Subcellular distributions of lipids in cultured BHK cells: evidence for the enrichment of lysobisphosphatidic acid and neutral lipids in lysosomes

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Abstract Homogenates of cultured hamster fibroblasts (BHK 21 cells) were fractionated by differential centrifugation into six main fractions: nuclear, mitochondrial, light mitochondrial, microsomal, soluble, and floating. The contents of several lipids and some marker enzymes were measured. According to the enzyme distributions, lysosomes were enriched both in the floating fraction and in the light mitochondrial fraction. Lysobisphosphatidic acid was enriched in the floating fraction more than tenfold relative to phospholipid. Cholesteryl esters and triglycerides were the main constituents of the fraction (70% of total lipids). Lysobisphosphatidic acid, triglycerides, and cholesteryl esters were enriched also in the light mitochondrial fraction. Their distribution patterns were different from those of the other lipids. Electron microscopy showed that the floating fraction contained numerous lipofuscin-like particles with darkly stained peripheries and with core regions staining like droplets of neutral lipids. Similar particles, frequently containing prominent multilamellar formations, were also common in intact cells. They contained cytochemically identified acid phosphatase. We conclude that lysobisphosphatidic acid was enriched in the lysosomes of the BHK cells and that the lysosomes also contained variable amounts of neutral lipids in the form of intralysosomal droplets.

Supplementary key words differential centrifugation · marker enzymes · triglycerides · cholesteryl esters · lipid morphology

Different membrane classes of mammalian cells display characteristic differences in their lipid compositions (for reviews see ref. 1, 2). For instance, cardiolipin seems to be present exclusively in the inner mitochondrial membrane whereas cholesterol and sphingolipids are predominantly in the plasma membrane. The possible functional roles of the membrane-specific lipids are poorly understood.

Recent studies have demonstrated the distinct organelle-specificity of a minor phospholipid, lysobisphosphatidic acid (LBPA) (3–6). It is highly enriched in the lysosomes of rat liver loaded with Triton WR 1339 (3, 4) and in those of the rabbit alveolar macrophages loaded with mineral oil (5). LBPA accumulates in the livers of patients suffering from Niemann-Pick disease (7), and it is enriched in the characteristic lipid-storing lysosomes in such livers (6). Thus it seems that LBPA is a specific lipid of secondary lysosomes, although there is evidence that its concentration in these particles is highly variable (8).

LBPA of cultured hamster fibroblasts (BHK 21 cells) has a curious stereochemical structure in the sense that both glycerol moieties are esterified with the phosphate via the number 1 carbon atoms (9).

In the present work we have employed analytical differential centrifugation (10, 11) to study the distributions of LBPA and other lipids in the BHK cell fractions. Our results indicate the enrichment of LBPA in the lysosomes. The lysosomes of the BHK cell also contain large amounts of triglycerides and cholesteryl esters.

MATERIALS AND METHODS

Cells

Hamster kidney fibroblast cells (BHK 21 cells, strain Wi-2) were grown as monolayers in glass bottles, which had flat bottom areas of about 120 cm², in 40 ml of culture medium as described earlier (12, 13).

Abbreviations: LBPA, lysobisphosphatidic acid or bis(monoacylglyceryl)phosphate.

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Cell fractionation

Cell layers from 10–40 bottles were washed once with a buffered 0.3 M sucrose solution containing 5 mM Tris-HCl and 0.5 mM EDTA, pH 7.4. The cells were scraped into the same buffer and sedimented at 1500 g_{av} for 1 min in a Sorvall centrifuge, rotor SS 34 (Ivan Sorvall, Inc., Norwalk, CT). The pellet was washed twice by resuspension and resedimentation in the same solution. This and the subsequent steps were done at 0–4°C. The yield of packed cells was 110 ± 30 mg wet weight/bottle (mean ± SD, n = 5) equivalent to 7.3 ± 1.1 mg protein/bottle (mean ± SD, n = 19).

Cells were suspended in 10–20 ml (depending on the cell yield) of a hypotonic sucrose solution (see, e.g., ref. 14) consisting of 0.1 M sucrose, 10 mM Tris-HCl and 1 mM EDTA, pH 7.4, and were then homogenized in a Dounce homogenizer (Thomas Co., Philadelphia, PA) by 10 strokes with the tight-fitting B-pestle. One volume of 0.5 M sucrose was added and the suspension was centrifuged at 2300 g_{av} for 1.5 min. The supernatant was decanted and the pellet was rehomogenized and sedimented as above. The pellet obtained was designated as the nuclear fraction. Virtually no breakage of the nuclei occurred during the homogenization, as evidenced by the high recovery of DNA (96 ± 4 (SD) %, n = 4) in the nuclear fraction.

The mitochondrial fraction was sedimented from the combined nuclear supernatant by spinning it first at 3300 g_{av} for 10 min and then increasing the speed to 9300 g_{av} for another 2 min (see ref. 15). The pellet was washed once with the 0.3 M buffered sucrose solution using the same centrifugation procedure. The light mitochondrial fraction was sedimented at 32,000 g_{av} for 10 min and washed once. The resulting supernatant was centrifuged at 82,000 g_{av} for 60 min in a Beckmann L3-50 centrifuge, rotor SW 27 (Spinco Div., Palo Alto, CA). The whitish yellow floating fraction was collected with a Pasteur pipette and diluted with the 0.3 M buffered sucrose solution. The clear, soluble fraction was poured off and the pelleted microsomal fraction was suspended in the 0.3 M buffered sucrose solution. The fractions were washed by recentrifugation as above.

In one experiment the floating fraction was purified from the bulk of the contaminating cytoplasmic protein by reflotation through a sucrose step gradient. The crude floating fraction was diluted with an equal volume of 1 M sucrose (in 5 mM Tris, 0.5 mM EDTA, pH 7.4) and ovarlaid with a layer of 0.4 M sucrose in the same buffer in a Beckmann SW 27 centrifuge tube. The tube was centrifuged at 82,000 g_{av} for 120 min and the floating fraction was collected with a Pasteur pipette.

The fractions (suspended in the 0.3 M buffered sucrose solution) and the washing solutions were divided into samples of convenient size and stored at −20°C. The washings were not combined with the previous supernatants during the fractionation to avoid an excessive dilution of the soluble fraction. Some partial fractionations were done by omitting one or several of the centrifugations.

Enzyme assays

Succinate dehydrogenase (succinate: ferricyanide oxidoreductase, E.C. 1.3.99.1) was measured by a procedure based on those presented in ref. (16), modified in order to increase the sensitivity of the assay of the turbid organelle suspensions and to allow the simultaneous processing of a large number of samples. The reaction mixture (1 ml) consisted of the enzyme sample, 4 mM potassium succinate, 2 mM potassium ferricyanide, 100 mM potassium phosphate buffer pH 7.5, 1 mM potassium cyanide, 0.1% bovine serum albumin, 0.1 μM rotenone and 0.1 μM carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (a gift from Prof. N. E. Saris, Department of Medical Chemistry, University of Helsinki). The mixtures were shaken for 60 min at 30°C in a water bath and the reaction was stopped by 3 ml of cold 10% trichloroacetic acid. The absorbance of the supernatant at 420 nm was subtracted from the absorbance of a blank mixture, without enzyme, run in triplicate along with each series of assays. The absorbance differences were converted to enzyme activities by means of a calibration curve prepared using different known amounts of enzyme and/or different incubation times (both were superimposable at least to 60 min incubation time). The standard deviation of the mean of duplicate analyses was 6%.

Glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase, E.C. 3.1.3.9) and acid β-glycerophosphatase (E.C. 3.1.3.2) were measured at 30°C as described previously (17). Sodium fluoride, 2 mM, was used to estimate the contribution of acid phosphatase to the apparent glucose-6-phosphatase activities (17,18) and the background of the fluoride-resistant soluble phosphatase (17) in the acid phosphatase assays. The inhibition of a possible alkaline phosphatase was ensured by 4 mM EDTA (18).

Acid β-glucuronidase (β-D-glucuronide glucuronohydrolase, E.C. 3.2.1.31) was determined at 30°C as described previously (12). The turbidity of the reaction mixtures containing the floating fraction was clarified by extraction with an equal volume of

chloroform. Control experiments with the whole homogenate showed that this did not affect the color yield.

Arylesterase (E.C. 3.1.1.2) was assayed in one experiment using indoxyl acetate as substrate (19) at 30°C.

Chemical analyses

Lipids were extracted from organelle suspensions by chloroform–methanol (20). When necessary, phospholipids were separated from neutral lipids by column chromatography on silicic acid (13). Phospholipid classes were separated by two-dimensional thin-layer chromatography and quantitated by phosphorus analysis (13). Neutral lipids were separated by one-dimensional thin-layer chromatography and quantitated by charring (13). The total neutral lipid content was calculated by summing up the individual lipid classes, but omitting the hydrocarbon-like material running near the solvent front and the monoglyceride spot that frequently was too close to the phospholipid spot near the origin to allow a reliable analysis.

Protein was assayed as described previously (12, 13). The values for the turbid floating fraction were corrected with appropriate blanks. Clarification of the reaction mixtures by extraction with chloroform gave identical results and did not affect the color yield obtained for the whole cell homogenate.

Electron microscopy

The cells were fixed in situ with 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.1, for 15 min. They were then scraped off, pelleted in a conical centrifuge tube and fixed in the same medium overnight at 4°C. The pellets were rinsed with cold 7.5% sucrose in 0.1 M cacodylate, pH 7.1, and they were stored 1–4 days in the same buffer at 4°C. After postfixation with 1.5% osmium tetroxide in 0.1 M phosphate buffer at pH 7.2, the cells were dehydrated with ethanol and propylenoxide and embedded in Epon 812. Thin sections were stained with uranyl acetate and lead citrate (21) which were diluted 10- or 20-fold with 0.1 M sodium hydroxide to avoid overstaining. Electron microscopy was performed with JEOL (JEOL USA, Inc., Medford, MA) JEM 100-B at 80 kV acceleration voltage. The uncalibrated magnifications are accurate to about ±10%.

The floating fraction was filtered on a Millipore filter (Millipore Co., Bedford, MA), 0.4 μm pore size, and the filter was fixed overnight in the 3% buffered glutaraldehyde at 4°C. The whole filter was processed further as above.

The cytochemical staining of acid phosphatase was done in the presence of dimethylsulfoxide to increase the permeability of the substrates (22). Nonstained sections were examined at 60 kV acceleration voltage.

RESULTS

Enzyme distributions

The distributions of enzymes, protein, total phospholipids, and total neutral lipids in two complete fractionation experiments are given in **Table 1.** Most of the cellular protein was recovered in the nuclear and soluble fractions. The nuclear fraction was contaminated by a considerable number of unbroken cells and cell debris as judged by the activity of succinate dehydrogenase, a nonnuclear enzyme. The large amount of protein in the supernatant demonstrates that the BHK cell contains a relatively sparse population of sedimentable organelles.

Fig. 1 shows the relative specific activities of the enzymes in the subcellular fractions drawn as a histogram against the cumulative protein content (assuming a 100% recovery for all the components) (10, 11). The distribution patterns display characteristic differences indicating a partial separation of the organelles containing the respective enzymes.

Succinate dehydrogenase was enriched about sevenfold in the mitochondrial fraction and had a shoulder in the light mitochondrial fraction consistent with its ubiquitous localization in the mitochondria of animal cells (1, 11, 23).

The acid β-glucuronidase was used as a potential lysosomal marker. It was markedly enriched in the light mitochondrial fraction but barely elevated in the mitochondrial fraction, thus providing a clear contrast to the pattern of succinate dehydrogenase. However, its specific activity in the microsomal fraction was almost equal to that in the light mitochondrial fraction. This could be due either to an unusually low average sedimentation coefficient of the BHK cell lysosomes rich in β-glucuronidase or to the enzyme also present in some microsomal components. The latter alternative is supported by the moderate β-glucuronidase activity observed in the purified endoplasmic reticulum of the BHK cells (12). In several other tissues, a considerable fraction of the acid β-glucuronidase activity resides in the microsomal fraction (10, 24–26).

An attempt to distinguish between the light mitochondrial and the microsomal fractions using arylesterase as a marker enzyme was unsuccessful; the distribution was practically identical to that of β-glucuronidase. Arylesterase is a good marker of the

TABLE 1. Distribution of protein, lipid, and enzymes between the BHK cell fractions[a]

	Experiment no.	Protein	Phospholipid	Neutral Lipid	Succinate Dehydrog.	β-Glucuronidase	Arylesterase	Glucose-6-phosphatase		
								Total	F-sensit.	F-resist.
		mg	mg	mg	μmol/hr	μmol/hr	μmol/hr	μmol/hr	μmol/hr	μmol/hr
Total Homogenate (40 culture bottles)	1	318	55.6	36.8	944	13.8	1470	158	65.2	92.8
	2	242	45.6	18.2	788	7.8	ND[a]	143	65.6	77.2
Nuclear fraction	1	41.6	31.4	24.2	31.4	29.0	31.3	34.8	29.8	38.4
	2	36.3	26.1	29.4	19.4	28.5	ND[b]	30.0	28.7	31.1
Mitochondrial fraction	1	3.0	7.6	4.5	26.1	4.4	2.9	3.9	6.7	1.9
	2	6.1	13.3	14.4	39.0	8.2	ND	8.3	11.4	5.6
Mitochondrial wash	1	2.2	6.2	ND	10.3	7.2	3.8	4.4	6.7	2.8
	2	2.8	8.1	ND	8.8	8.8	ND	6.5	7.7	5.4
Light mito. fraction	1	5.0	16.8	11.8	27.0	17.7	12.2	13.8	22.7	7.6
	2	3.8	14.6	22.3	14.4	16.1	ND	12.3	15.0	10.0
Light mito. wash	1	2.9	9.6	ND	0.5	10.5	7.8	5.6	5.8	5.4
	2	2.2	8.5	ND	0.4	9.0	ND	6.1	5.5	6.7
Microsomal fraction	1	3.5	16.0	7.8	0.4	11.8	8.4	9.6	12.6	7.4
	2	5.1	21.0	17.4	0.3	20.2	ND	15.6	13.3	17.6
Microsomal wash	1	2.3	2.0	ND	0.1	5.8	3.2	1.0	2.4	0.0
	2	1.9	0.7	ND	0.0	1.0	ND	0.6	0.5	0.6
Soluble fraction	1	35.3	3.4	ND	1.0	9.8	ND	3.8	7.3	1.3
	2	26.6	1.1	ND	1.6	6.2	ND	3.0	2.5	3.3
Floating fraction	1	1.0	0.4	8.8	ND	2.3	1.2	0.8	2.0	0.0
	2	0.3	0.1	7.9	0.0	1.4	ND	0.3	0.3	0.3
Recovery	1	96.8	93.4	57.0[c]	96.7	98.5	71.2[c]	77.7	96.0	64.8
	2	85.1	93.5	91.4[c]	83.9	99.4	ND	82.7	84.9	89.6

[a] Expressed as percentages of total.
[b] ND, not determined.
[c] Sum of the fractions assayed.

liver microsomal fraction (19, 23, 27), but there is also a distinct lysosomal activity (27). In spleen the enzyme is essentially lysosomal (28).

The distribution of the total glucose-6-phosphatase was similar to that of β-glucuronidase and arylesterase. However, about 40–50% of the total activity was inhibited by 2 mM fluoride and the inhibitable activity had a peak in the light mitochondrial fraction with considerable shoulders on both sides. Acid phosphatase is known to be sensitive to fluoride (17, 18) whereas the true microsomal glucose-6-phosphatase is not (18). The acid phosphatase activity of a crude lysosomal preparation of the BHK cells extends well into the pH range of the glucose-6-phosphatase assay (pH 6.5) whereas the acid phosphatase of the BHK cell plasma membrane has a negligible activity at this pH (17). Our conclusion is that the fluoride-sensitive glucose-6-phosphatase activity probably was a manifestation of the lysosomal acid phosphatase and that the light mitochondrial fraction was enriched in lysosomes. Lysosomes of several other tissues have similar sedimentation characteristics (10, 23, 26–30).

The fluoride-resistant glucose-6-phosphatase appeared to be a fairly adequate marker of the BHK cell microsomal fraction as it is in liver, kidney, and hamster intestine (10, 18, 23).

The floating fraction contained less than 1% of the cellular protein and phospholipid but more than 8% of the neutral lipid (Table 1). After a more extensive purification, the protein content of the floating fraction was reduced to about 0.2% of the total (**Table 2**). This preparation was strongly enriched in the lysosomal marker enzymes, including acid β-glycerophosphatase (Table 2), which indicates the presence of higher purified lysosomal material in the floating fraction. The lower enrichments in the previous experiments (Table 1 and Fig. 1) were evidently due to the residual cytoplasmic protein in the preparations.

The relatively low percentages of the activities of the hydrolases in the soluble fraction (Tables 1 and 2) indicate that only little damage was caused to the lysosomes by the hypotonic homogenization medium, which was used to achieve an efficient disruption of the cells (14).

Phospholipids of the cell fractions

Table 3 shows the phospholipid contents and compositions of the subcellular fractions. Phosphatidylcholine and phosphatidylethanolamine accounted for 70–85% of the lipid phosphorus of the BHK cell and its subcellular fractions. The rest was divided mainly between sphingomyelin, phosphatidylserine, cardiolipin, phosphatidylinositol, and LBPA.

The floating fraction exhibited the largest single deviation from the average cellular composition, since LBPA contributed almost 20% of its lipid phosphorus. The increase in LBPA was compensated mainly by the decreased percentage of phosphatidylcholine.

Of the sedimentable fractions, the light mitochondrial fraction contained a significantly elevated amount of LBPA, about 3% of phospholipids. This fraction was also enriched in sphingomyelin and phosphatidylserine, with a corresponding decrease in phosphatidylcholine.

The microsomal fraction, too, had elevated concentrations of sphingomyelin and phosphatidylserine, although it had less than those of the light mitochondrial fraction. Only the mitochondrial fraction was enriched in cardiolipin.

Fig. 2 shows the relative concentrations[2], on a protein basis, of some phospholipids in the subcellular fractions. This allows a direct comparison with the enzyme distributions.

Lysobisphosphatidic acid had a pronounced peak in the light mitochondrial fraction and another in the floating fraction. Thus its distribution resembled more that of the fluoride-sensitive glucose-6-phosphatase than of any of the other enzymes, which suggests that it was specifically located in lysosomes. The light mitochondrial fraction was the largest contributor of LBPA; it contained about 33% of the postnuclear LBPA. Only a small proportion of the cellular LBPA (about 4% of the amount in the postnuclear supernatant) was recovered in the floating fraction, in spite of its high enrichment among the phospholipids of the fraction.

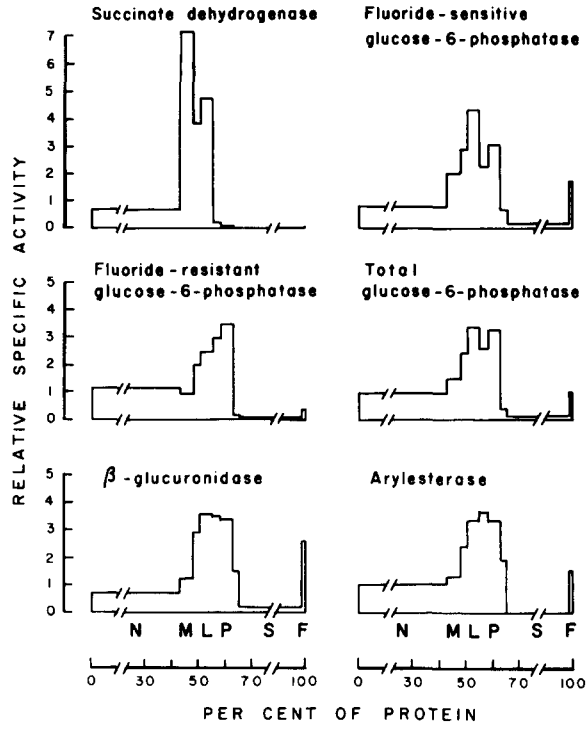


Fig. 1. Distributions of marker enzymes in experiments 1 and 2 (Table 1). Symbols of the cell fractions: N, nuclear fraction; M, mitochondrial fraction; L, light mitochondrial fraction; P, microsomal fraction; F, floating fraction. The small unnamed fractions are the washings of the preceding fractions.

[2] The relative concentration is defined analogously to the relative specific activity of the enzymes (10, 11) as the percentage of the total cellular content of a compound in a cell fraction divided by the percentage of protein in the fraction.

TABLE 2. Enrichment of hydrolases in the purified floating fraction of the BHK cells[a]

	Protein	Glucose-6-P-ase (F-sensitive[b])		Acid Phosphatase (F-sensitive[c])		β-Glucuronidase	
Total Homogenate (50 culture bottles)	440 mg	88.1 μmole/hr		135.4 μmole/hr		16.1 μmole/hr	
	%	%	r.s.a.[d]	%	r.s.a.	%	r.s.a.
Nuclear fraction	25.1	26.8	1.07	11.5	0.46	10.9	0.43
Particulate fraction	21.0	59.9	2.85	70.0	3.33	71.7	3.41
Soluble fraction	51.8	7.03	0.14	14.7	0.28	10.9	0.21
Purified floating fraction	0.18	3.14	17.2	5.77	31.4	1.47	8.0
Recovery	98.1	96.9		102.0		95.0	

[a] The postnuclear supernatant was centrifuged at 82,000 g_{av} for 60 min to obtain a crude floating fraction, a soluble fraction, and a particulate fraction. The floating fraction was purified by reflotation through 0.4 M sucrose as described in Materials and Methods.
[b] 50.2% of the total activity of the homogenate.
[c] 88.8% of the total activity of the homogenate.
[d] r.s.a., relative specific activity.

TABLE 3. Phospholipid compositions of the BHK cell fractions. Means ± standard deviation of n analyses[a]

	Cell	Nuclear Fraction	Mitochondrial Fraction	Light Mitochondrial Fraction	Microsomal Fraction	Floating[b] Fraction
Phospholipid/Protein (g/g)	0.16 ± 0.02	0.15 ± 0.03	0.40 ± 0.03	0.55 ± 0.10	0.72 ± 0.08	0.88
n	14	3	4	5	5	1
Phosphatidylcholine	55.1 ± 2.9	60.8 ± 1.8[c]	44.9 ± 4.5[c]	47.6 ± 1.9[c]	56.8 ± 2.2	40 ± 4[c]
Phosphatidylethanolamine	25.7 ± 1.2	24.6 ± 0.2	30.7 ± 2.2[c]	27.6 ± 2.1	24.8 ± 1.4	30 ± 6
Sphingomyelin	6.5 ± 0.7	4.9 ± 0.9	6.3 ± 1.7	9.0 ± 0.5[c]	7.7 ± 0.8[c]	7 ± 3
Phosphatidylserine	4.3 ± 1.0	3.3 ± 0.6	5.1 ± 2.5	6.3 ± 0.5[c]	5.7 ± 1.1[c]	2 ± 2
Cardiolipin	3.2 ± 1.1	2.2 ± 0.4	7.1 ± 1.6[c]	3.3 ± 1.0	0.2 ± 0.2[c]	0 ± 1[c]
Phosphatidylinositol	2.3 ± 0.9	2.6 ± 0.4	2.2 ± 0.5	2.3 ± 0.8	2.8 ± 0.6	2 ± 3
LBPA	1.7 ± 0.6	1.0 ± 0.8	2.5 ± 1.4	3.0 ± 1.1[c]	1.1 ± 0.8	19 ± 6[c]
Lysophosphatidylcholine	0.6 ± 0.4	0.3 ± 0.2	1.1 ± 0.4	0.7 ± 0.3	0.7 ± 0.2	0
Phosphatidic acid	0.6 ± 0.5	0.3 ± 0.3	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.2	0
n	7	3	4	4	4	3

[a] Percent of lipid phosphorus in lipid classes. The figures include the alkenylacyl and alkylacyl types of phosphoglycerides (13).

[b] The analytical data of the floating fraction were less accurate than in the case of the other fractions due to the smaller amounts of material.

[c] Differences to homogenate significant at the confidence level of >0.95 in the rank–sum test (31).

Cardiolipin followed closely the distribution of succinate dehydrogenase in accordance with its exclusive localization in mitochondria from other sources (1,2). Sphingomyelin and phosphatidylserine were distributed about equally between the microsomal and the light mitochondrial fractions, suggesting the enrichment of plasma membrane fragments (13) in both fractions. Phosphatidylcholine and phosphatidylinositol had distributions (not shown) similar to that of the fluoride-resistant glucose-6-phosphatase.

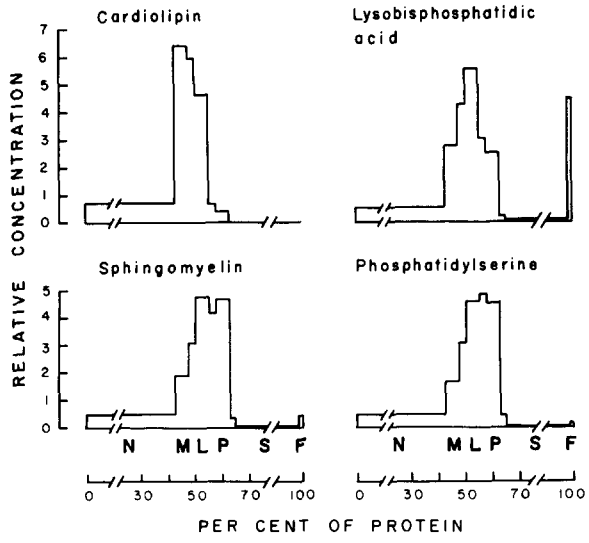


Fig. 2. Distribution patterns of phospholipids in experiments 1 and 2 (Table 1). Symbols of cell fractions as in Fig. 1. Average recoveries of the two experiments were: cardiolipin, 95%; lysobisphosphatidic acid, 109%; sphingomyelin, 91%; phosphatidylserin, 130%.

Neutral lipids of the cell fractions

About a third of the total BHK cell lipid extract consisted of neutral lipids (**Table 4**). Triglycerides, cholesteryl esters, and free cholesterol amounted to about 70–80% of the neutral lipid fraction.

The floating fraction consisted mainly of neutral lipids (Tables 1 and 4) consistent with the low density of the particles. The neutral lipid/phospholipid ratio of three preparations was 18 ± 5 (SD). About 70–80% of the neutral lipids were triglycerides and cholesteryl esters whereas free cholesterol was a smaller component (Table 4).

All sedimentable fractions contained large amounts of neutral lipids of which 30–50% were cholesteryl esters and triglycerides (Table 4). The highest neutral lipid/phospholipid ratio occurred in the light mitochondrial fraction.

The distributions of the individual neutral lipids in relation to protein are shown in **Fig. 3.** Triglycerides and cholesteryl esters were highly enriched in the floating fraction and also in the light mitochondrial fraction. The distribution patterns resembled the profiles of lysobisphosphatidic acid and the fluoride-sensitive glucose-6-phosphatase, except that the balance between the floating and the sedimentable fractions was different.

The pattern of free cholesterol was similar to those of sphingomyelin and phosphatidylserine, with about equal enrichment in both the microsomal and the light mitochondrial fractions.

Morphology of the neutral lipid-rich particles

Fig. 4A shows a general view of the floating fraction of the BKH cells fixed on a filter. Few of the

TABLE 4. Neutral lipid compositions of the BHK cell fractions

	Cell		Nuclear Fraction		Mitochondrial Fraction		Light Mito. Fraction		Microsomal Fraction		Floating Fraction	
Experiment no.	1	2	1	2	1	2	1	2	1	2	1	2
$\frac{\text{Neutral lipid}}{\text{Phospholipid}}\left(\frac{g}{g}\right)$	0.66	0.40	0.51	0.44	0.39	0.43	0.46	0.60	0.32	0.33	15.9	23.7
$\frac{\text{Cholesterol}}{\text{Phospholipid}}\left(\frac{mol}{mol}\right)$	0.30	0.22	0.23	0.21	0.23	0.21	0.34	0.36	0.30	0.28	1.9	1.9
Percent of neutral lipid in lipid classes	%		%		%		%		%		%	
Cholesteryl esters	24	23	24	18	18	27	19	24	17	14	50	20
Triglycerides[a]	23	27	25	26	26	26	23	19	15	16	24	58
Cholesterol	22	28	22	24	30	25	36	30	47	43	6	4
Diglycerides	13	8	15	15	11	10	10	11	11	13	9	7
Fatty acids	17	15	14	16	16	13	13	15	9	14	11	12

[a] Including alkyldiacyl glycerols (13).

particles had the appearance of a typical "naked" lipid droplet (32, 33). Most particles had an irregular shape and were surrounded by a darkly stained layer of variable thickness that often formed large dark bulges on the sides of the pale gray areas of neutral lipid. In favorable sections, the particles were seen to be enclosed by a trilaminar membrane. At a higher magnification, the dark material often was resolved into prominent multilamellar structures (Fig. 4B).

The same types of particles were observed also in the intact cells, where their morphology was easier to preserve than in the purified preparations. Occasional naked cytoplasmic lipid droplets were encountered (**Fig. 5A**); however, many of the droplets showing lipid-like staining properties were enclosed in membrane-bound vacuoles among different amounts of additional materials. A series of these particles is shown in Figs. 5B–5F. Some of the particles contained little else but the pale gray droplet and the membrane (Fig. 5B) whereas in others the droplet(s) constituted a smaller fraction of the visible contents (Figs. 5C–5E). The additional material in the vacuoles appeared either as a finely granular or flocculent matrix or as striking multilamellar arrays (Figs. 5C–5E). The trilaminar units of these arrays frequently seemed to originate from the gray area in neatly curving closely stacked arcs (Fig. 5C). In other cases the lamellar material formed apparently open-ended curls in the vacuoles (Fig. 5D). In some cases an electron-lucent "halo" was visible beneath the outer membrane of the particles (Fig. 5E), although in many of the other pictures the intravacuolar materials reached close to the envelope.

Acid glycerophosphatase activity could be demonstrated cytochemically in some of the lipid-rich vacuoles (Fig. 5F), which suggests that they were lysosomes.

DISCUSSION

Our results show that lysobisphosphatidic acid (LBPA) was highly enriched in the floating neutral lipid-rich fraction of the BHK cell homogenates, constituting about 20% of the phospholipids of three preparations.

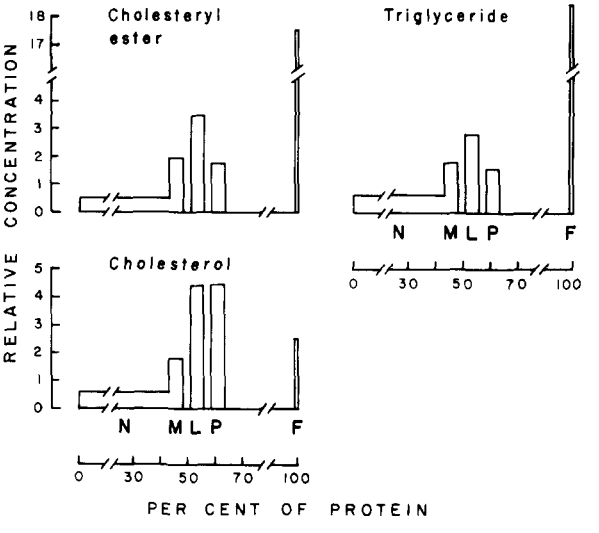


Fig. 3. Distribution patterns of neutral lipids. Symbols of cell fractions as in Fig. 1. The wash fractions were not analyzed. The average partial recoveries of the analyzed fractions in the two experiments were: cholesteryl ester, 71%; triglyceride, 71%; cholesterol, 78%.

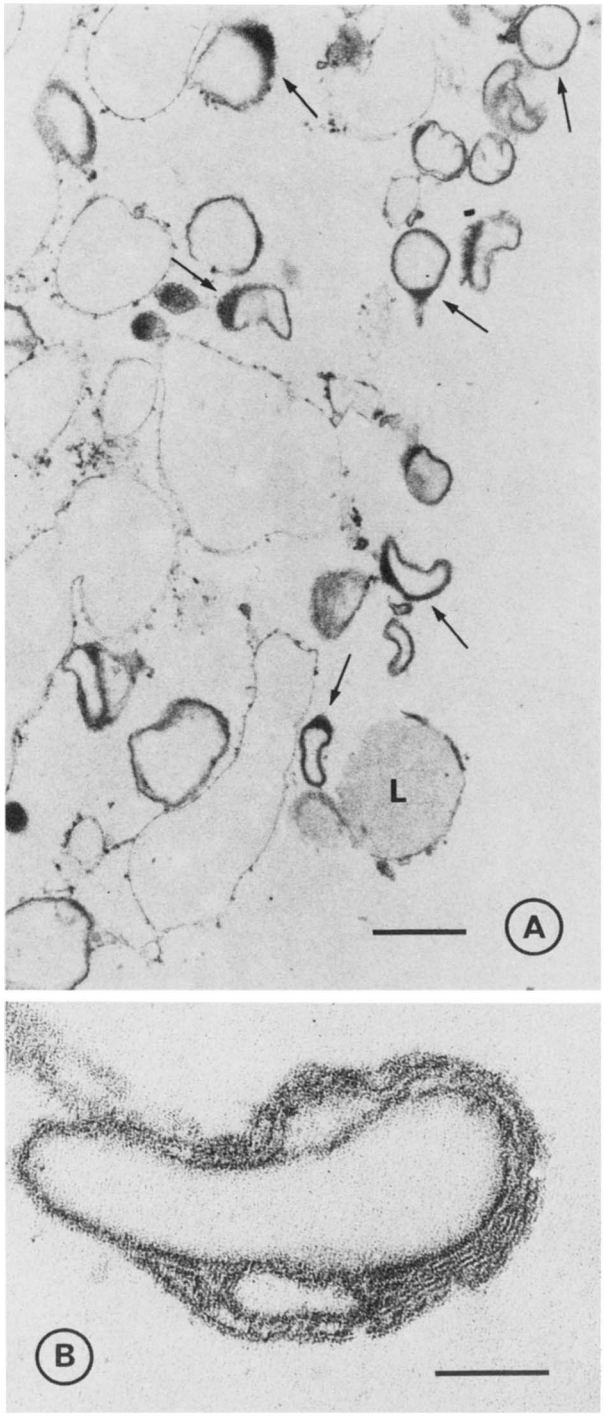


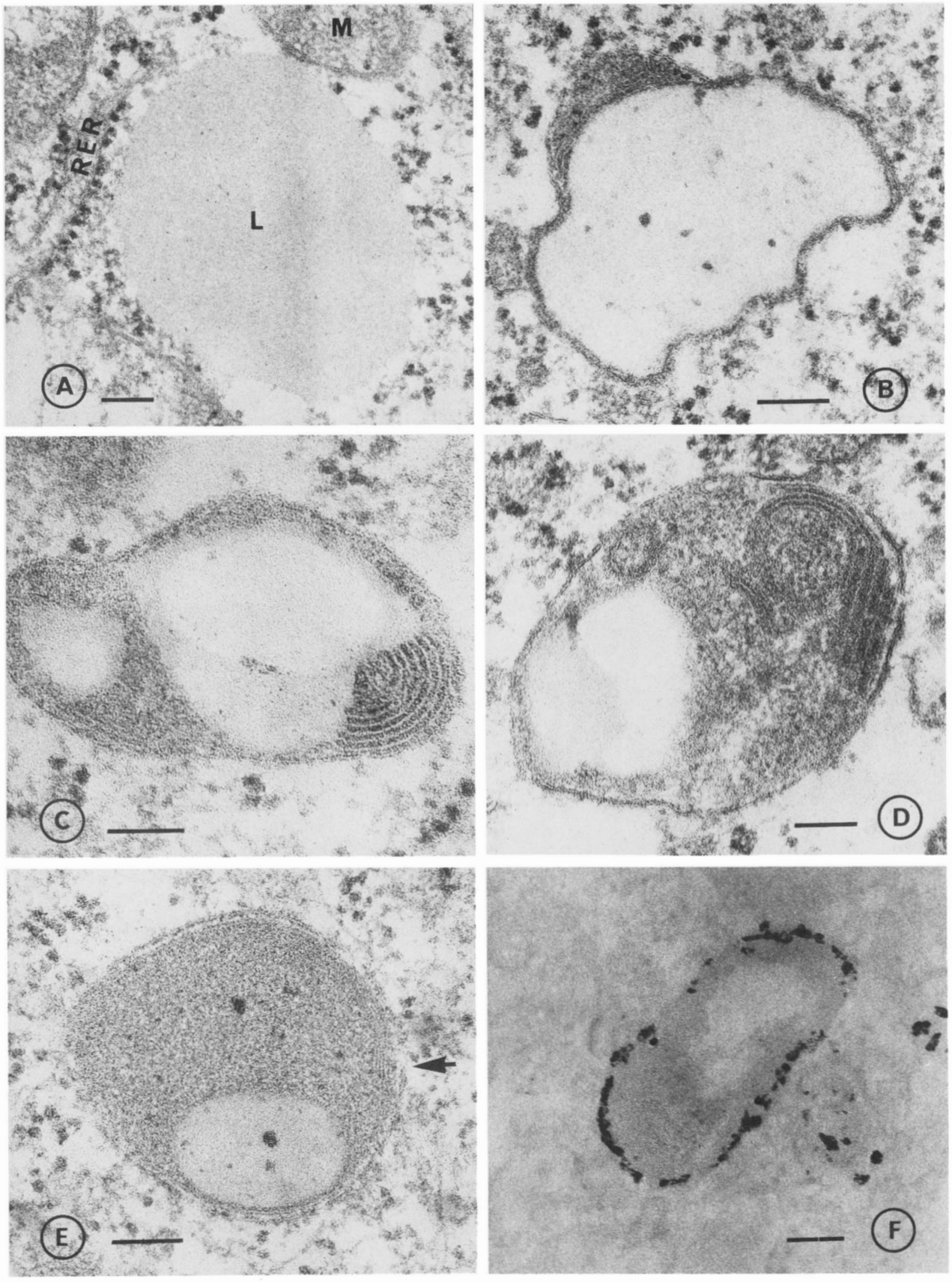
Fig. 4. Floating lipid-rich particles fixed on a Millipore filter. (*A*) A survey micrograph. The surface region of the filter runs through the right half of the picture. The filter material is visible as thin, smoothly curving contours. A thin layer of particles remains on the surface of the filter. An apparently naked lipid droplet is denoted by L. The other particles are believed to be lipid-rich lysosomes because of the presence of electron-dense peripheral material shown by arrows in some cases. Scale = 0.5 μm. Magnification 26,000. (*B*) A lipid-rich particle partially enveloped by several membrane-like layers. At the left side of the particle only one membrane is seen. Scale = 0.1 μm. Magnification 160,000.

The purified floating fraction contained fluoride-sensitive glucose-6-phosphatase, acid β-glycerophosphatase, and acid β-glucuronidase at specific activities 8–30 times higher than the cellular averages. This indicates that lysosomes were highly enriched in the floating fraction. Acid phosphatase is also present in the plasma membranes of the BHK cells and β-glucuronidase in the endoplasmic reticulum (12, 17). However, the possibility of an appreciable contamination of the floating fraction by these membranes can be excluded because of the relatively low concentrations of the characteristic phospholipids of the plasma membrane (sphingomyelin and phosphatidylserine) and endoplasmic reticulum (phosphatidylcholine and phosphatidylinositol) (13) in the floating fraction.

Approximately 70% of the lipids of the floating fraction were triglycerides and cholesteryl esters. These lipids are generally the main constituents of the cytoplasmic lipid droplets that are recovered from the cell homogenates by flotation. However, a qualitative morphological examination of the floating preparations of the BHK cells revealed relatively small numbers of the "naked" lipid droplets.

The appearance of most particles was compatible with that of a lysosome, which is filled with a droplet of neutral lipid; the featureless pale gray area of neutral lipid (32) was surrounded by a membrane and darkly stained amorphous or multilamellar material. Furthermore, similar vacuoles in the intact cells displayed acid phosphatase activity and an occasional "halo" inside the surrounding membrane.

Fig. 5. Morphology of neutral lipid-like material in the BHK cells. (*A*) A cytoplasmic lipid droplet (L). Note the absence of any distinct surface layer. M, mitochondrion; RER, rough endoplasmic reticulum. Scale = 0.1 μm. Magnification 92,000. (*B*) A particle similar to that in 5A but completely surrounded by a membrane. The layer of dark material inside the membrane forms a bulge on one side of the particle. In the bulge, faint lamellar formations are visible. Scale = 0.1 μm. Magnification 128,000. (*C*) A membrane-bound vacuole containing two regions of neutral lipid and multilamellar arrays with an apparent distance of 10–14 nm between the adjacent layers. The lamellae seem to arise directly from the homogeneous material. Scale = 0.1 μm. Magnification 140,000. (*D*) A vacuole containing a small lipid droplet and multilamellar material with two apparent periodicities, 11–12 nm and 20–21 nm. These may possibly represent the same substance sectioned at different angles. The lamellae seem to arise diffusely from the flocculent matrix. Scale = 0.1 μm. Magnification 112,000. (*E*) A dense body-type of lysosome with a lipid droplet. Note the electron-lucent "halo" inside the membrane and the faint lamellar structures marked with an arrow. Scale = 0.1 μm. Magnification 128,000. (*F*). The cytochemical demonstration of acid phosphatase in a lipid-rich vacuole. The electron-opaque reaction product is characteristically concentrated along the periphery of the vacuole. Scale = 0.1 μm. Magnification 100,000.



These hallmarks allow the identification of these organelles as secondary lysosomes (34). The name "lipolysosome" was given to analogous lipid-rich lysosomes by Nehemiah and Novikoff (34).

It seems reasonable to assume that lysobisphosphatidic acid was associated with the lysosomes of the floating fraction. High concentrations of LBPA, 7–27% of phospholipids, have been observed in lysosomes purified from other mammalian sources (3–6). The possibility that the LBPA-rich material in the floating fraction was derived mainly from lysosomes disrupted during the homogenization seems unlikely because of the high activities of the soluble lysosomal enzymes, which remained associated with the particles.

The enrichment of acid hydrolases has demonstrated the presence of lysosomes in the floating neutral lipid-rich cell fractions isolated from kidney (33) and atherosclerotic aorta (25). In the former case, particles of "lipofuscin-like" morphology were present in the preparations and were the probable source of the phosphatase activity (33). Particles resembling those in the floating fraction of the BHK cells have been purified by the same method from heart (35) and atherosclerotic aorta (36, 37).

The reports on the phospholipid compositions of floating fractions isolated from a number of mammalian tissues (35, 38–41) do not reveal signs of the presence of LBPA-like lipids. Apart from the possibility that LBPA remained undetected even if present, this may mean either that none of the preparations contained significant numbers of lysosomes or that the lysosomes present were devoid of LBPA. Several examples are known of lysosomes that contain only little, if any, LBPA (5, 6, 8).

The floating lysosomes formed only a small part of the total lysosomal population of the BHK cells, judged by the high percentage of the total hydrolase activities recovered in the other cell fractions. Similar large proportions of LBPA and, more unexpectedly, triglycerides and cholesteryl esters were associated with the sedimentable fractions. These three lipids, unlike the other lipids studied, were enriched preferentially in the light mitochondrial fraction. Thus, their distribution patterns resembled that of the lysosomal marker enzyme, fluoride-sensitive glucose-6-phosphatase, and were different from those of the mitochondrial and microsomal markers. It appears therefore, that LBPA and the neutral lipids were enriched also in that population of lysosomes that had a higher average density than that of the fractionation medium.

The lysosomes of the BHK cells probably form a continuum of varying density caused by a variable content of neutral lipids, the floating lysosomes representing an arbitrary cut at the low-density end of the population. It is probable that a large proportion of the total cellular triglycerides and cholesteryl esters were associated with the lysosomes. In the atheromatous aorta the cholesteryl ester-rich lysosomes, too, form a broad density distribution, the most lipid-rich particles floating on top of the density gradients (25). Interestingly, Warburton and Wynn (42) recently found in another line of hamster fibroblasts a population of lysosomes rich in triglycerides and cholesteryl esters. These may be identical to the lipid-rich lysosomes of the BHK cells.

The reasons for the high content of neutral lipids in the lysosomes of the BHK cell are not known. It is possible that the lysosomes are deficient in lipases with the resultant accumulation of undigested lipids and the formation of multilamellar "lipofuscin pigment" (43). This is the etiological cause of Wolman's disease (44) and, possibly, atherosclerosis (45). On the other hand, the lysosomes of the BHK cells may be engaged in an active autophagic digestion of cytoplasmic lipid droplets in order to mobilize their fatty acids for metabolic needs. In this case the lamellar material around the intralysosomal lipid droplets may be composed of the hydrolysis products of the neutral lipids, such as those observed around chylomicrons digested by lipoprotein lipase (46).

The high concentration of lysobisphosphatidic acid in the lysosomes of the BHK cells may be associated with the presence of the intralysosomal neutral lipids. This notion is supported by the finding that the cellular contents of LBPA and triglycerides increase simultaneously when a hypertrophy of lysosomes occurs in the BHK cells.[3] The biological significance of this possible association is not known. The floating fraction appears to be a convenient source of highly purified lipid-rich lysosomes for further studies of these questions.

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