Lipid composition of mitochondria from bovine heart, liver, and kidney

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ABSTRACT Highly purified preparations of mitochondria from bovine heart, liver, and kidney were isolated and characterized by electron microscopy, oxidative phosphorylation ability, cytochrome *c* reductase activity, and cytochrome content. Components of lipid extracts of the preparations were determined by thin-layer chromatography, diethylaminoethyl-cellulose column chromatography, and spectrophotometric procedures. The major phospholipids were identified by their chromatographic behavior, IR spectrometry, and paper chromatography of their hydrolysis products.

The lipid content of the mitochondria paralleled that of the components of the electron transfer chain, heart mitochondria being richest and liver mitochondria poorest in lipid. Heart mitochondria contain equal concentrations of coenzyme Q and cholesterol (1%); the highest cholesterol content (4.7%) was found in mitochondria from kidney. The phospholipids of mitochondria from the three organs were qualitatively and quantitatively very similar. The major polar lipid components (cardiolipin, choline glycerophosphatides, and ethanolamine glycerophosphatides) were present in a molar ratio of 1:4:4.

It is suggested that mitochondria from different sources contain characteristic lipids, mainly phospholipids, of which cardiolipin is particularly diagnostic of the source of the mitochondria.

KEY WORDS mitochondria · preparation · purification · lipid composition · enzymic activity · cytochromes · phospholipids · separation · analysis · cardiolipin · coenzyme Q · cholesterol

Abbreviations: TLC, thin-layer chromatograpny; DEAE-cellulose, diethylaminoethyl cellulose; cardiolipin, tetra-acyl (alkyl) glycerol diphosphatide; MPL, mitochondrial phospholipid.

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LIPIDS ARE important major components of subcellular particles. Interest in one organelle, the mitochondrion, is particularly great because lipids have been demonstrated to be essential for mitochondrial functions (1, 2). The problems associated with the determination of the lipid composition of subcellular particles have recently been considered in detail and the literature has been reviewed (3). It was pointed out that difficulties associated with the isolation of pure particles, their characterization by electron microscopy and chemical means, and the determination of their lipid composition have prevented accurate definition of lipid composition of subcellular particles, including mitochondria. This report presents procedures suitable for isolation of very pure mitochondria from three bovine organs; evidence of their purity, morphological integrity, and functional ability; procedures for lipid analysis; and the lipid composition of the preparations.

MATERIALS AND METHODS

Preparation of Mitochondria

The large-scale procedure used for preparation of mito chondria was essentially that described by Crane, Glenn, and Green (4) for bovine heart mitochondria, with appropriate modifications for kidney and liver mitochondria (Table 1).

The organs were obtained fresh—directly from the killing floor of the slaughterhouse—and transported to the laboratory in crushed ice. All general operations were carried out in a cold room at 4°C and centrifugations were performed in refrigerated centrifuges. The left ventricle of the heart and the renal cortex (easily

TABLE 1 CONDITIONS FOR PREPARATION OF MITOCHONDRIA BLENDING

Source	Weight of Tissue	Volume of Sucrose	Blending Time	Rheostat Setting	Low Speed Sedimentation Speed*	Low Speed Sedimentation Time
	g	*liters*	*sec*		*rpm*	*min*
Heart	400	1.2	45	120	1900	11
Liver	500	1.5	20	65	1400	8
Kidney	500	1.5	35	80	1400	8

* The speeds are for the 13 liter International centrifuge. 1900 rpm is equivalent to 1600 *g* maximal force at the bottom of the cup.

seen and separated in bovine kidney) were selected for preparation of mitochondria. Tissues were trimmed free from adipose tissue and encapsulating outer integuments.

The trimmed tissue was cut into cubes and ground in an electric meat grinder (containing a plate of $^3/_{32}$ inch holes). The liver cubes were washed several times with 0.25 M sucrose–0.01 M phosphate buffer before they were placed in the meat grinder. The paste was then blended in 0.25 M sucrose–0.01 M phosphate buffer (pH 7.4) in the w/v ratios shown in Table 1, in an overhead blender with a 0.5 hp motor (Dunmore Co., Racine, Wis.) fitted with a 25 cm long shaft, and a three pointed blade which described a circle of radius 7 cm. The blending time and speed, controlled with a powerstat, were adjusted for each tissue (Table 1).

The homogenate was sedimented at low speed to remove debris, unbroken cells, and nuclei, with either a 13 liter International refrigerated centrifuge with aluminum cups (Table 1), or the International PR-2 (276A rotor and 353A cups holding four 1 liter plastic bottles; modified in the factory to attain a speed of 2500 rpm at full load, 12 min at 2500 rpm), or the M.S.E. Mistral 6 liter refrigerated centrifuge (12 min at 2200 rpm for heart mitochondria; smaller forces were used for kidney and liver mitochondria).

The red supernatant fraction from the centrifugations was decanted through four layers of cheesecloth and diluted with 0.25 M sucrose (heart 8:3 liters; kidney and liver, 1:1). The diluted solution was centrifuged in a continuous flow refrigerated Sharples supercentrifuge (Model TIP) at 50,000 rpm (62,000 *g*) at a flow rate of 150–200 ml/min ($^3/_{32}$ inch nozzle) to give the mitochondrial fraction. "Ice" cubes made with 0.25 M sucrose were added to maintain the temperature at 0°C.

The crude mitochondrial paste was purified further, essentially according to Hatefi and Lester (5). It was suspended by homogenization with 6 volumes of 0.25 M sucrose [made 0.01 M with respect to Tris buffer [tris (hydroxymethyl) amino methane] that had been adjusted to pH 7.8 at 0°C] in a loose-fitting homogenizer (Teflon pestle) of the Potter-Elvehjem type (approximately 0.010 inch net clearance rather than the usual 0.006 inch clearance). The suspension was sedimented at 18,000 rpm for 10 min in a Spinco preparative centrifuge (No. 30 rotor). Two distinct sedimented layers were obtained. The upper layer was lighter in color and consisted of swollen mitochondria, mitochondrial fragments, and contaminating microsomes and lysosomes. The lower layer, designated as the heavy mitochondrial fraction, was deep brown. The upper layer was separated from the lower layer by means of a stirring rod and discarded. The lower layer was then removed from the small dark button at the bottom of the tube. The heavy fraction was resuspended in half the original volume and the suspension sedimented as before. Any residual upper layer as well as any lower dark button was again discarded. The purified mitochondria were finally resuspended in 0.25 M sucrose.

Yields were somewhat variable. Seven ventricles yield roughly 100 g wet weight of mitochondrial paste (from the Sharples bowl) and about 3.0 g (calculated from protein determinations) of highly purified bovine heart mitochondria. It is important to work as rapidly as possible to obtain the best preparations, especially in the preparation of liver mitochondria. The preparation of crude mitochondria takes about 1.5 hr from the time of blending and the highly purified mitochondria are obtained about 1.5 hr later.

Electron Microscopy

The mitochondria were first sedimented into a pellet. The supernatant fraction was replaced by a 2% solution of ice-cold unbuffered osmium tetroxide. The pellets were fixed for 2 hr and then rapidly dehydrated in a graded series of methanol–water mixtures, and allowed to return to room temperature in absolute methanol. They were then embedded in Epon 812. Sections were cut on a Porter-Blum ultramicrotome with a glass knife and counterstained with lead by the lead citrate method of Reynolds (6). Thin sections were examined in an RCA EMU 3E electron microscope.

Determination of Total Lipid, Coenzyme Q, Cholesterol, Total Unsaturation, Plasmalogen, Cytochromes a + a_3 and b, Total Protein, and Enzyme Assays

Lipid for TLC analysis was extracted from the mitochondrial suspension in 0.25 M sucrose with chloroform–methanol 2:1 (7–9) under an atmosphere of pure nitrogen to minimize autoxidation. Nonlipid was removed from extracts used for TLC by Sephadex column chromatography (6a) or by partition according to the method of Folch, Lees, and Sloane Stanley (7) using 0.017% $MgCl_2$ in the first partition. The lower lipid layer was then washed three times with "upper phase"

and evaporated to dryness at room temperature in a flash evaporator. The almost dry sample was dried in vacuo for 15 min over KOH pellets and the amount of lipid was determined by weighing.

DEAE-cellulose column chromatography was performed on lipids extracted with chloroform–methanol 2:1, but not washed (10). The extract was evaporated to dryness and the residue reextracted with a small volume of chloroform–methanol 2:1, by means of a Potter-Elvehjem homogenizer equipped with a Teflon pestle to insure efficient extraction. The insoluble residue was sedimented in a clinical centrifuge, washed several times with chloroform–methanol 2:1, and then discarded. The lipid extracts were combined and stored under nitrogen at −20°C. Extracts were applied to columns both prior to and after removal of nonlipid contaminants by Sephadex column chromatography.

The neutral lipid was defined as the fraction eluted from DEAE-cellulose with chloroform. CoQ (11) and cholesterol (12) were determined in the neutral lipid fractions.

Cytochrome *a* (+ a_3) and cytochrome *b* were determined spectrophotometrically (Cary Model 14) on mitochondria clarified by the addition of sodium deoxycholate to a final concentration of 0.2% (13, 14). The cytochrome values for kidney and liver mitochondria are approximate because the reduced chromogens are unstable.

Protein was determined by the biuret procedure (15) with bovine serum albumin as protein standard. Lipid phosphorus was determined by a modification of the phosphorus method of Chen, Toribara, and Warner (16). Unsaturation was determined by measuring bromine uptake spectrophotometrically (17). Plasmalogen was determined by the *p*-nitrophenylhydrazone reaction, the molar extinction of higher fatty acid aldehyde *p*-nitrophenylhydrazones being taken as 23,800 (18).

Succinate was assayed in cytochrome *c* reductase (EC 1.3.99.1) activity (19) and reduced NAD-cytochrome *c* reductase (EC 1.6.99.3) activity (20) with or without the addition of lipid [mitochondrial phospholipids were added as aqueous micelles (21, 22)]. Oxidative phosphorylation was measured with the use of hexokinase to convert ATP to the more stable glucose 6-phosphate. The oxygen consumed was measured manometrically; the esterified phosphate was determined by the Martin and Doty method as modified by Lindberg and Ernster (23).

Quantitative TLC of Phospholipids

One- and two-dimensional TLC were carried out with an adsorbent (24) composed of silicic acid (Silica Gel plain, Warner-Chilcott Laboratories, Richmond, Calif.) mixed with 10% by weight magnesium silicate (Allegheny Industrial Chemical Co., Butler, N.J.). The adsorbent (200 g), mixed with about 80 porcelain grinding balls $^1/_2$ inch in diameter (total weight about 300 g) in a 2 quart glass jar, was heated at 150°C for 6 hr and the jar was capped, cooled, and rotated for 2 hr on a ball mill. A slurry of 20 g of adsorbent in 60–73 ml of water was used to spread five 8 × 8 inch glass plates to a thickness of 250 μ. Plates were air-dried, activated at 120°C for 30 min, and cooled for 20 min just prior to sample application. Mitochondrial lipids in chloroform–methanol 2:1 or 9:1 (5–10 mg/ml) were applied as a row of small spots over a length of about 1 cm. Immediately, plates were placed in chromatography chambers completely lined on all four vertical walls with Whatman 3 mm filter paper saturated with solvent.

Four developing solvents were routinely used: I, chloroform–methanol–water 65:25:4; II, 1-butanol–acetic acid–water 60:20:20; III, chloroform–methanol–28% by weight aqueous ammonia 65:35:5; and IV, chloroform–acetone–methanol–acetic acid–water 5:2:1:1:0.5. For two-dimensional TLC solvent I followed by II or solvent III followed by IV were used. Chromatograms were dried for 10 min between development in the first and second dimensions, usually in chromatography chambers flushed with dry nitrogen.

General spray reagents used were rhodamine 6G (10), and sulfuric acid–potassium dichromate for charring (24). The molybdenum (25) and ninhydrin (26) spray reagents were used for specific detection of substances containing phosphorus and free amino groups respectively.

Quantitative analysis by TLC was accomplished by charring and transmission densitometry (24) with standards of pure preparations of mitochondrial choline and ethanolamine glycerophosphatides, and cardiolipin isolated by DEAE-cellulose column chromatography. Purity of the phospholipid standards was judged by TLC and IR spectrophotometry. This analysis was combined with phosphorus assay of spots (27) as an additional means for determination of the molar ratios of phospholipids.

Isolation of Phospholipids by TLC and Sephadex Column Chromatography

Small amounts (2 mg or less) of phospholipids were isolated by preparative TLC in solvent I. Spots were detected with a water spray and lipids were eluted from the moist adsorbent (by vigorous shaking) with 10 ml of chloroform–methanol 2:1 saturated with water. The adsorbent was filtered on a sintered glass filter and again extracted. The combined filtrates were evaporated in the cold in a rotary evaporator initially flushed with pure nitrogen (10). The lipid thus obtained was contaminated with adsorbent, which was removed by Sephadex column

TABLE 2 SCHEME OF ELUTION OF MITOCHONDRIAL LIPIDS FROM DEAE-CELLULOSE COLUMNS

Fraction No.	Eluting Solvent	Volume	Components
		ml	
1	$CHCl_3$	450	Neutral (nonionic) lipids*
2	$CHCl_3$–CH_3OH, 9:1	250	Choline glycerophosphatide (lecithin)
3	$CHCl_3$–CH_3OH, 7:3	425	Ethanolamine glycerophosphatide (PE)
4	CH_3OH	400	Nonlipid (primarily sucrose)
5	$CHCl_3$–acetic acid	325	"Altered" PE† and free fatty acid‡
6	Acetic acid	425	Serine glycerophosphatide, if present
7	CH_3OH	250	Little or no lipid used to remove HAc
8	$CHCl_3$–CH_3OH–NH_3§	450	Cardiolipin
9	$CHCl_3$–CH_3OH–NH_3§ + 0.01 M NH_4Ac‖	450	Inositol glycerophosphatide
10	CH_3OH	400	Cardiolipin and breakdown products¶, and phosphatidyl inositol
11	HAc	500	Restore column to the acetate cycle

Total mitochondrial lipid (200–240 mg), containing approximately 25% by weight of sucrose, was placed on a column 2.5 (I.D.) × 20 cm high. A 4.5 cm (I.D.) column allows a 2.5 times greater loading factor. Column volumes for 2.5 and 4.5 cm (I.D.) columns, respectively, are 75 and 230 ml; corresponding flow rates are 3 and 10 ml/min. A similar elution pattern was obtained with 190 mg of lipid from which nonlipids were first removed by passing the mixture through a Sephadex column (28). For these studies, Selectacel DEAE, type 20 (lot No. 1680), containing 0.82 meq/g was used.

* Does not include some acidic materials such as free fatty acids which are eluted with $CHCl_3$ from silicic acid columns.

† See Discussion for explanation of term.

‡ Free fatty acid may be lost during evaporation of the solvent.

§ Chloroform–methanol 4:1 containing 20 ml/liter of 28% by weight aqueous ammonia. The aqueous ammonia was prepared by bubbling gaseous ammonia into distilled water at 0–4 °C.

‖ Ammonium acetate prepared by mixing appropriate amounts of redistilled acetic acid and freshly prepared 28% ammonia as in (§).

¶ The acidic breakdown products seem to be formed by decomposition of cardiolipin since rechromatography of pure cardiolipin gives rise to the same components.

chromatography (6a) using 0.8 cm (I.D.) × 10 cm columns. The lipid was dissolved in chloroform–methanol 19:1 saturated with water and eluted with 35 ml of the same mixture, and the column was cleared with 35 ml of methanol–water 1:1 before reuse.

DEAE-Cellulose Column Chromatography

The general procedure has been described (10, 26). Regular grade Selectacel DEAE (Brown Co., Berlin, N.H.) was washed on a sintered glass filter with three cycles of aqueous 1 N HCl (3 column volumes), water (to neutrality), aqueous 0.1 N KOH (3 column volumes), and water (to neutrality). The ion exchanger was then washed with 3 column volumes of glacial acetic acid and 3 of methanol. The adsorbent was air-dried and then dried to constant weight in a desiccator over KOH pellets. Portions of the dry adsorbent, 15 g for 2.5 (I.D.) × 20 cm and 45 g for 4.5 (I.D.) × 20 cm columns, were allowed to stand overnight in glacial acetic acid, packed into chromatography tubes (25–30 cm long), and washed with 4 column volumes of methanol, 4 of chloroform–methanol 1:1, and 4 of chloroform–methanol 9:1 or chloroform. The column was tested for uniform packing (26). Samples (not more than 250 mg for 2.5 × 20 cm columns) were applied in 10–30 ml of chloroform or chloroform–methanol 9:1. Various elution schemes were explored. The recommended procedure is shown in Table 2. Pure phosphatidyl choline is eluted from DEAE columns with chloroform–methanol 9:1 when chloroform is first used for elution of neutral lipids. A trace of sphingomyelin is eluted with phosphatidyl choline from extracts of less pure mitochondrial preparations. The chloroform–methanol 9:1 is continued until a positive ninhydrin is obtained on an aliquot of the eluate (9). This signifies that all of the lecithin has been eluted and phosphatidyl ethanolamine is then more efficiently eluted with chloroform–methanol 7:3.

The elution of acidic lipids may vary somewhat, according to the effectiveness of the washing of the DEAE-cellulose, the lipid load, and whether or not solvents are free of impurities. Under optimal conditions, most of the cardiolipin is eluted with solvent 8 (Table 2), and the inositol glycerophosphatide with solvent 9; solvent 10 elutes more phosphatidyl inositol and another portion of diphosphatidyl glycerol and its breakdown products. For example, in a typical run, fraction 9 contained diphosphatidyl glycerol (16.22% of total phosphorus), fraction 10 contained phosphatidyl inositol (1.72%), and fraction 11 contained 4.85% of diphosphatidyl glycerol, its breakdown products, and more phosphatidyl inositol.

For preparative purposes we have found it convenient to divide the run into 2 days' work. Phosphatidyl choline

and phosphatidyl ethanolamine are prepared on the 1st day, and diphosphatidyl glycerol and phosphatidyl inositol on the 2nd day. Elution solvent 5 (Table 2) is usually omitted when the method is used for preparative purposes. Fractions 8, 9, and 10 contain ammonium acetate and are readily desalted with the use of small Sephadex columns (0.8 × 20 cm) (6a).

IR Spectrophotometry

Lipid (100–200 μg) was weighed on a Cahn microbalance, mixed with 10 mg of potassium bromide, and pressed into a pellet. Pellet thickness was measured with a micrometer and spectra were obtained by means of a Beckman IR-4 double beam instrument (sodium chloride optics) equipped with a beam condenser. Spectra were compared with those obtained from phospholipids from other sources (10).

Characterization of Hydrolysis Products

Glycerophosphatides of choline, ethanolamine, inositol, and cardiolipin were further characterized by hydrolysis. After acid hydrolysis with 2 N or 3 N HCl (2 mg of lipid per ml of acid) in a sealed tube at 100°C for 2–8 hr, fatty acids and fatty aldehydes were extracted into hexane and examined by TLC or gas chromatography. The GLC data were reported previously (3). The aqueous phase was then evaporated to dryness, water was added, the evaporation was repeated, and the solids were weighed on a Cahn microbalance and dissolved in water. The hydrolysates were examined by paper chromatography for the presence of glycerophosphate, choline, ethanolamine, and inositol. IR spectra of weighed amounts of the mixtures of water-soluble hydrolysis products were obtained by the potassium bromide pellet technique. Spectra were compared with those obtained from the same lipid classes hydrolyzed under the same conditions, or with equimolar amounts of glycerophosphate and choline or ethanolamine exposed to the same hydrolysis and extraction procedures (10).

The presence of inositol in the aqueous hydrolysate was checked by ascending paper chromatography (28) in either propanol–ethanol–water 5:3:2 or butanol–acetic acid–water 4:1:5 (upper phase) on Whatman No. 1 paper, developed to a height of 22 cm at room temperature (about 6 hr). Sugars were detected with a silver nitrate–sodium hydroxide dip reagent (6a).

RESULTS

Electron Microscopy

Typical electron micrographs of the mitochondrial preparations are shown in Figs. 1–3. Heart and kidney mitochondria (Figs. 1 and 2) are replete with cristae; no contaminants are visible. The liver mitochondria shown in Fig. 3 had shorter and fewer cristae than mitochondria from heart and kidney. The lesser degree of contrast between cristae and matrix arises from the denser matrix of liver mitochondria. The cristae appeared to be well preserved. Small vesicles of uncertain origin were observed (<5% by volume of the preparation), even in our best liver mitochondrial preparations.

The characteristics of one of our early preparations of liver mitochondria are shown in Fig. 4 for contrast. Even though the shearing force (45 sec, powerstat 85) used for homogenization was lower than that suitable for heart, it was too large for liver and was subsequently reduced. It may be noted in the electron micrograph that cristae were absent. The mitochondria of this early preparation from liver were swollen and fragmented, lacked matrix, and were incapable of oxidative phosphorylation. It cannot be overly stressed that conditions described for the preparation of mitochondria from one organ may not be applied indiscriminately to another organ.

Oxidative Phosphorylation and Cytochrome c Reductase Activity

All mitochondria used were capable of oxidative phosphorylation with succinate as well as with a mixture of pyruvate and malate as substrates. Electron transfer capacity, as evaluated by measuring succinate-cytochrome *c* reductase activity and NADH-cytochrome *c* reductase activity, is shown in Table 3. The capacity was highest for heart and lowest for liver mitochondria. The higher electron transfer activity is similarly reflected in a greater content of the electron transfer carriers (cytochromes $a + a_3$) and the coenzyme Q content (Table 4).

Total Lipid of Mitochondria

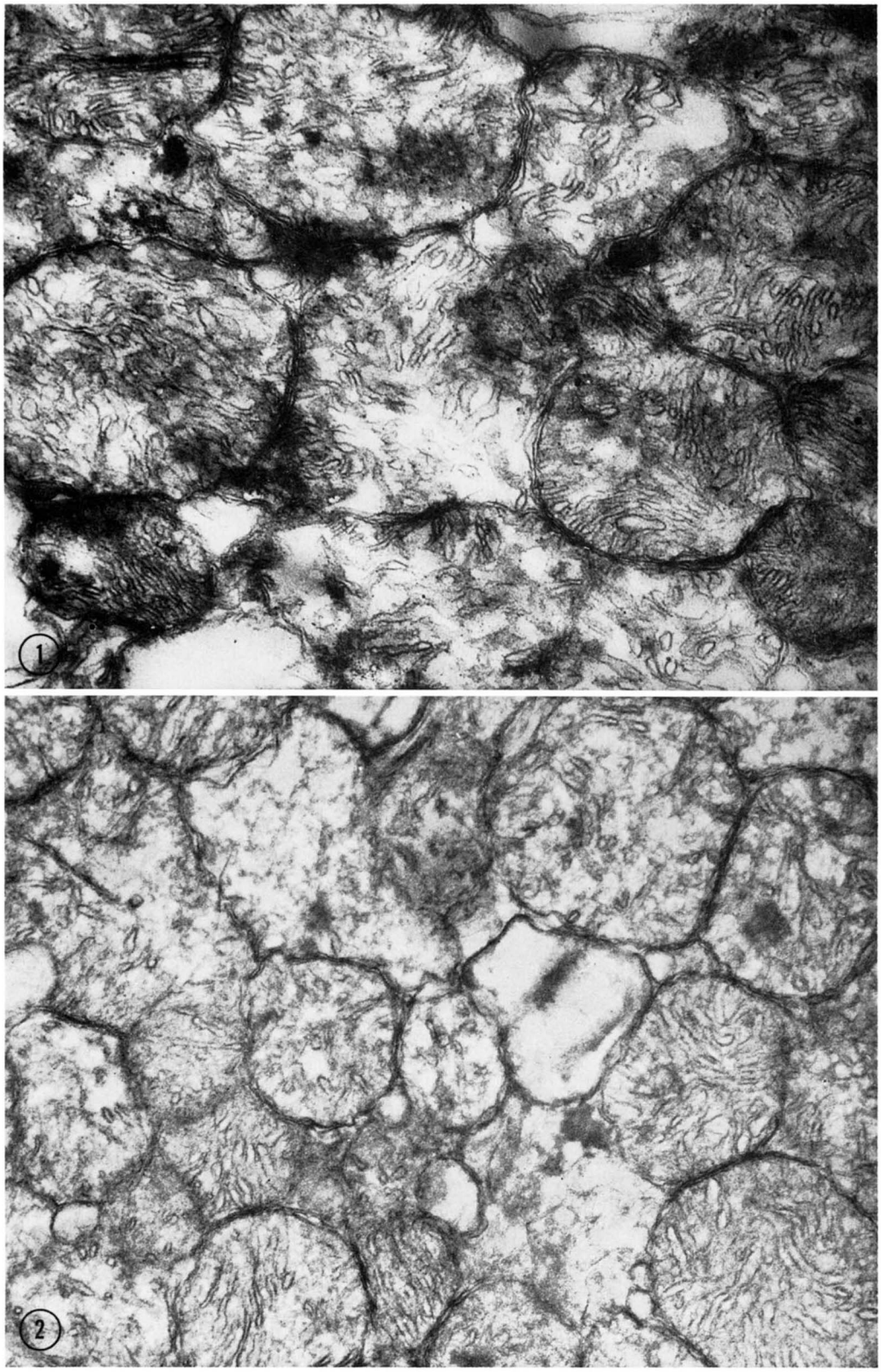
The total lipid contents of mitochondria from the three organs were very different (Table 4). Heart mitochondria contained nearly twice as much lipid as those from liver. Phospholipid accounted for 61–65% of the phosphorus of our mitochondrial preparations.

TABLE 3 CYTOCHROME CONTENT AND ELECTRON TRANSFER ACTIVITY OF MITOCHONDRIA

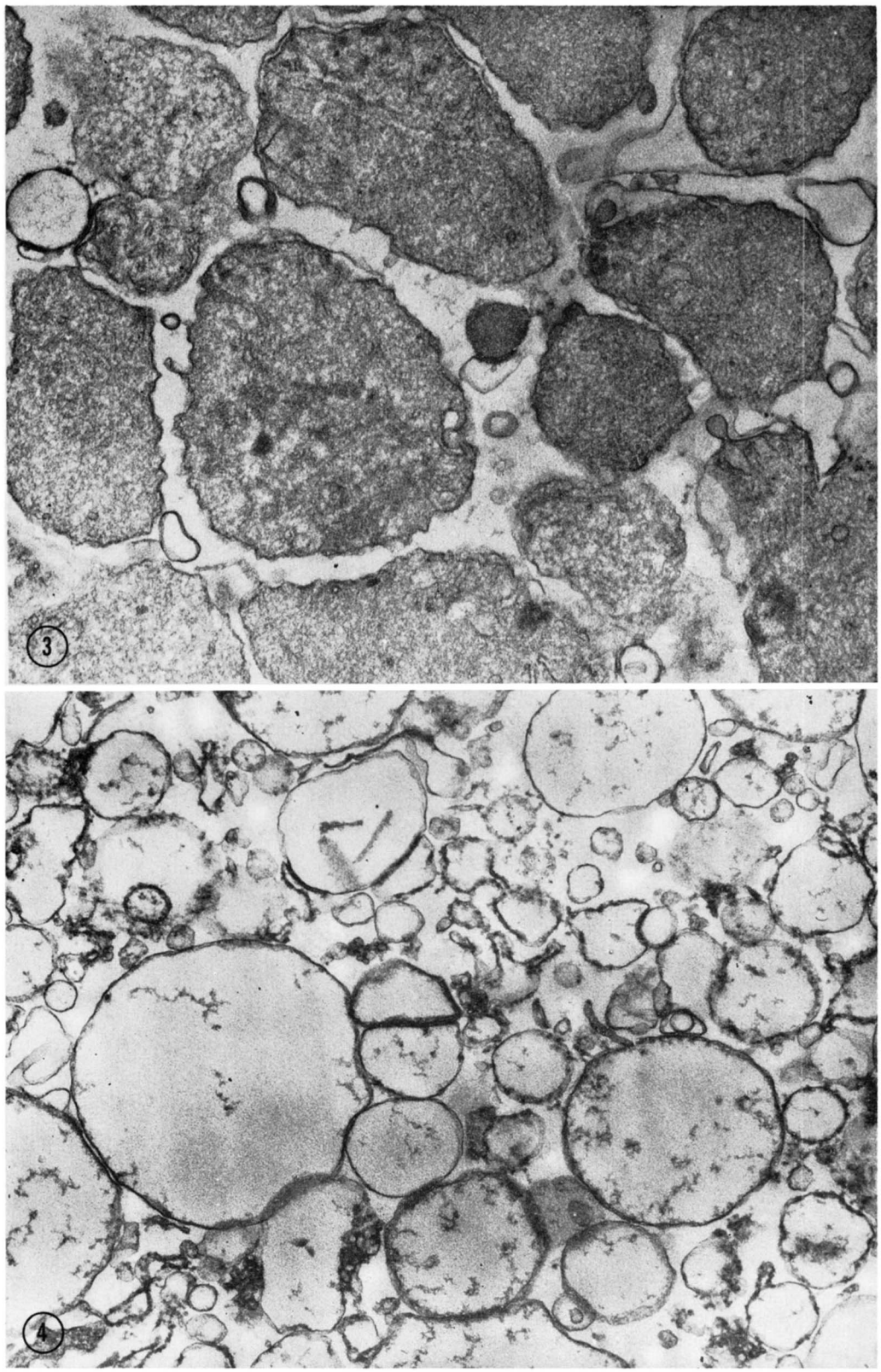
	Heart	Kidney	Liver
Cytochromes $a + a_3$, *mμmoles/mg protein*	1.3	0.23	0.09
Cytochrome *b*, *mμmoles/mg protein*	0.64	0.16	0.11
Succinate-cytochrome *c* reductase*	1.1	0.91	0.40
Succinate-cytochrome *c* reductase* + (Co Q + MPL)†	1.1	1.1	0.50
NADH-cytochrome *c* reductase*	1.8	0.58	0.28
NADH-cytochrome *c* reductase* + (Co Q + MPL)†	2.0	1.0	0.32

* The rates are expressed as μmoles of cytochrome *c* reduced per min per mg of protein at 32°C.

† Assayed in the presence of added coenzyme Q and mitochondrial phospholipids (21).



FIGS. 1 and 2. Electron micrographs (× 27,000) (osmium and lead staining) of highly purified bovine heart mitochondria (above) and kidney mitochondria (below).



FIGS. 3 and 4. Electron micrographs (osmium and lead staining) of highly purified liver mitochondria (above, × 27,000) and of damaged liver mitochondria (below, × 23,000).

TABLE 4 LIPIDS OF MITOCHONDRIA

	Heart	Kidney	Liver
Total phosphorus, *μg/mg protein*	18.5	11.5	9.7
Lipid P, *μg/mg protein*	11.3	7.6	5.8
Lipid P, *μg/mg lipid*	33*	31	33
Lipid, *mg/mg protein*	0.32	0.24	0.18
Neutral lipid†, *% of total lipid*	5.7	7.2	9.0
Coenzyme Q, *% of total lipid*	1.06	0.57	0.43
Coenzyme Q, *mμmoles/mg protein*	3.94	1.58	0.89
Cholesterol, *% of total lipid*	1.1	4.7	2.3

* A value of 35 was obtained after Sephadex column chromatography (28).

† The "neutral lipid" is defined as the fraction which is eluted from DEAE-cellulose columns with chloroform.

Less Polar Lipids of Mitochondria

Most of the lipid of mitochondria is phospholipid; the neutral lipid values (6–9%) were lowest for heart mitochondria and highest for liver mitochondria (Table 4). Heart mitochondria contained equal concentrations of coenzyme Q and cholesterol, although these concentrations represented the greatest percentage of coenzyme Q and the smallest of cholesterol in the three preparations. The greatest concentration of cholesterol was found in kidney mitochondria.

Phospholipids of Mitochondria

The scheme of elution for separating mitochondrial lipids by DEAE-cellulose chromatography is shown in Table 2. Excellent lipid recoveries, averaging 101%, are obtained.

DEAE-cellulose column chromatography of lipids extracted from less highly purified preparations of heart, kidney, and liver mitochondria showed somewhat higher percentages of choline and ethanolamine glycerophosphatides and lower diphosphatidyl glycerol values than in highly purified preparations. Some of the less pure preparations also contained a trace of sphingomyelin.

The purified mitochondrial phospholipids and the products obtained by acid hydrolysis of the major phospholipids of heart mitochondria had the same characteristics on paper chromatography, TLC, and IR spectrometry as those from corresponding lipid classes from brain, heart, and soybean.

Each lipid class gave only one spot on TLC if the lipids (200 μg) were applied immediately after preparation; the limit of detection is 1 μg. Each of the purified phospholipids contained almost exactly 4% phosphorus.

Fig. 5 shows typical IR spectra obtained from choline and ethanolamine glycerophosphatides and from cardiolipin after separation by TLC and removal of impurities derived from the adsorbent by Sephadex column chromatography. The spectra are in good agreement with those of mitochondrial lipids obtained by DEAE-cellulose column chromatography as well as those of the same lipid classes isolated from brain (10).

Thin-layer chromatograms of the phospholipids of a highly purified preparation of heavy bovine heart mitochondria are shown in Figs. 6 and 7. Similar results were

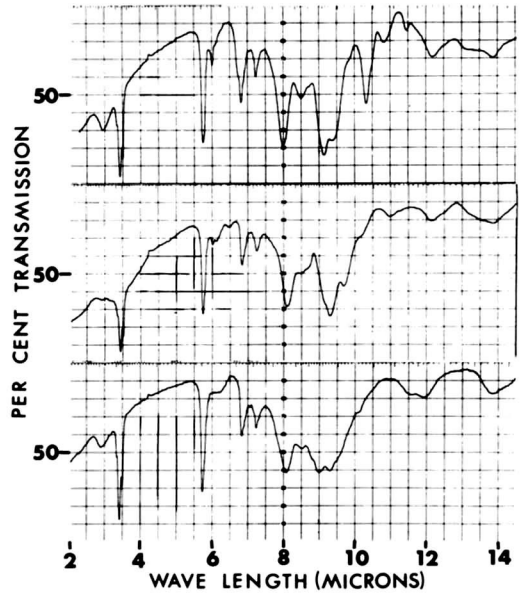
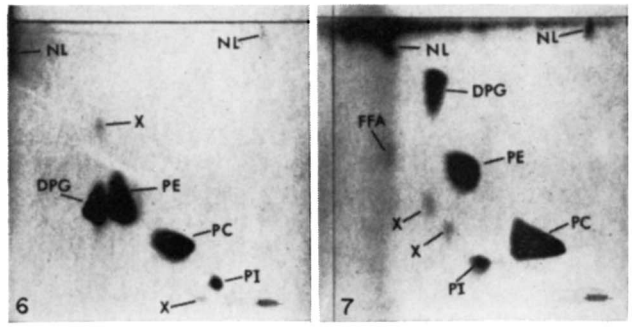


FIG. 5. Infrared spectra of choline glycerophosphatides (top, 1.54% in KBr, pellet 0.16 mm thick), ethanolamine glycerophosphatides (middle, 1.44% in KBr, pellet 0.12 mm thick), and diphosphatidyl glycerol (cardiolipin) (bottom, 1.59% in KBr, pellet 0.12 mm thick) from bovine heart mitochondria. Samples were eluted from thin-layer chromatograms and purified by passage through Sephadex.



FIGS. 6 and 7. Two-dimensional TLC of lipids from highly purified heavy mitochondria of bovine heart (600 μg applied). The chromatogram in Fig. 5 was developed with solvent III (chloroform–methanol–28% by weight aqueous ammonia, 65:35:5) followed by solvent IV (chloroform–acetone–methanol–acetic acid–water, 5:2:1:1:0.5), while that in Fig. 6 was developed with solvent I (chloroform–methanol–water, 65:25:4) followed by solvent II (1-butanol–acetic acid–water, 60:20:20) (see text). Chromatography was performed in chromatographic chambers ($10^3/_4 \times 2^3/_4 \times 10^1/_2$ inches, l × w × h) completely lined on all four vertical walls with Whatman 3 mm filter paper, saturated with solvent about 30–45 min before insertion of the plates. Fresh solvent was used for each chromatogram. Spots were detected with 55% sulfuric acid–0.6% potassium dichromate spray and heat.

Abbreviations: *NL*, neutral lipid; *DPG*, diphosphatidyl glycerol (cardiolipin); *PE*, phosphatidyl (and phosphatidal) ethanolamine; *PC*, phosphatidyl (and phosphatidal) choline; *PI*, phosphatidyl inositol; *FFA*, free fatty acid; *X*, uncharacterized.

TABLE 5 PHOSPHOLIPID COMPOSITION OF MITOCHONDRIA

Lipid Class	Heart			Kidney	Liver
	Phosphorus Analysis*				
	A	B	Charring†	Charring†	Charring†
			% of total phospholipid		
Diphosphatidyl glycerol	20.6 ± 0.4	20.4 ± 0.8	19.1 ± 0.8	19.2 ± 0.4	17.2 ± 0.5
Ethanolamine glycerophosphatides	35.5 ± 0.4	36.8 ± 0.3	37.4 ± 0.4	38.1 ± 0.4	34.5 ± 0.4
Choline glycerophosphatides	40.3 ± 0.5	39.9 ± 0.6	40.8 ± 0.6	39.6 ± 0.3	43.4 ± 0.3
Minor components‡	3.2 ± 0.9	2.9 ± 0.8	2.7 ± 0.7	3.5 ± 0.8	4.9 ± 0.6

* Determined by phosphorus analysis after TLC; values in column A by two-dimensional TLC as described in Fig. 6 and values in column B as described in Fig. 5. Each determination is the average and standard deviation (SD) of four analyses.

† Determined by charring and transmission densitometry. Each determination is the average and SD of eight analyses.

‡ The most abundant minor component in all preparations is phosphatidyl inositol (about 3% of the total phospholipid). Traces only (0.1–0.3%) of phosphatidyl serine and uncharacterized acidic lipids were present in heart and kidney mitochondria.

TABLE 6 PLASMALOGEN AND DOUBLE BOND CONTENT OF THE PHOSPHOLIPIDS OF BOVINE HEART MITOCHONDRIA

	Plasmalogen	Double Bonds
	μmole/μmole P	*No./μmole P*
Choline glycerophosphatides	0.50	2.7
Ethanolamine glycerophosphatides	0.41	3.7
Cardiolipin	0.095	3.5

obtained with kidney and liver mitochondria. The relative amounts of the phospholipids are shown in Table 5. Mitochondrial phospholipids from the three organs have very similar compositions. The weight and phosphorus ratios are very nearly 1:2:2 for cardiolipin:ethanolamine glycerophosphatide:choline glycerophosphatide from heart and kidney mitochondria (i.e., the molar ratio of phospholipids is 1:4:4). Liver mitochondria have relatively more choline glycerophosphatide. The minor phospholipids, primarily phosphatidyl inositol, comprise only about 3% of the total phospholipid.

The high plasmalogen content of choline and ethanolamine glycerophosphatides from heart mitochondria is shown in Table 6. Only a small amount of plasmalogen was found in cardiolipin. All three lipids were found to be rather highly unsaturated, in agreement with the fatty acid compositions previously reported (3, 8).

DISCUSSION

Pure mitochondria contain three major phospholipid classes: choline and ethanolamine glycerophosphatides and cardiolipin. Inositol glycerophosphatide is a minor component. A trace (0.1% or less) of serine glycerophosphatide was found in our highly purified preparations. Sphingomyelin, phosphatidyl glycerol, and cerebrosides, which have been reported to be mitochondrial components, were not detected (<0.1% of the total lipid). The lower limit of detection is made possible with the combined use of column chromatography for concentration of minor lipid fractions and TLC for determination of the purity of the phospholipid. Traces of uncharacterized lipids occur in mitochondria (see Figs. 6 and 7). Other components may arise either from contamination of mitochondria with other particles or by decomposition of the native lipids. These problems have been considered in detail in a recent review (3). Earlier, less pure preparations of mitochondria gave higher values for choline and serine glycerophosphatides and for sphingomyelin. Contamination with microsomes or nuclear lipid could produce this result since phosphatidyl choline is a major component of both these fractions.[1] The higher percentage of choline glycerophosphatide in our liver mitochondrial preparations may be due to contamination by vesicles of unknown origin (Fig. 4). Since our earlier preparations of liver mitochondria contained even more phosphatidyl choline and less diphosphatidyl glycerol, it seems probable that when pure liver mitochondria are available the phospholipids will be found to be present in exactly the same ratios in the three organs.

Decomposition of native mitochondrial lipids occurs readily, and substances are produced that appear as additional components in TLC and column chromatography. Cardiolipin may decompose, with the production of extra spots that migrate ahead of or behind the native lipid on TLC. Oxidation products of ethanolamine glycerophosphatide can be mistaken for lysophosphatidyl ethanolamine and a series of artifacts migrating behind the native lipid can be produced. Cardiolipin can decompose during one-dimensional paper chromatography (3), and the artifacts produced migrate with other components, giving rise to erroneous values for all the lipid classes. We have observed mitochondrial lipid extracts to change on storage in solution at −20°C, with a decrease of diphosphatidyl glycerol and phosphatidyl ethanolamine in particular and the production of more polar artifacts. All extracts should therefore be analyzed as soon as possible.

[1] Fleischer, S., G. Rouser, and B. Fleischer. Unpublished work.

The percentage by weight and the phosphorus content of diphosphatidyl glycerol, and ethanolamine and choline glycerophosphatides are very nearly in a ratio of 1:2:2 for both heart and kidney mitochondria; those for the somewhat less pure liver mitochondria give a ratio that is only slightly different. This ratio was found by Siakotos and Rouser[2] for bovine brain mitochondria. Our unpublished studies[3] suggest that nuclei also have a characteristic lipid pattern, which is different from that of other organelles.

The uniform pattern of phospholipids of mitochondria may reflect the presence of a repeating unit composed of one molecule of diphosphatidyl glycerol with four molecules each of phosphatidyl ethanolamine and phosphatidyl choline. Cholesterol is not a major component of mitochondria, as it is of myelin; thus the cholesterol–phospholipid complex postulated for myelin (29) either does not exist at all in mitochondria or occurs in only limited portions of the membranes (possibly only in the outer membrane). The great differences in cholesterol percentage in mitochondria from the different organs may arise from differences in the outer environment of the mitochondrion. Since the molecular weight of cholesterol is about one-half that of a phospholipid, the maximum amount of cholesterol–phospholipid complex would be 3.3% of the total lipid for heart, 14.1% for kidney, and 6.9% for liver mitochondria.

Previous studies, the results of which have been considered in detail elsewhere (3), failed to show this simple and characteristic mitochondrial lipid pattern, probably because the mitochondria were not sufficiently purified and because lipid decomposition falsified the analytical results. The application of a procedure for isolation of mitochondria to different organs without careful checking of purity of the organelles has probably been responsible for the deficiencies of some studies.

DEAE-cellulose column chromatography provides a very convenient means for separation of mitochondrial lipids. In general, pure lipids can be recovered without decomposition if all work is carried out in an atmosphere of pure nitrogen, evaporations are carried out in the cold, and lipids are not allowed to remain for more than a few minutes spread over glass surfaces in the dry state. Exposure to air, particularly when the lipids are in the solid state and spread over a glass surface, is associated with the appearance of altered products.

Two major difficulties of DEAE-cellulose chromatography exist: the presence of what appears to be ethanolamine glycerophosphatide in the chloroform–acetic acid eluate, and a slight variability of elution of acidic phospholipids. The reason why "altered" phosphatidyl ethanolamine is eluted with chloroform–acetic acid, rather than with chloroform–methanol 7:3 as is characteristic for native phosphatidyl ethanolamine, has not been determined. The term "altered" phosphatidyl ethanolamine was introduced for the second fraction because oxidized preparations of phosphatidyl ethanolamine are eluted in part with chloroform–acetic acid (10), although ethanolamine glycerophosphatide eluted with chloroform–acetic acid after chloroform–methanol 7:3 is not an oxidation product.

The elution of the acidic phospholipids from DEAE-cellulose columns (fractions 8–10, Table 2) is sensitive to small changes in elution conditions and must be controlled with great care.

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