Lipid composition of heart muscle homogenate

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SUMMARY Using cytochrome oxidase and esterase assays as a guide, mitochondria and microsomes were prepared from ox heart homogenate with about 25% cross-contamination of phospholipid. By the same criteria, the lipid complement of well washed myofibrils was essentially microsomal in origin. Approximately 60% of the phospholipid of the whole homogenate was found to be associated with microsomes, about half of this being firmly bound to myofibrils.

Mitochondrial lipids were characterized by a higher degree of unsaturation of the free fatty acids and higher contents of cardiolipin, cholesterol, and coenzyme Q than in microsomes, where choline-containing phospholipid, especially sphingomyelin, formed a greater proportion of the total phospholipid than in mitochondria. The outstanding difference was the virtual localization of ethanolamine plasmalogen in microsomes, in contrast to the equal distribution of choline plasmalogen between mitochondria and microsomes. Myofibril lipids resembled more closely microsomal than mitochondrial lipids, but contained in addition phosphatidyl serine and phosphoinositide, which were not detected in mitochondria and microsomes.

KEY WORDS heart muscle · mitochondria · microsomes · myofibrils · phospholipids · cardiolipin · plasmalogen · sphingomyelin · phosphoinositide · fatty acids · cholesterol · coenzyme Q · ox

THE DISTRIBUTION of complex lipids in subcellular organelles has been the subject of a number of investigations, aimed at providing an analytical basis for understanding the functions of lipids in cellular membrane systems (1–5). For example, the greater abundance of cardiolipin in heart and breast muscle compared with skeletal muscle (6) reflects both a localization of cardiolipin in mitochondria (1, 3) and a higher density of mitochondria per cell in the first two types of muscle (7). On the other hand, the significance of the unusually high plasmalogen content of muscle is not understood. The present study provides more complete data on the subcellular distribution of lipids in heart muscle, including myofibrils as well as mitochondria and microsomes.

It is known that the differential centrifugation procedure applied to sucrose homogenates does not work as well with muscle (9) as it does with liver (8). Some kind of evaluation of the procedure is necessary. Hulsmans (10) has demonstrated the use of cytochrome oxidase and esterase as markers, in heart muscle, for mitochondria and microsomes, respectively. Hulsmans pointed out that 75% of the microsomes of heart muscle remained bound to the "residue" fraction, consisting chiefly of myofibrils. Using Hulsmans' approach, it has been possible to isolate heart muscle mitochondria and microsomes with about 25% cross-contamination of phospholipid, and also to show that microsomes constitute the major phospholipid compartment in heart muscle homogenate. In confirmation of Hulsmans' finding, we have found that approximately half the microsomes were firmly bound to myofibrils, accounting for virtually all the phospholipid in this fraction. Major differences in composition between mitochondrial and microsomal lipids were seen in the distribution of cholesterol, coenzyme Q, unsaturated free fatty acids, and cardiolipin (all of which were higher in mitochondria), as well as of ethanolamine plasmalogen and sphingomyelin (both of which were higher in microsomes). Significant amounts of inositol-containing phospholipid were found only in myofibrils.

METHODS

Tissue Fractionation

Ox hearts were obtained on the killing floor of a slaughter house and transported in ice to the cold room. Portions of ventricle were passed through a hand meat grinder and the mince was rinsed with 0.25 M sucrose–0.002 M EDTA, pH 6.9. Finer fragmentation was obtained in a

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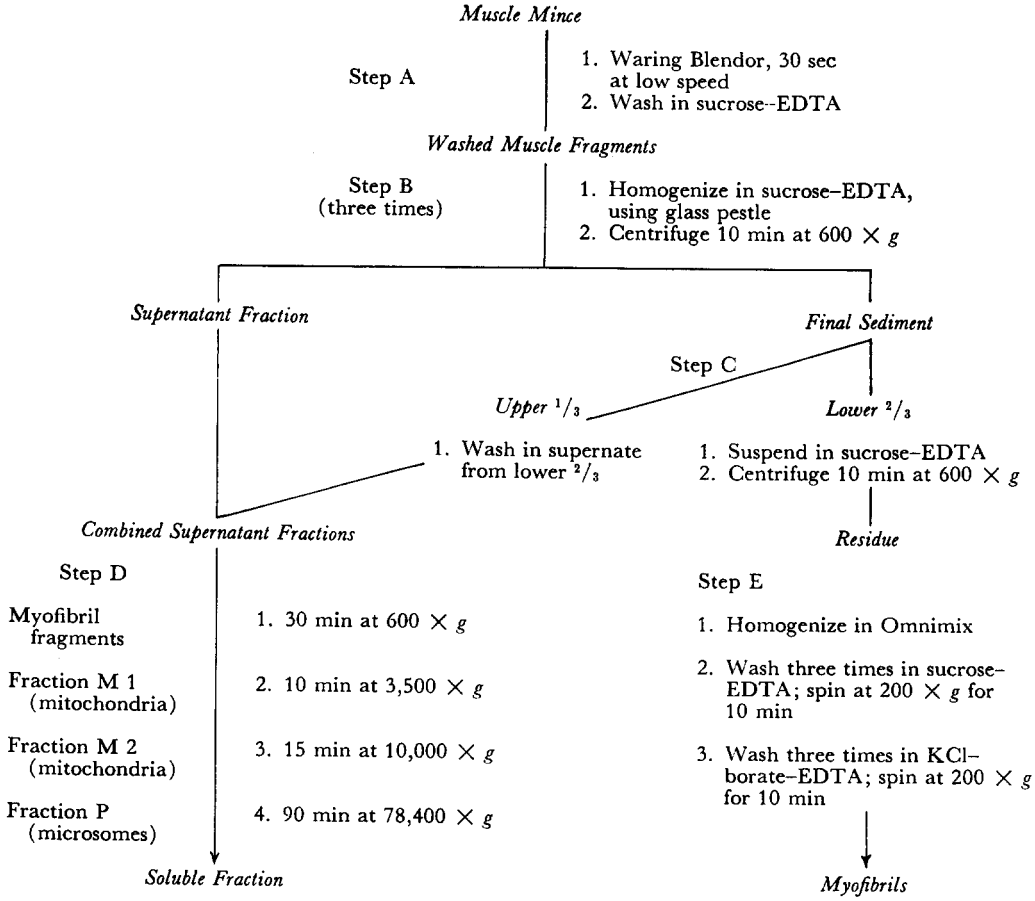

FIG. 1. Fractionating scheme for isolation of main components of heart muscle homogenate.

Waring Blendor (30 sec at low speed) in the presence of sufficient $KHCO_3$ to maintain the pH at 6.9–7.0. The muscle fragments were recovered by low speed centrifuging and washed several times by resuspension in the sucrose–EDTA.

Procedure 1. The final suspension was diluted with 5 volumes of sucrose–EDTA and homogenized in portions in a Potter-Elvehjem glass homogenizer, the pestle being driven once to the bottom of the tube and the packed residue under the pestle discarded. From the supernatant fluid, mitochondria (M fraction) were recovered at 10,000 × *g* for 15 min (Lourdes Angle Centrifuge, Model AT; time does not include acceleration). Microsome fractions P1 and P2 were sedimented at 10,000 × *g* for 60 min and at *g* max = 78,000 for 120 min, respectively (Spinco Ultracentrifuge, Model L).

Procedure 2 is shown diagrammatically in Fig. 1. The washed muscle fragments obtained from the Waring Blendor were thoroughly homogenized in 5 volumes of sucrose–EDTA with the glass pestle (step B) and centrifuged for 10 min at 600 × *g*. The resulting sediment was suspended in fresh sucrose, rehomogenized and spun down twice more. Successive extracts were combined and pieces of connective tissue discarded. The final sediment was divided into two portions, the upper layer being distinguished by its pale pink color. (Nuclei were also concentrated in the upper layer, which contained 0.25 μmole of DNA-phosphorus per mg of protein-nitrogen, compared with 0.08 in the lower portion). The lower layer (approximately 2/3) was washed once in sucrose–EDTA and spun down (10 min at 200 × *g*); the washings were used to resuspend the upper portion, which was then also centrifuged (10 min at 600 × *g*). The supernatant portion was combined with previous extracts to give the combined supernatant fluid.

A reconstituted homogenate (from which samples were taken for the determinations described below) was prepared by combining upper and lower sediments and supernatant fluid in amounts proportional to the original homogenate. A reconstituted "debris" fraction was similarly prepared by combining the upper and lower sediments.

Myofibrils were then prepared as follows (step E); first the cytochrome oxidase activity of the lower sediment was measured by the spectrophotometric method (see below); the sediment was then homogenized at 0° in the "Servall Omnimixer," until there was no further increase in cytochrome oxidase activity (2–3 min). The

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homogenate was diluted with sucrose–EDTA, then centrifuged at 200 × *g* for 10 min. After two further washes with sucrose–EDTA, the residue was washed three times with 0.025 M KCl–0.039 M borate–0.004 M EDTA, pH 6.8 (11), centrifuging as before.

Analytical Methods

Protein. Samples were prepared according to Cleland and Slater (12) for analysis either by Kjeldahl digestion followed by distillation of ammonia (for the results shown in Table 5), or by the biuret reaction, using crystalline egg albumin as standard (for the results shown in Table 2). In the latter case, the factor of 0.16 was used to convert protein to protein-N.

Phospholipid. Samples (0.5–2.0 ml) of the particle suspensions were homogenized with 25 ml of chloroform–methanol 2:1 (v/v) and centrifuged, and the aqueous layer was discarded. The chloroform phase was washed twice with 5.0 ml portions of 0.88% KCl (13), dried over Na_2SO_4, and filtered, and the filtrate was taken to dryness under reduced pressure. The lipid residue was taken up in chloroform and analyzed for phosphorus (14).

RNA and DNA. Samples of the particle suspensions (2–5 ml) were homogenized with an equal volume of 10% trichloroacetic acid (TCA) and the precipitates washed twice with 5% TCA at room temperature. The precipitates were then made to volume in 5% TCA and heated for 30 min at 100°. The hydrolysates were analyzed for pentose (15) and deoxypentose (16).

Enzyme Determinations. Cytochrome oxidase activities were measured at room temperature by the spectrophotometric method of Cooperstein and Lazarow (17), and at 30° by the manometric method of Slater (18). For the spectrophotometric method, samples were homogenized for 3 min at 0° in the Servall Omnimixer. Activity was determined over a range of protein concentration for each fraction. Room temperature varied by no more than 2° throughout. One unit of activity was taken as the amount of cytochrome oxidase which caused the ferrocytochrome concentration to decrease by one order of magnitude per minute. In the manometric procedure, determinations were made at cytochrome *c* levels between 9.25×10^{-5} M and 3.7×10^{-4} M. In all determinations, activity was virtually constant between 2.77 and 3.7×10^{-4} M, so that extrapolation to infinite cytochrome *c* concentration was not necessary. One unit of activity is equivalent to 1 μl of oxygen taken up per hr. The results in Table 2 were obtained by the manometric method and those in Table 5 by the spectrophotometric method.

Nonspecific esterase activity was measured at 24° with β-naphthyl acetate as substrate, according to Seligman and Nachlas (19). One unit of enzyme activity is defined as 1 μmole of β-naphthol released per hr.

Calculation of Phospholipid Distribution between Mitochondria and Microsomes. The calculation employed was that of Hulsmans (10), except that specific activities (S.A.) of cytochrome oxidase and esterase were expressed in terms of phospholipid instead of protein. Thus, the percentage of phospholipid in the M fraction which is of mitochondrial origin (x), and the per cent mitochondrial phospholipid in the P fraction (y) were derived from the equations:

$$x = f_1 y \text{ and } y = \frac{100\text{-}100\, f_2}{f_1 - f_2},$$

where

$$f_1 = \frac{\text{Cytochrome oxidase S.A. of M fraction}}{\text{Cytochrome oxidase S.A. of P fraction}},$$

and

$$f_2 = \frac{\text{Esterase S.A. of M fraction}}{\text{Esterase S.A. of P fraction}}.$$

The "true" cytochrome oxidase specific activity of mitochondria in M fraction =

$$\frac{\text{Cytochrome oxidase S.A. of M fraction}}{\%\text{ Mitochondrial phospholipid in M fraction}}.$$

Similarly, the "true" esterase specific activity of microsomes in the P fraction =

$$\frac{\text{Esterase S. A. of P fraction}}{100 - \%\text{ mitochondrial phospholipid in P fraction}}.$$

The latter values were used to determine the proportions of mitochondrial and microsomal phospholipid in the homogenate and myofibrils. In the case of the homogenate, it was necessary to subtract the amount of nonparticulate esterase, which was taken as equal to that which was recovered in the soluble fraction. Thus, per cent microsomal phospholipid in homogenate =

$$\frac{\text{"Particulate" esterase S.A. of homogenate}}{\text{"True" esterase S.A. of microsomes}}.$$

Analysis of Lipid Extracts

Total lipid extracts (13) of beef heart muscle were resolved into their major components by a single chromatographic procedure, illustrated in Fig. 2. Silicic acid (Mallinckrodt Chemical Works, Jersey City, N.J.), sifted to 100–260 mesh, was dehydrated by washing it with acetone and diethyl ether and dried under an infrared lamp. Columns of it were prepared in hexane. Resolution of neutral lipids essentially followed the scheme of Hirsch and Ahrens (20), except that columns were maintained at 5°. Analytical methods used were: glycerol in glycerides (21), cholesterol (22), free fatty acids (23), coenzyme Q (24), ester (25), total phos-

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phorus (14) and unsaponifiable phosphorus (26), lipid amino-nitrogen (27), and plasmalogen (28). Lysocephalin was taken as amino-nitrogen eluted together with lecithin, forming a small peak at the leading edge of the lecithin peak (29). Sphingomyelin was estimated as unsaponifiable phosphorus in the tailing portion of the lecithin peak, and subsequent fractions; lysolecithin in these fractions was calculated by interpolation from saponifiable phosphorus and ester values (4). Inositol-containing lipids were found in both the tailing portion of the amino-nitrogen peak and the leading part of the lecithin peak; these fractions were analyzed by hydrolysis followed by spectrophotometric determination of inositol (30). Serine and ethanolamine were estimated as their 2,4-dinitrophenyl (DNP) derivatives (31).

Because of the poor solubility of the mitochondrial and microsomal lipids in hexane, they could not be applied as a whole to the column; instead, they were first separated into neutral and phospholipid fractions on silicic acid as described by Borgström (32). After separation of free fatty acids from the neutral lipid fraction (33), the remaining neutral lipids and the phospholipids were chromatographed separately.

Phospholipid samples were methanolyzed before saponification to remove aldehydes (34). Triglycerides were refluxed in equal parts of methanol and 1 N KOH for 3 hr. The isolated fatty acids were converted to methyl esters by heating in 0.5 N HCl in anhydrous methanol for 60 min at 60° and were then analyzed by gas–liquid chromatography on an ethylene glycol succinate polyester column at 195°. Esters were identified by comparison with standards and their degree of unsaturation was assessed by the method of James (35). The relative areas of the peaks were obtained by triangulation. Quantitative results with the National Heart Institute Fatty Acid Standards (mixtures A through F) agree with stated composition data with a relative error of less than 5% for major components (>10% of total mixture) and less than 15% for minor components (<10% of total mixture).

Composition of Whole Heart Muscle

Whole heart ventricle, freed of adhering fat, had the following lipid composition (millimoles of each constituent per kilogram fresh weight): triglycerides, 8.9; free fatty acids, 0.46; cholesterol, 2.38; cholesterol ester, 0.09; coenzyme Q, 0.10; total phospholipid, 23.8; cardiolipin, 2.2; total aminophospholipid, 7.0; cephalin plasmalogen, 3.6; lysocephalin, 0.14; total lecithin, 10.9; lecithin plasmalogen, 6.0; lysolecithin, 0.10; phosphoinositide, 0.50; spingomyelin, 1.07. Total cholesterol was 2.6 mmoles/kg. Free fatty acids and coenzyme Q showed some variation in three hearts analyzed; free fatty acids ranged from 0.25 to 0.74 meq/kg and coenzyme Q from 0.06 to 0.18 mmole/kg. The lowest content of free fatty acid found was equal to the total amount

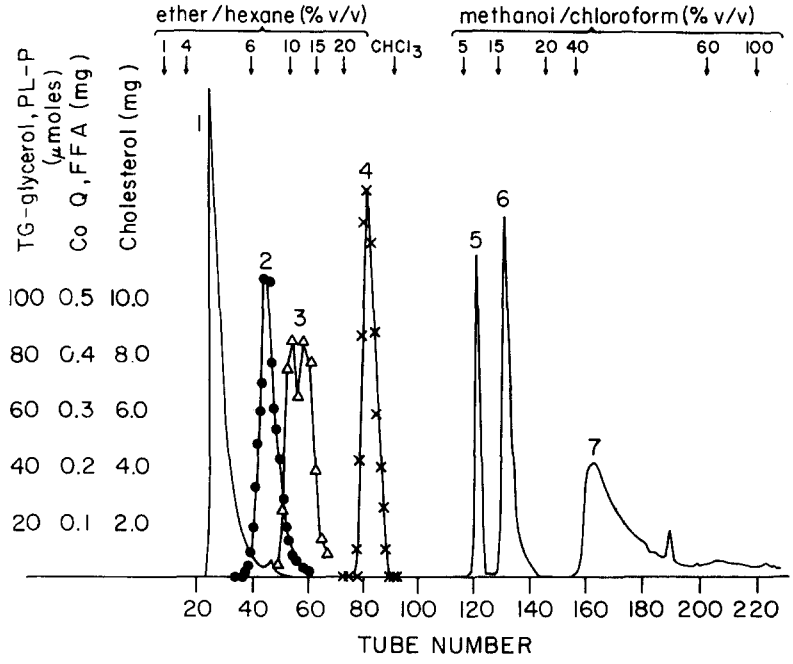

FIG. 2. Resolution of total lipids of heart muscle on silicic acid. Peaks are as follows: *1*, triglycerides (solid line = glycerol); *2*, free fatty acids; *3*, coenzyme Q; *4*, cholesterol; *5*, cardiolipin; *6*, amino-phospholipids ("cephalin"); and 7, choline-containing phospholipids (solid line = phosphorus).

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TABLE 1 FATTY ACID COMPOSITION OF LECITHIN AND "CEPHALIN" FROM WHOLE HEART MUSCLE

	Fatty Acid Composition									
	16:0	16:1	18:0	18:1	18:2	18:3	20:3	20:4	20:5	22:5
	% by weight									
Lecithin	19.1	2.5	7.2	18.9	31.0	7.2	2.3	7.1	2.6	1.5
"Cephalin"	3.6	1.0	21.4	6.9	22.0	4.2	2.3	30.5	6.3	1.8

Both ester and plasmalogen forms are included. "Cephalin" equals phosphatidyl ethanolamine + phosphatidyl serine.

TABLE 2 RESOLUTION OF HEART MUSCLE MITOCHONDRIA AND MICROSOMES*

Fraction	Phospholipid-P: Protein-N	Cytochrome Oxidase Specific Activity	Esterase Specific Activity	% Mitochondrial Phospholipid in Fraction	% Microsomal Phospholipid in Fraction
	μmoles/mg	*units of enzyme/μmole of phospholid-P*			
Mitochondria	M 2.96	6.05	2.88	76	24
Microsomes	P1 3.51	1.77	9.20	22	78
	P2 4.33	0.65	10.70	8	90

* Results of procedure 1.

of lysophospholipid (0.24 mmole/kg). The fatty acid composition of lecithin and cephalin from whole muscle is shown in Table 1.

Composition of Mitochondria and Microsomes

Mitochondrial (M) and microsomal (P) fractions, isolated as described in procedure 1, had the properties shown in Tables 2 and 3. It can be seen that the extent of cross-contamination of mitochondria and microsomes in fractions M and P1 was about equal, amounting to approximately 25%. However, of the total phospholipid recovered in all fractions, 79% was present in the M fraction, while 11 and 6.9% were found in fractions P1 and P2, respectively. Thus, although fraction M was predominantly mitochondrial, it actually contained most of the microsomes as well; only a small part of the total microsomes appeared in the P fractions.

The pooled lipids of fractions P1 and P2 (together calculated to be 83% microsomal) and the lipid of fraction M (calculated to be 76% mitochondrial) were analyzed, giving the results shown in Table 3 and presented graphically in Fig. 3. The following observations may be made from these analyses:

(*a*) The higher phospholipid content of microsomes compared with mitochondria was due chiefly to a higher content of choline-containing phospholipids, especially phosphatidyl choline and sphingomyelin. On the other hand, there was slightly more phosphatidal choline in mitochondria.

(*b*) The "cephalin" fraction of both mitochondria and microsomes consisted almost entirely of ethanolamine phosphoglycerides, no more than traces of serine and inositol phosphoglycerides being present. In microsomes, the "cephalin" fraction consisted predominantly of phosphatidal ethanolamine. The diester form of this phosphoglyceride was present at a much higher level in mitochondria.

TABLE 3 LIPID COMPOSITION OF HEART MUSCLE HOMOGENATE COMPONENTS

Constituent	Fraction: Mitochondria	Fraction: Microsomes	Supernatant Fraction	Myofibrils
	mole/g-atom of phospholipid-P			
Free fatty acids	0.161	0.156	0.50	0.060
Triglycerides	0.053	0.131	1.61	0.062
Diglycerides	0.007	0.013	0.12	0.007
Cholesterol	0.66	0.28	0.35	0.144
Coenzyme Q	0.0071	0.0023	—	0.0038
Cardiolipin	0.203	0.092	0.035	0.085*
Total aminophospholipid	0.336	0.304	0.28	0.300
Phosphatidyl ethanolamine	0.155	0.058	...	0.105
Phosphatidal ethanolamine	0.146	0.218	...	0.151
Lysophosphatidyl ethanolamine	0.030	0.015	...	0.01
Phosphatidyl serine†	0.01	0.01	...	0.037
Phosphoinositide	0.01	0.01	...	0.057
Total choline-containing phospholipid	0.461	0.605	0.69	0.537
Phosphatidyl choline	0.162	0.19	...	0.174
Phosphatidal choline	0.290	0.259	...	0.270
Lysolecithin	0.02	0.065	...	0.02
Sphingomyelin	0.030	0.089	...	0.090

* This fraction contained 0.13 g-atom of amino-nitrogen per g-atom of phospholipid-P: see text.

† Includes phosphatidal serine, if present.

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TABLE 4 FATTY ACID COMPOSITION OF LIPID FRACTIONS

Constituent	Fatty Acid Composition								
	16:0	16:1	18:0	18:1	18:2	18:3	20:3	20:4	20:5
	% by weight								
Free Fatty Acids									
Mitochondria	7.0	1.3	8.1	11.4	26.2	0.9	5.2	36.1	3.6
Microsomes	17.6	2.9	14.0	18.7	19.1	1.9	3.1	19.7	1.7
Supernatant fraction	17.6	5.4	11.0	21.9	16.1	2.0	2.1	16.7	2.0
Myofibrils	17.1	3.4	13.9	17.3	19.3	2.8	1.4	19.7	3.4
Triglycerides									
Mitochondria	21.2	2.4	26.0	42.0	7.1	1.2	0.0	0.0	0.0
Microsomes	21.0	2.5	26.1	40.5	7.3	1.8	0.0	0.0	0.0
Supernatant faction	21.4	2.3	23.1	42.6	7.7	1.7	0.0	0.0	0.0
Myofibrils	20.8	1.5	24.6	31.9	8.3	1.4	0.0	8.7	0.0
Cardiolipin									
Mitochondria	1.3	2.5	tr.	9.0	84.0	2.7	0.0	0.0	0.0
Microsomes	3.3	3.7	2.9	10.7	76.7	2.6	0.0	0.0	0.0
*Cephalin**									
Mitochondria	1.8	tr.	32.8	4.4	15.6	0.8	3.6	36.3	4.7
Microsomes	4.5	tr.	18.5	7.1	22.0	tr.	7.7	35.6	4.6
Lecithin†									
Mitochondria	22.6	2.2	5.5	13.5	36.7	2.5	5.9	9.7	1.1
Microsomes	21.3	3.6	10.3	14.7	31.8	2.5	6.2	9.4	tr.

Trace amounts were ignored in the summation of peak areas; docosapentaenoic acid was not determined.
* Phosphatidyl + phosphatidal ethanolamine account for at least 95% of this fraction.
† Phosphatidyl + phosphatidal choline.

(*c*) Cardiolipin was found in substantially higher concentration in mitochondria than in microsomes or in the soluble fraction.

(*d*) Both mitochondria and microsomes contained significant amounts of lysophospholipids. In the mitochondria, this was mainly lysophosphatidyl ethanolamine, while in microsomes, lysophosphatidyl choline predominated.

(*e*) Fatty acid composition (Table 4) appeared to be characteristic of the class of phosphoglyceride and quite independent of its distribution within the cell. Slight differences between mitochondria and microsomes with respect to the degree of unsaturation of the acids of a particular phospholipid probably reflected varying proportions of diester and ester-ether forms: thus, microsomal "cephalin" was lower in stearic acid content than mitochondrial "cephalin," agreeing with the higher content in microsomes of phosphatidal ethanolamine, in which the fatty acids are at least 90% unsaturated (36).

(*f*) Free fatty acids were most abundant in the soluble fraction. In microsomes and mitochondria, the proportions of free fatty acid to total phospholipid were similar. In mitochondria, free fatty acids were 85% unsaturated, and included much more arachidonic and linoleic acid than the free fatty acids of microsomes, which were only 69% unsaturated.

(*g*) Of the neutral lipids, cholesterol and coenzyme Q were present in larger amounts in mitochondria than in microsomes or soluble fraction. Cholesterol esters were not detected in any fraction. Triglycerides, the major lipid in the soluble fraction, were present in smaller amounts in the particulate fractions, but were similar in fatty acid composition. Diglycerides were present at approximately 10% of the triglyceride level. Monoglyceride was not detected in any fraction.

Lipid Composition of Myofibrils

The lipid composition of myofibrils, shown in Table 3 and Fig. 3, resembled more closely that of microsomes than that of mitochondria. This is seen especially in the proportions of the major components: cholesterol, cardiolipin, and choline-containing phospholipid. The composition of the free fatty acids was also strikingly

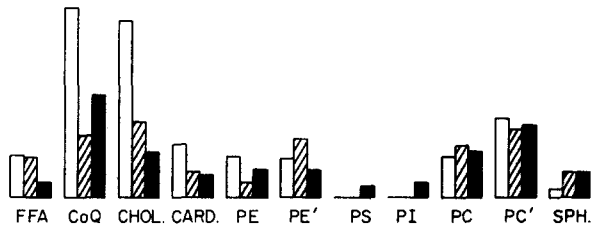

FIG. 3. Graphic representation of data in Table 3. FFA = free fatty acids. CoQ = coenzyme Q. Chol. = cholesterol. Card. = cardiolipin. PE = phosphatidyl ethanolamine. PE′ = phosphatidal ethanolamine. PS = phosphatidyl serine. PI = inositol-containing phospholipid. PC = phosphatidyl choline. PC′ = phosphatidal choline. Sph. = sphingomyelin. Height of columns represents molar proportion of each constituent with respect to total phospholipid. Open, hatched, and shaded columns correspond to mitochondria, microsomes, and myofibrils, respectively.

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TABLE 5 COMPOSITION OF HEART MUSCLE HOMOGENATE*

Fraction		Protein (% Homogenate)	Phospholipid-P: Protein-N	RNA-P: Protein-N	Cytochrome Oxidase Specific Activity	Esterase Specific Activity	% Mitochondrial Phospholipid in Fraction	% Microsomal Phospholipid in Fraction
			μmoles/mg		*units enzyme/μmole phospholipid-P*			
Homogenate		100	0.77	0.16	32.6	15.7 (12.2)†	39	67
Mitochondria	M1	8.3	1.79	0.16	50.4	7.6	61	44
	M2	8.5	2.53	0.18	54.4	5.9	66	34
Microsomes	P	2.9	3.85	0.33	19.3	13.3	23	77
Soluble		19.0	0.0	0.27	0.0	—	0.0	0.0
Myofibrils		–	0.47	0.13	7.0	14.6	8.4	83

* Results of procedure 2 (see Fig. 1). Recoveries in the "debris" fraction were as follows (as % homogenate): protein, 63.3; phospholipid, 38.7; RNA 42.0; cytochrome oxidase, 13.8; esterase, 39.4.

† Value in parenthesis is particulate esterase activity. This was obtained by subtraction of esterase in soluble fraction, which was 23.4% of that in the homogenate.

similar to that of microsomes. However, myofibrils differed from mitochondria and microsomes in the following respects: (*a*) the presence of phosphatidyl serine and phosphatidyl inositol, which were not above the limit of detection in either mitochondria or microsomes; (*b*) the presence of ninhydrin-positive material in the cardiolipin fraction [upon hydrolysis of this fraction and reaction of the hydrolysate with 2,4-dinitro-1-fluorobenzene (31), at least three dinitrophenyl derivatives were detected—none of these corresponded to dinitrophenyl serine or dinitrophenyl ethanolamine]; and (*c*) the absence of lysophospholipids.

Distribution of Phospholipid in Heart Muscle Homogenate

To determine the distribution of phospholipid between mitochondria and microsomes in whole muscle, a more thorough homogenization of the tissue (procedure 2) was used in order to ensure accurate sampling of the homogenate. Consequently, the M fractions (Table 5) were not uniform in composition, M1 probably containing fragments of myofibrils. Both the "heavy" and "light" mitochondrial fractions were more contaminated with microsomes than the mitochondria obtained by procedure 1. Relative amounts of phospholipid attributable to either mitochondria or microsomes in the homogenate and myofibrils were calculated from the "true" cytochrome oxidase and esterase activities of mitochondria and microsomes in fractions M2 and P (see Methods).

Since esterase was also found in the soluble fraction, the assumption was made that two forms of esterase occur in heart muscle: a soluble form and a form bound to microsomes. To calculate the proportion of microsomal phospholipid in the homogenate, it was necessary to subtract the contribution of soluble esterase. This procedure showed that microsomes contained 67% of the phospholipid in the homogenate, which is in contrast to the very small quantity of microsomes obtained by centrifugation (Table 5, fraction P). An explanation of this apparent discrepancy is seen in the high proportion of total esterase recovered in the "debris" fraction and particularly in the high specific esterase activity of the myofibrils, which constituted the bulk of the "debris" fraction. Actually, esterase activity in the myofibrils was sufficient to account for 83% of the myofibril lipid as due to contamination with microsomes, while cytochrome oxidase activity could account for only a trace of mitochondrial contamination (Table 5).

DISCUSSION

Hulsmans (10) obtained from rat heart homogenate an "M" fraction calculated to be 86% mitochondrial protein and a "P" fraction in which 58% of the protein was microsomal, the remainder being attributed to fragments of mitochondria. In the present study, the extent of cross-contamination of phospholipid in these fractions was found to be about equal, a result which could be explained by a higher content of phospholipid in microsomes than in mitochondria. A higher ratio of phospholipid-P to protein-N was in fact found in the P fractions (Tables 2 and 5). The true phospholipid content of heart muscle mitochondria and microsomes may be calculated approximately from the content of mitochondria and microsomes in the M and P fractions, using the data in Table 2. Mitochondria in fraction M contained 2.6 μmoles of phospholipid-P per mg of protein-N, or approximately 25% phospholipid, if dry weight is taken as the sum of protein and phospholipid; similarly, microsomes in fractions P2 contained 4.6 μmoles of phospholipid-P per mg of protein, or 36% of dry weight. These values are in fair agreement with those obtained directly for mitochondria and microsomes

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isolated from liver homogenate (37), where the degree of cross-contamination is very much less.

The column chromatographic approach to lipid analysis employed in this work was based on that used by Gray and Macfarlane (28), and the results for whole muscle phospholipids agree with this earlier work. Of the neutral lipids, the cholesterol level was found to be within the range given by Bloor and Snider (38). Coenzyme Q fell in the range reported by Crane, Lester, Widmer, and Hatefi (24).

Earlier data on the phospholipid composition of heart muscle mitochondrial and microsomal preparations have been obtained by different methods, including paper chromatography (2) and mild alkaline hydrolysis (3). Neither of these procedures gave any information on the distribution of plasmalogens or fatty acids. The main point of difference from the present results concerns the level of phosphatidyl inositol. The paper chromatographic method yielded considerably higher values both for whole heart muscle (39) and for heart muscle mitocohondria and microsomes (2), while the hydrolytic method gave intermediate values (3). In the present work, significant amounts of inositol-containing phospholipid were found only in the myofibrils. Choline-containing phospholipids were found at a higher level in microsomes than in mitochondria. Data in Table 3 show that in heart muscle, this difference decreased in the order sphingomyelin; phosphatidyl choline; phosphatidal choline. While lecithin plasmalogen was almost equally distributed between mitochondria and microsomes, ethanolamine plasmalogen was relatively more abundant in microsomes. Total plasmalogen was thus higher in microsomes than in mitochondria. A similar distribution of total plasmalogen has been found for homogenates of rat liver (40) and rat brain (4).

The amounts of lysophospholipids found in the homogenate fractions, weighted so as to represent whole homogenate, were two to three times higher than found by direct extraction of whole ventricle. This suggests that phospholipids were hydrolyzed during fractionation of the homogenate. However, there was five times as much free fatty acid (on a molar basis) as lysophospholipid in mitochondria and twice as much in microsomes, which means that free fatty acids cannot have been derived solely from phospholipid. Furthermore, hydrolysis of phospholipid, if resulting from phospholipase action, would be expected to enrich the free fatty acid fraction with unsaturated acids (41). Only in mitochondria were the free fatty acids predominantly unsaturated. In microsomes, myofibrils, and soluble cytoplasm, they included a large proportion of palmitic and stearic acids. These findings might be explained by the occurrence of two pools of free fatty acids. One pool would be common to all cell constituents, while in mitochondria a superimposed chain-lengthening and desaturation process (42), or a more labile exchange of unsaturated acids with phospholipid (43), would account for the higher content of unsaturated acids.

The presence of phospholipid in heart muscle myofibrils suggests that a membrane structure is quite firmly bound to the contractile unit. The present results distinguish this as mainly microsomal in nature, both on account of the high specific esterase activity of myofibrils and the close resemblance to microsomes in lipid composition. However, the term "microsomes" takes no account of the morphological complexity of the structure which may give rise to it when the tissue is disintegrated. There is evidence that muscle microsomes are derived from the sarcoplasmic reticulum (9), but this structure is highly differentiated under the electron microscope, much of it being in intimate association with myofibrils (44). The myofibrillar localization of inositol-containing lipid is interesting in view of the suggestion that phosphatidyl inositol may be a relaxing factor in muscle (45). Phosphatidyl inositol has also been implicated in the ATP-induced contraction of mitochondria (46).

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