the precipitate. The extraction of lipids and the purification of lipid extracts were carried out by the method of Folch and coworkers[8]. The lipid extracts adjusted as described were used for the determination of total lipid content[9], cholesterol content[10], lipid phosphorus[11] using a conversion factor for phospholipids 25, for the analysis of the distribution of individual phospholipid fractions by thin-layer chromatography on silica gel[12], and for chromatographic separation of lipids from the supernatant by thin-layer chromatography on silica gel in the system light petroleum–ether–acetic acid (85 : 15 : 2). The spots on the chromatograms were detected by iodine vapors and by phosphomolybdic acid. Acid hydrolysis of the eluate was effected in 6M-HCl, 48 h at 105°C.

RESULTS

The chemical composition of lipoproteins obtained from the supernatant fraction of guinea pig and bovine brains was investigated. Since a small quantity of blood is retained by the brain tissue after the animals have been sacrificed and could affect the analyses of the supernatant, we examined also the chemical composition of lipoproteins from the supernatant fraction of guinea pig brains which had been washed with 0·9% sodium chloride and thus most of the residual blood had been removed.

The supernatant fraction of bovine and guinea pig brain was analyzed for the content of proteins, total lipids, phospholipids, and cholesterol. The results of the analyses (Table I) show that the composition of the supernatant fraction of bovine brain differs only little from the composition of the supernatant fraction of guinea pig brain. The traces of blood remaining in the tissue practically do not interfere with the results of the analyses of proteins, total lipids, and phospholipids yet they partly increase the cholesterol values.

The supernatant brain fraction is very poor in lipids. The quantity of lipids per 1 mg of proteins is a little more than 30 μg, that is the ratio of lipid to protein content

is approximately 0·03. The quantity of phospholipids and cholesterol per 1 mg of protein is likewise very low; the ratio of phospholipid to protein content is approximately 0·006, the ratio of cholesterol to protein content approximately 0·004. The analysis of the lipid moiety of lipoproteins from the supernatant fraction showed that there was only about 150–200 μg of phospholipids and cholesterol per 1 mg of lipids in this fraction (Table II). Whereas phospholipids together with cholesterol represent more than 70% of the lipid extract of whole brain, these lipids participate only by a little more than 30% on total lipids of the supernatant. The lipid content of the supernatant fraction of brain washed with 0·9% sodium chloride contained even less cholesterol in total lipids than bovine brain and unwashed guinea pig brain (Fig. 1). In view of this result we tried to find the cause of this low phospholipid and cholesterol content. With regard to the methods used, we considered the possibility

TABLE I

Quantity of Proteins, Total Lipids, Phospholipids, and Cholesterol in Brain Supernatant

Examined value	N	Guinea pig brain		Bovine brain
		washed	unwashed	
Proteins mg/ml supernatant	13	2·43 ± 0·33	2·36 ± 0·37	2·31 ± 0·11
Total lipids μg/mg proteins	12	32·38 ± 9·45	30·42 ± 9·11	38·12 ± 7·80
Phospholipids μg/mg proteins	11	6·33 ± 1·14	6·37 ± 1·16	7·11 ± 1·92
Cholesterol μg/mg proteins	11	3·18 ± 0·83	4·16 ± 0·80	5·07 ± 1·01

TABLE II

Composition of Lipid Moiety of Lipoproteins of Brain Supernatant

Value examined μg/mg total lipids	N	Guinea pig brain		Bovine brain
		washed	unwashed	
Phospholipids	11	194·85 ± 25·18	208·52 ± 26·17	186·12 ± 24·63
Cholesterol	12	98·26 ± 21·86	136·61 ± 25·44	133·52 ± 23·00

of formation of artifacts caused by tanging of lipoproteins from the supernatant during precipitation by trichloroacetic acid. We also regarded as possible that other lipids in addition to phospholipids and cholesterol participate to a considerably higher degree than in membrane lipoproteins on the lipid moiety of the lipoprotein fraction of the supernatant.

To elucidate this problem, we resolved the lipid extract of brain supernatant by thin-layer chromatography on silica gel in the system chloroform–methanol–water (65 : 25 : 4); we analyzed lipids extracted from the protein precipitate and lipids extracted directly from the supernatant by a mixture of chloroform and methanol. It can be seen on the chromatograms (Fig. 2*) that the method of extraction is without effect on the spectrum of the lipid extract of the supernatant. The same fractions of phospholipids and glycolipids were separated from the lipid extracts of the supernatant prepared by both methods; neither did the spectrum of phospholipids and glycolipids differ qualitatively from that of the lipid extract of the whole brain tissue. The chromatogram clearly shows, however, that a characteristic lipid fraction separates from the lipid extracts of the supernatant; this fraction moves almost with the front of the solvent and its concentration in the total brain extract is very low.

The subsequent part of our study was devoted to the determination of the quantitative distribution of certain phospholipid fractions in total phospholipids and to the identification of lipids which moved with the front of the solvent in the course of separation of phospholipids. The distribution of individual phospholipid groups in total phospholipids is given in Table III. Since we were not able to separate in a sufficiently pure state inositolphosphatides from sphingomyelins and phosphatidylcholines from serinephosphatides, we give common values for these two fractions. The fraction of ethanolamine phospholipids includes also phosphatidylethanolamines. For reasons of comparison we also give in Table III the distribution in per cent of these fractions in the whole brain tissue. When we compare the distribution in per

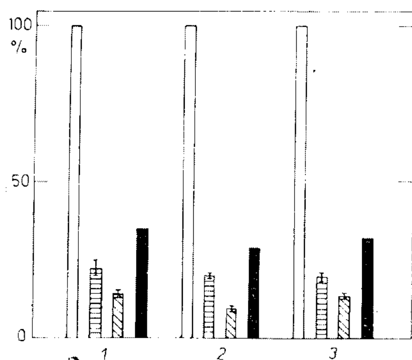

FIG. 1

Distribution in Per Cent of Phospholipids and Cholesterol in Total Lipids of Brain Supernatant

1 Supernatant of unwashed guinea pig brain; *2* supernatant of guinea pig brain washed with 0·9% sodium chloride and *3* supernatant of bovine brain; Empty column-total lipids, horizontally hatched column — phospholipids, diagonally hatched column-cholesterol, full column — phospholipids + cholesterol.

* See insert facing p. 1460.

cent of the individual phospholipid types in total phospholipids of the supernatant and in the unfractionated brain tissue, it becomes obvious that this distribution is very different as regards the fraction of phosphatidylcholines + phosphatidylserines and phosphatidylethanolamines. Whereas phosphatidylethanolamines are prevalent in the whole brain tissue, the fraction of phosphatidylcholines + phosphatidylserines is clearly dominant in phospholipids of the supernatant. It is likely that the distribution of inositolphosphatides and sphingomyelins will be also different, our separation technique, however, does not permit us to make any conclusions in this respect.

The lipid fractions of the supernatant, which moved with the solvent front, were eluted and the eluates were treated in two manners: one part was subjected to acid hydrolysis with subsequent determination of amino acids, the other part was again separated by thin layer chromatography on silica gel. The electrophoretic and chromatographic resolution of the hydrolysate of the eluate revealed the presence of eight amino acids, of which we determined aspartic acid, glutamic acid, lysine, and phenylalanine. The presence of alanine, tyrosine, proline, and leucine is probable. We assume that these amino acids are derived from a lipopeptide of markedly nonpolar character and readily soluble even in pure chloroform. We have not analyzed as yet the lipid moiety of this substance.

The separation of the eluates by thin-layer chromatography on silica gel was carried out in the system light petroleum–ether–acetic acid (85 : 15 : 2). Additional lipid fractions, *i.e.* mono-, di-, and triglycerides, free fatty acids and cholesterol esters were obtained by separation of the eluates in this solvent system. The separation

TABLE III

Distribution in Per Cent of Individual Phospholipid Groups in Total Phospholipids of Lipoproteins of Brain Supernatant

Examined value	Total phospholipids, %	
	supernatant N = 8	whole brain N = 10
Lysophosphatides	5·37 ± 1·91	11·12 ± 3·30
Inositolphosphatides + Sphingomyelins	21·33 ± 4·21	20·52 ± 5·24
Phosphatidylcholines + Phosphatidylserines	55·24 ± 5·42	33·56 ± 5·15
Phosphatidylethanolamines	20·21 ± 3·08	35·63 ± 6·71

of lipid extracts of brain under these conditions is shown in Fig. 3*. As can be seen in the Figure, the washing of the brain with 0·9% sodium chloride is without any influence on the separation of lipids in this solvent system as regards their qualitative spectrum.

DISCUSSION

The results of our studies on the chemical composition of brain supernatant provided additional evidence showing that this fraction is very poor in lipids. Whereas the quantity of proteins in the brain supernatant fraction represents according to our results, which are in good agreement with the data of other authors[3,13], about 15% of total brain proteins, only about 1% of total lipids of the brain are found in the supernatant. Lapetina and coworkers[3] report that the ratio of lipids to proteins in the supernatant fraction is about 0·08, according to our findings this ratio is even lower, *i.e.* approximately 0·03. We obtained generally lower values in this study also for phospholipids and cholesterol found in the supernatant. The highest values of these two types of lipids have been reported by Lapetina and coworkers[3] (1·1 mg per 1 g of brain for phospholipids and 0·4 g per 1 g of brain for cholesterol).Other authors[1,5] relate values for phospholipids and cholesterol in the supernatant which are relatively very similar, about 0·7 mg per 1 g of brain for phospholipids and about 0·2 mg per 1 g of brain for cholesterol. Our data when expressed per 1 g of brain account for approximately 0·3 mg of phospholipids and 0·12 mg of cholesterol; the value of phospholipids is practically identical with our earlier findings[4]. We assume that the different values of the quantity of lipids in the supernatant can be ascribed to differences in the technique of extraction of proteins and lipoproteins from the brain tissue (sucrose, buffers, concentration of salts) and also to differences in the treatment of the supernatant.

The results of the analyses of the supernatant fraction of guinea pig brain which had been washed with 0·9% sodium chloride showed that the residual blood in the brain tissue interfered only little with the analyses of the supernatant fraction and partly increased the values of cholesterol. It follows from the data on the volume of blood in brain and from the information on lipemia in quinea pigs[14] that plasma lipids found in the supernatant can represent merely less than one tenth of total lipids of the supernatant. Considering our results and these data we postulate that the presence of residual blood in the brain tissue cannot affect any substantially the general image of the composition of the supernatant fraction.

The distribution of the individual phospolipid groups in total phospholipids of brain supernatant differred according to our results mainly in the fractions of phosphatidylcholines + serine phosphatides and ethanolamine phosphatides compared

* See insert facing p. 1462.

to their ratios in the whole brain tissue. Our finding of high quantities of phosphatidylcholines + serine phosphatides is in good agreement with the data recorded in literature[5,15].

The investigation of the lipid moiety of lipoproteins of the supernatant revealed an interesting fact that phospholipids and cholesterol represent only 35% of total lipids of the supernatant whereas their quantity in the lipid extract of the whole brain is more than 70% of total lipids. A similar composition of lipids of the supernatant has been observed also by other authors[5]. An additional analysis of lipids of the brain supernatant showed that the supernatant fraction contains – in addition to phospholipids, free cholesterol, and glycolipids – also mono-, di-, and triglycerides, free fatty acids, and cholesterol esters in concentrations considerably higher (compared to phospholipids) than those in the lipid extract of the whole brain tissue. We obtained identical qualitative results also with supernatants of guinea pig brains free of blood. We also observed that the lipid extract of brain supernatant contains a lipopeptide soluble even in pure chloroform and we determined partly its amino-acid composition.

The present concepts of the presence of neutral lipids in brain are considerably different. First, the data on this subject recorded in literature are very meagre and second, the data available are very dispersed from the quantitative viewpoint. So far only the problem of cholesterol esters in brain has been known in more detail[16–19]. Alling and Svennerholm[18] report that the quantity of cholesterol esters in human brain is 0·1 – 2% of total cholesterol, its quantity being largest at the stage of myelinization and lowest immediately after myelinization. Since the authors found considerable differences in fatty acid content of cholesterol esters in brain compared to cholesterol esters in serum, it can be assumed that these lipids in low concentrations represent a normal component of the brain tissue. According to other authors[20], neutral lipids constitute approximately 11% of total lipids of rabbit brain; this group of lipids contains predominantly triglycerides and free fatty acids.

Our results show that neutral lipids represent a considerable part of lipids of the supernatant of guinea pig brain and their concentration in this fraction is markedly higher than in the whole brain tissue. We assume that in view of the higher amount of total cholesterol in brain containing residual blood, only a small portion of cholesterol esters is present in the supernatant as a result of contamination with plasma. Glomset[21] points to the possibility of formation of cholesterol esters *via* the acyltransferase reaction *post mortem*; however, in view of the prompt treatment of material we do not suspect formation of cholesterol esters *via* this reaction in our experiments.

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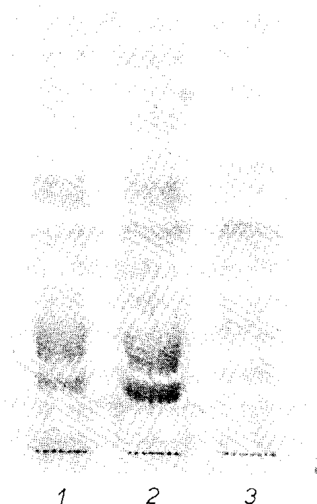

Fig. 2

Separation of Lipid Extract of Supernatant and of Lipid Extract of Whole Bovine Brain

1 Lipid extract of supernatant after preceding precipitation of proteins of supernatant by trichloroacetic acid; *2* lipid extract of supernatant obtained by direct extraction with chloroform–methanol, and *3* lipid extract of whole brain. Starting from the origin to the front *a* lysophosphatides; *b* inositolphosphatides + sphingomyelins; *c* phosphatidylcholine + phosphatidylserines; *d* phosphatidylethanolamines; *e* cerebrosides; *f* unidentified lipids; *g* cholesterol, and *h* unidentified lipids.

Fig. 3

Separation of Lipid Extracts of Supernatant of Guinea Pig Brain after Washing (*2*) and without Washing (*3*) with 0·9% Sodium Chloride Compared to Separation of Lipid Extract of Whole Brain Tissue (*1*)

Thin layer chromatography on silica gel in the system light petroleum–ether–acetic acid (85 : 15 : 2). *a* Monoglycerides + phospholipids + glycolipids; *b* 1,2-diglycerides; *c* free cholesterol + 1,3-diglycerides; *d* free fatty acids; *e* triglycerides, and *f* cholesterol esters.