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Uneven Distribution of Phospholipids in Rat Liver Inner Mitochondrial Membranes *

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Ultrasonic treatment of rat liver mitochondria followed by centrifugation in a discontinuous sucrose gradient gave rise to three subfractions. The sediment contained enzyme markers of inner membrane and of mitochondrial matrix; the subfraction present at the interface between 1.32 M and 0.76 M sucrose layers contained the totality of monoamine oxidase, the enzyme marker of outer membrane, and part of the enzyme activities belonging to inner membranes. The soluble subfraction contained almost exclusively enzymes of the matrix. The fragments belonging to the inner membrane obtained at the interface had a high proportion of unsaturated phospholipids, mainly phosphatidylethanolamine and cardiolipin together with a high activity of ATPase and of cytochrome oxidase.

The existence of a heterogenety of the inner mitochondrial membrane in the sense of presenting areas with different morphological aspect, and lipid and enzyme composition has been suggested after studying the submitochondrial fragments obtained by the lytic action of inducers of peroxidation reactions (8, 9, 11, 12) or by ultrasonic treatment (16). According to previous reports (8, 9) the different molecular species of phospholipids would be grouped rather selectively in areas of the inner membrane depending on their degree of unsaturation. The respiratory chain components would be located preferentially in the areas more unsaturated (9, 12).

In the work here reported it was found that when mitochondria were subjected to ultrasonic treatment fragments belonging to the inner membrane were separated; the phospholipids present in those fragments were highly unsaturated and the proportion of phosphatidylethanolamine and of cardiolipin was also very high. ATPase and cytochrome oxidase were also enriched in those fragments.

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Materials and Methods

Male Wistar rats weighing approximately 200 g were used in all the experiments. Liver were homogenized in 0.25 M sucrose and mitochondria isolated according to the method of HOGEBOOM (2). Outer mitochondrial membranes were prepared as described by PARSONS *et al.* (7). Mitochondrial subfractions were obtained following the method of SOTTOCASA *et al.* (13); mitochondria were sonicated in a MSE sonifier at 0° C during 15 seconds, at an amplitude of 6.7 microns.

Proteins were determined by the method of LOWRY *et al.* (3). Phospholipids were extracted from the samples, after precipitation with $HClO_4$ to give a final concentration of 0.3 N, as described previously (11) and separated by thin layer chromatography according to NESKOVIC *et al.* (5). Lipid phosphorus was determined by the method of BARTLETT (1). Methyl esters of the fatty acids of the different phospholipids present in the lipid extract were prepared through direct methylation with 14 % FB_3 in methanol according to MORRISON and SMITH (4). Methyl esters were then analyzed with a Beckman GC4 gas chromatograph, using a double column, with a 1/8 inch diameter and a 6 ft length; the liquid phase was 20 % DEGS, and the solid phase, Chromosorb W; particle size, 42/60 μ diameter; hydrogen and air flows were respectively 50 cc/min and 250 cc/min; column temperature, 160° C, and that of the detectors, 280° C. Methylation of fatty acids still bound to protein after lipid extraction was carried out as previously described (11).

The following components and enzyme activities were determined according to techniques already described in the literature; cytochromes a, b, c_1 and c (17), monoamine oxidase (15), malate dehydrogenase (6), cytochrome oxidase (13), ATPase (10) and succinate cytochrome c reductase (14).

Results and Discussion

The specific activity of mitochondrial enzymes were determined in the different subfractions from liver mitochondria obtained by ultrasonic treatment and subsequent centrifugation in discontinuous gradient. Figure 1 shows the results obtained. It may be seen that subfraction A, corresponding to the sediment, contained enzymes belonging to the inner membrane. The relative specific activity of both succinate-cytochrome c reductase and cytochrome oxidase was greater than 1 as referred to the original mitochondria (Table I). However, ATPase had a lower relative specific activity. Some malate dehydrogenase activity was also present in this subfraction. Subfraction B, which corresponded to the interface between 0.76 M and 1.32 M sucrose, contained nearly the totality of monoamine oxidase activity; it had also a high relative specific activity of ATPase and of cytochrome oxidase. Most of the total cytochrome content was distributed between subfractions A and B. Subfraction C, corresponding to the soluble components present in the 0.45 M sucrose layer, contained predominantly malate dehydrogenase activity and negligible activity of enzymes associated with either inner or outer membranes. It is obvious that the

Table I. *Enzyme activities and cytochrome content in mitochondria.*

Cytochrome oxidase	210 $m\mu mol \times min^{-1} \times mg\ protein^{-1}$
Succ. cyt. c reductase	270 $m\mu mol \times min^{-1} \times mg\ protein^{-1}$
ATPase	0.7 $\mu mol\ Pi\ min^{-1} \times mg\ protein^{-1}$
Malate dehydrogenase	1.6 $\mu mol \times min^{-1} \times mg\ protein^{-1}$
Monoamine oxidase	20 $m\mu mol \times min^{-1} \times mg\ protein^{-1}$
Cytochromes	0.7 $m\mu mol \times mg\ protein^{-1}$

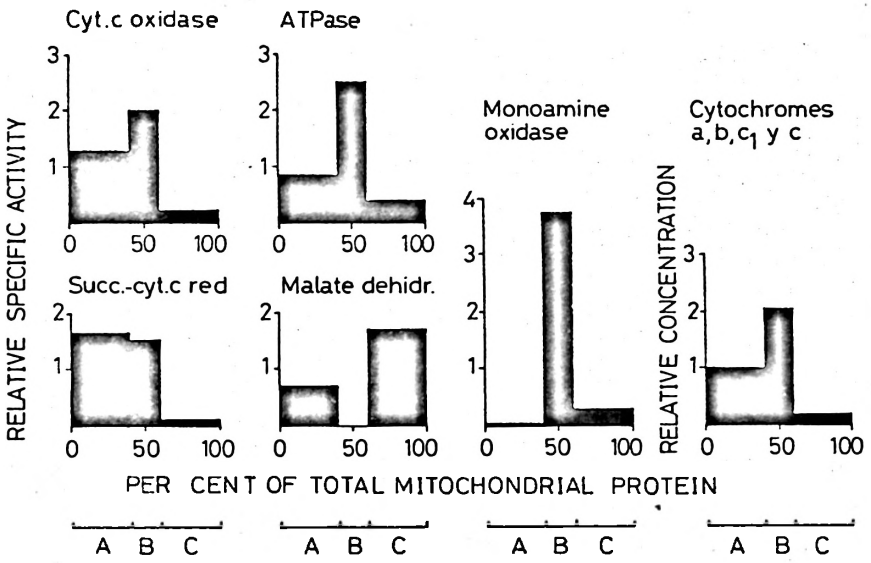

Fig. 1. *Distribution of mitochondrial activities in subfractions obtained by ultrasonic treatment.*
Subfraction A corresponded to the sediment; subfraction B to the interface between 1.32 M and 0.76 sucrose; and subfraction C to the soluble components present in the 0.45 M sucrose layer. The ordinates represent relative specific activities referred to the mitochondria before separation of the subfractions. The abscissas indicate the percentages of the total mitochondrial protein in each of the subfractions.

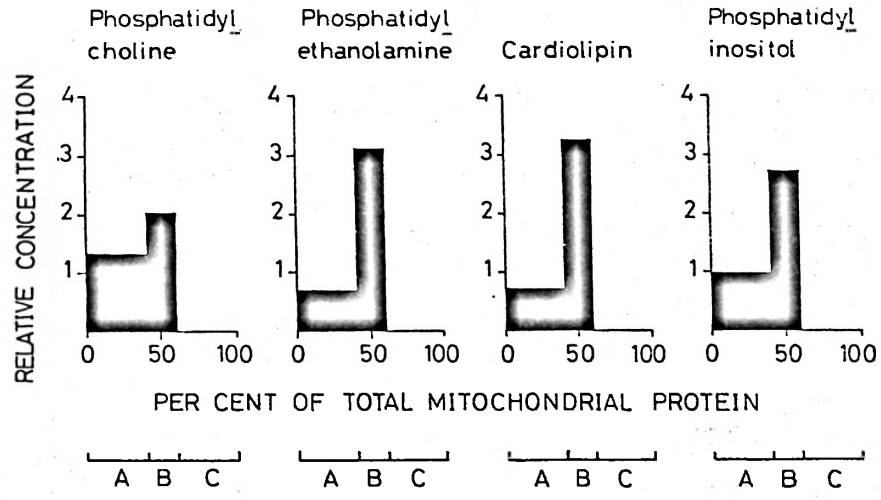

Fig. 2. *Distribution of phospholipids in subfractions obtained by ultrasonic treatment.* See figure 1 for identifications of subfractions. The ordinates represent relative concentration of lipid P referred to the mitochondria before separation of the subfractions. The abscissas indicate the percentages of the total mitochondrial protein in each of the subfractions.

Table II. *Phospholipid composition of mitochondria.*

Phospholipids	μg Lipid P/ mg protein
Lipid P	5.1
Phosphatidylethanolamine	1.7
Phosphatidylcholine	1.9
Phosphatidylinositol	0.6
Cardiolipin	0.6
Phosphatidylserine	0.1
Sphingomyelin	0.1
Fatty acids in total phospholipid extract	Area*
Saturated	49
Unsaturated	51

* The areas are referred to the sum of all the areas in the chromatogram of the fatty acids present in total phospholipid extract taken as 100.

outer membranes were present in subfraction B, since its enzyme marker monoamine oxidase was recovered almost exclusively associated with it. Judging from their specific enzyme markers it may be concluded that the inner membranes were distributed between subfractions A and B. The mitochondrial matrix was found preferentially in the soluble subfraction C; a small amount was also present in subfraction A.

The phospholipid content in each subfraction was also determined. Figure 2 shows the relative concentration with respect to the original mitochondria (Table II) for the different phospholipids in each subfraction. Phospholipids were not detected in subfraction C. In subfractions B, the relative concentration of phosphatidylethanolamine and of cardiolipin with respect to the original mitochondria had values over 3 and slightly lower values for phosphatidylcholine and phosphatidylinositol. In subfraction A the relative concentration of phosphatidylethanolamine and of cardiolipin was below 1.

The relative concentrations with respect to the original mitochondria (Table II) of saturated and unsaturated fatty acids present in the total phospholipid extract of each subfraction are shown in figure 3. It may be seen that the values of both saturated and unsaturated fatty acids are higher in subfraction B than in subfraction A.

The components of subfraction B belonged both to outer and inner membranes. Methods, such as that of PARSONS *et al.* (7), are available for the isolation of a purified outer membrane fraction, permitting the determination of the specific activity of the enzyme marker monoamine oxidase as well as its phospholipid content (Table III); these data may be used in establishing the quantitative participation of outer membranes and of all their components in subfraction B. Figure 4 shows the relative concentration of each phospholipid with respect to the original mitochondria, differentiating in subfraction B whether they belong to the inner (B_i) or to the outer membrane (B_o).

Table III. *Phospholipid composition and specific activity of monoamine oxidase in outer membranes.*

Phospholipids	μg Lipid P/ mg protein
Lipid P	20.0
Phosphatidylethanolamine	8.0
Phosphatidylcholine	6.3
Phosphatidylinositol	3.4
Cardiolipin	2.0
Phosphatidylserine + Sphingomyelin	0.3
Fatty acids in total phospholipid extract	Area*
Saturated	58
Unsaturated	42
Monoamine oxidase	Activity
	100 mμmol × min^{-1} × mg $protein^{-1}$

* The areas are referred to the sum of all the areas in the chromatogram of the fatty acids present in total phospholipid extract taken as 100.

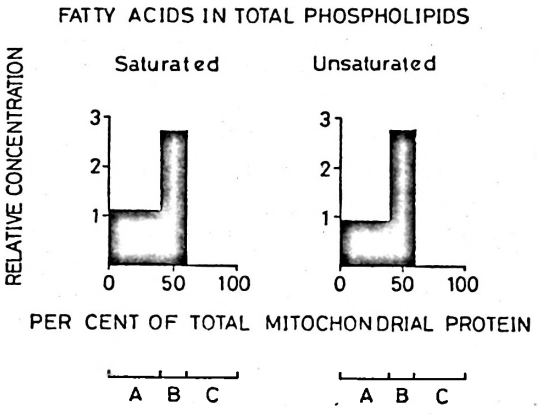

Fig. 3. *Distribution of saturated and unsaturated fatty acids present in total phospholipids extract in subfractions obtained by ultrasonic treatment.*

See figure 1 for identification of subfractions. The ordinates represent relative concentration of fatty acids saturated or unsaturated referred to the mitochondria before separation of the subfractions. The abscisas indicate the percentages of the total mitochondrial protein in each of the subfractions.

It may be observed that the component of the inner membrane present in subfraction B (B_i) had a very high relative concentration of cardiolipin and of phosphatidylthanolamine; on the other hand the value corresponding to phosphatidylcholine was rather low. In figure 5 it has been represented the relative concentrations of saturated and of unsaturated fatty acids present in the total phospholipids assigning them within subfraction B to either the inner membrane component (B_i) or to the outer membranes (B_o). It may be seen that the inner membrane component was enriched in unsaturated phospholipids with respect to the outer membranes.

In subfraction A components belonging to inner membranes and to the mitochondrial matrix were present. All the phospholipids in this subfraction corresponded exclusively to inner membranes since these compounds are absent in the matrix.

Comparing subfraction A with the in-

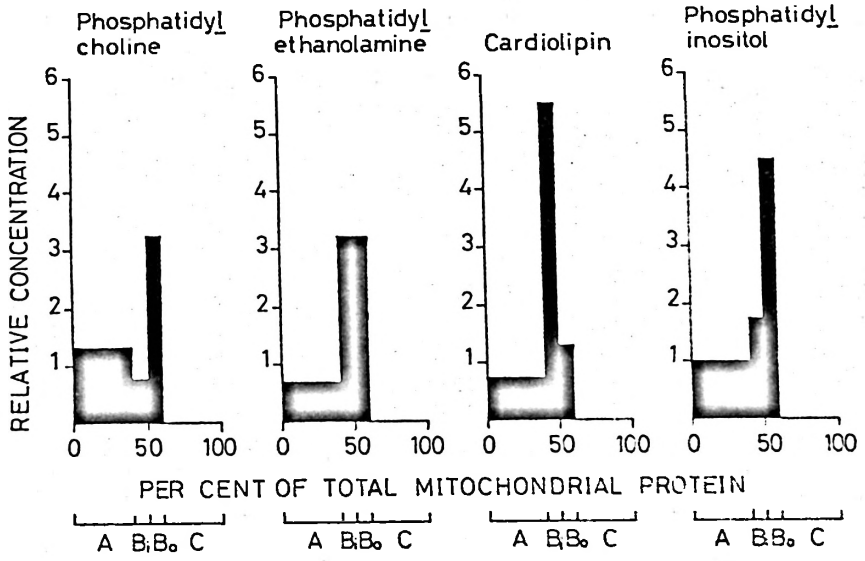

Fig. 4. *Distribution of phospholipids in subfractions obtained by ultrasonic treatment differentiating in subfractions B the fraction belonging to inner membranes (B_i) or to outer membranes (B_o).*

See text for other details. The ordinates represent relative concentration of lipid P referred to the mitochondria before separation of the subfractions. The abscisas indicate the percentages of the total mitochondrial protein in each of the subfractions.

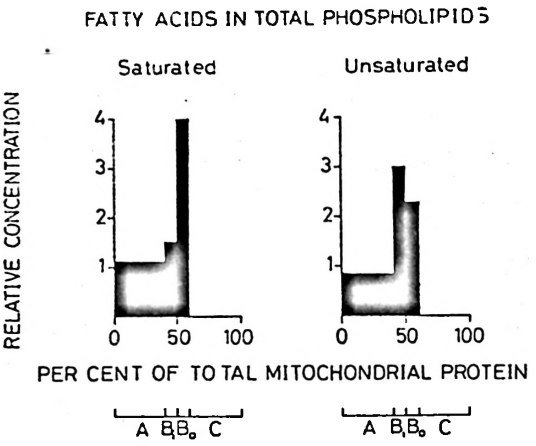

Fig. 5. *Distribution of saturated and unsaturated fatty acids present in total phospholipids extract in subfractions obtained by ultrasonic treatment differentiating in subfraction B the fraction belonging to inner membranes (B_i) or to outer membranes (B_o).* See text for other details. The ordinates represent relative concentration of fatty acids saturated or unsaturated referred to the mitochondria before separation of the subfractions. The abscissas indicate the percentages of the total mitochondrial protein in each of the subfractions.

ner membrane component present in subfraction B (B_i) in figures 4 and 5 it may be observed that the inner membrane fragments of subfraction B were richer in phosphatidylethanolamine and cardiolipin than those of subfraction A, and besides, that all its phospholipids were also more unsaturated. The inner membrane fragments present in subfraction B were also enriched in ATPase and in cytochrome oxidase.

These results strongly support the idea that in the inner membrane there are areas with different degrees of unsaturation as it has been previously suggested (8). The phospholipids present in the more unsaturated areas would be predominantly phosphatidylethanolamine and cardiolipin. The unsaturated areas would also be richer in ATPase and in cytochrome oxidase.

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Resumen

El tratamiento de las mitocondrias de hígado de rata con ultrasonidos y posterior centrifugación en gradiente discontinuo de sacarosa dio origen a tres subfracciones mitocondriales. El sedimento obtenido contenía enzimas marcadores de membrana interna y de matriz mitocondrial; la subfracción presente en la interfase de las capas de sacarosa 1,32 M y 0,76 M contenía la totalidad de la actividad del enzima marcador de membrana externa, monoamina oxidasa y parte de las actividades enzimáticas pertenecientes a la membrana interna; la fracción soluble contenía casi exclusivamente enzimas de matriz mitocondrial. Los fragmentos de la membrana interna obtenidos en la interfase tenían una alta proporción de fosfolípidos insaturados, principalmente fosfatidiletanolamina y cardiolipina, junto con una alta actividad ATPasa y citocromo oxidasa.

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