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EFFECT OF CHOLESTEROL CONTENT ON SOME PHYSICAL AND FUNCTIONAL PROPERTIES OF MITOCHONDRIA ISOLATED FROM ADULT RAT LIVER, FETAL LIVER, CHOLESTEROL-ENRICHED LIVER AND HEPATOMAS AH-130, 3924A AND 5123

F FEO, R A CANUTO, R GARCEA and L GABRIEL

Instituto di Patologia generale dell' Universita di Torino, Cso Raffaello 30, 10125, Torino (Italy)

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SUMMARY

The cholesterol to phospholipid ratio in mitochondria from hepatomas AH-130, 3924A and 5123 is higher than in the particles isolated from adult or fetal rat livers Nearly all the cholesterol of hepatoma mitochondria is located in membranes. As in liver mitochondria, in the particles isolated from hepatoma AH-130 there is more cholesterol in the outer than in the inner membrane.

In mitochondria from cholesterol-enriched liver and hepatomas, there occurs a decrease in extent of hypoosmotic and phosphate-induced swelling and a decrease of conformational changes linked to energy states The phenomenon is more marked in particles which exhibit higher cholesterol to phospholipid ratios. A statistically significant negative correlation exists between the cholesterol to phospholipid ratio and extent of volume or conformational changes. No significant modifications of these parameters were found in fetal liver mitochondria

Cholesterol content does not influence K^+ uptake by cholesterol-enriched or hepatoma mitochondria. Nor does cholesterol content affect the respiratory increment related to this uptake. As a consequence of K^+ uptake, total mitochondrial water exchangeable with tritiated water rises 20 % while sucrose-impermeable water rises 42–48 % in both adult rat liver and hepatoma AH-130 mitochondria Absorbance changes linked to ion uptake do not correspond merely to variations in mitochondrial water content Water content is apparently not influenced by the cholesterol to phospholipid ratio. However, the ratio is significantly correlated to both extent and initial rate of absorbance decrease of mitochondrial suspensions during K^+ uptake The higher the ratio, the lower the extent and initial rate of absorbance decrease

INTRODUCTION

Cholesterol and phospholipid are important constituents of biological membranes There exist cholesterol to phospholipid ratios each peculiar to a particular membrane [1]. It has been observed that the relative amounts of the hydrocarbon fatty acids chains and the interactions between phospholipid and cholesterol may in-

fluence permeability and physical resistance of natural and artificial membranes (for review, see ref. 2). The protein conformation also seems to be greatly influenced by the lipid composition of membranes [3]

In recent years, different functional and physical alterations have been demonstrated in mitochondria from hepatomas. These organelles were shown to be less prone to volume and conformational changes than normal mitochondria [4–7]. They exhibit great lability of their phosphorylating apparatus [8]. Interestingly, a similar pattern of alterations has been described by Graham and Green [9] in mitochondria 'enriched' in vitro with cholesterol. Mitochondria from different hepatomas are characterized by cholesterol to phospholipid ratios higher than in normal liver mitochondria [10–12]. In the present paper we investigated the influence of the modifications of cholesterol content on some functional and physical properties of mitochondria from Yoshida ascites hepatoma AH-130, and Morris hepatomas 3924A and 5123. For comparison, mitochondria from cholesterol-enriched and fetal livers were studied. The results indicate that a close relationship exists between cholesterol to phospholipid ratio and the extent of large-amplitude swelling or conformational changes of mitochondria No clear influences of changes in cholesterol content on K^+ uptake or energy coupling were observed.

METHODS

Animals and tumours. Long-Evans (150–220 g), female Buffalo (150–250 g) and female ACI (150–200 g) rats were used as source of normal livers, as well as to maintain hepatomas Yoshida ascites AH-130, Morris 5123 and 3924A, respectively Fetal liver was obtained from 19–20-day-old fetuses of Long-Evans rats. The animals were housed no more than three to a cage and given tap water ad libitum. The tumour-bearing animals and the corresponding controls were fed a semisynthetic diet (Piccioni, Brescia, Italy). Male Wistar rats (200–250 g) were used as controls or to induce hypercholesterolemia. All animals were fasted 16–18 h before killing.

Hypercholesterolic treatment. This was performed by using different mixtures of a hypercholesterolic and a stock diet. The hypercholesterolic diet contained 10 % peanut oil, 31 % coconut oil, 5 % cholesterol, 2 % sodium cholate, 20 % casein, 19 7 % sucrose, 2 % vitamin diet fortification mixture 1369 (Nutritional Biochemical Corporation, Cleveland, Ohio), 1 % choline chloride, 4 % salt mixture W-1139 (Nutritional Biochemical Corporation) and 6 % alphocel. The stock diet was a mixture of 5 % peanut oil, 20 % casein, 58.8 % sucrose, 2 % vitamin diet fortification mixture 1369, 0.2 % choline chloride, 4% salt mixture W-1139 and 15% cellulose. The animals were fed a 1 : 3 mixture of hypercholesterolic and stock diets during the first week of treatment, and a 1 : 1 mixture during the following 8–9 weeks. 2–3 weeks before killing, the rats were fed a 3 : 1 mixture Controls were fed the stock diet alone. The entire treatment lasted 11–13 weeks; at this time cholesterolemia reached its highest value.

Fractionation procedures. The slowly growing hepatoma 5123 was transplanted monthly and used 20–25 days after transplantation. The fast growing hepatomas 3924A and AH-130 were used 12–14 days and 6–7 days after transplantation, respectively. The medium and procedures for suspension, homogenization and isolation of mitochondria from adult rat liver, cholesterol-enriched liver, and hepatomas 5123 and 3924A were as previously described [8], except that a 133 000 $g \cdot$ min mitochon-

drial fraction was prepared. The cells from hepatoma AH-130 were separated from the ascitic fluid by low-speed centrifugation, washed in the isolation medium and resuspended in a volume of the same medium corresponding to 2 5 times their weight The suspensions were homogenized in a Dounce grinder with 15 strokes of a tight pestle. Homogenates were diluted to 20 % (w/v) and centrifuged at 6000 *g* min Sediments were resuspended, submitted to an additional 10 strokes of homogenization and centrifuged at 10 000 *g* · min. From collected supernatants, mitochondria were isolated at 133 000 *g* min. Fetal liver was homogenized in a Potter-Elvehjem homogenizer with one stroke of a manually-driven, loose pestle. Fetal liver mitochondria were isolated in the same way as the adult rat liver organelles All mitochondrial fractions were washed three times (unless otherwise stated). Microsomes were isolated as previously described [12] Purity of mitochondrial preparations was routinely evaluated by determining glucose-6-phosphatase and NADPH-cytochrome *c* reductase as markers of microsomes From the specific activities of the marker enzymes in the two subcellular fractions, concentrations of microsomes in mitochondria ranging between 4 9 % and 7.6 % were calculated

The soluble mitochondrial constituents were separated from the insoluble constituents according to Mitchell [13] Outer and inner membranes from 5-times-washed normal or cholesterol-enriched mitochondria were isolated and purified according to the method of Sottocasa et al. [14] as modified by Jones and Jones [15]. In order to isolate the same mitochondrial subfractions from hepatoma AH-130, slight modifications of the above method were introduced Five-times-washed mitochondria (7–10 mg protein/ml) were suspended in 10 mM sodium phosphate buffer (pH 7 4) After 20 min incubation at 0 °C, KCl, ATP and $MgCl_2$ were added to a final concentration of 480.5, 5 and 5 mM, respectively [15] After an additional 10 min incubation, the suspensions were submitted to 30 s of sonic oscillation with the use (at 60 % of its maximum output) of a Biosonik III sonifier (Bronwill Scientific, Rochester, N Y) provided with a standard tip. Mitochondria were finally centrifuged through a discontinous sucrose gradient [14] In some experiments, inner membrane plus matrix subfractions (5 mg protein/ml) were submitted to further purification by incubating them for 10 min at 0 °C, with gentle mixing, in a medium containing 290 mM sucrose, 20 mM Tris · HCl buffer (pH 7.4) and 0.125 % digitonin (twice recrystallized from ethanol). The suspensions were then centrifuged at 95 000 *g* min. Sediments, washed once in digitonin-free medium, constituted the purified inner membrane fractions. Inner mitochondrial membranes free of matrix were prepared by submitting the inner membrane plus matrix subfractions to 3 min sonic oscillation at 0 °C, using the Biosonik III sonifier at its maximum output and then centrifuging at 21 10^{-6} g min

Enzyme assays. The spectrophotometric determinations of rotenone-insensitive NADH-cytochrome *c* reductase (EC 1.6.99 3), NADPH-cytochrome *c* reductase (EC 1.6 99.1) and malate dehydrogenase (EC 1 1 1.37) were performed according to Schnaitman and Greenawalt [16] Cytochrome *c* oxidase (EC 1 9.3.1) was determined according to De Duve et al. [17], succinate dehydrogenase (EC 1.3.99.1) according to Pennington [18], and glucose-6-phosphatase (EC 3 1.3.9) according to Swanson [19].

Swelling and conformational changes. Hypoosmotic swelling was determined by recording the absorbance decrease of mitochondrial suspensions (1 0 mg protein/ml) in 10 mM Tris HCl buffer (pH 7 4) Phosphate-induced swelling was determined in a reaction system containing, in 3 ml, 140 mM KCl, 20 mM Tris · HCl buffer (pH 7 4)

and mitochondria (1.0 mg protein/ml). The swelling was initiated by addition of Tris/phosphate to a final concentration of 5 mM. Hypoosmotic and phosphate-induced swellings were followed until their rates corresponded to that of spontaneous swelling in a medium containing 140 mM KCl and 20 mM Tris · HCl buffer (pH 7.4). Valinomycin-induced swelling was evaluated in a medium containing, in 3 ml, 10 mM KCl, 2 mM Tris · HCl buffer (pH 7.4), 2 mM Tris/phosphate, 3 mM Tris/glutamate, 1.5 mM Tris/malate, 280 mM sucrose and mitochondria (1.0–1 6 mg protein/ml). The reaction was started by addition of valinomycin to a concentration of $1 \cdot 10^{-3}$ mM. Small amplitude absorbance changes were recorded by suspending mitochondria (1.0–1.6 mg protein/ml) in 3 ml of a reaction mixture containing 100 mM sucrose, 3 mM Tris/EDTA (pH 7.8), 3 mM L-glutamate and 1,5 mM L-malate. When the absorbance was steady, sodium phosphate (pH 7.8) was added to a concentration of 4 mM. When the absorbance reached its minimum, ADP was added to a concentration of 0.1 mM.

The absorbance changes were followed in a Beckman Acta CIII spectrophotometer, at 250 nm, at 23 °C.

Respiratory rate and acceptor control ratios. Mitochondrial respiration, oxidative phosphorylation and acceptor control ratios were measured as previously described [8]. The stimulation of respiration by K^+ plus valinomycin was determined under the same conditions adopted for the measurements of valinomycin-induced swelling.

Potassium uptake. K^+ concentrations were measured with a selective ion electrode (Beckman, 39137) using the same conditions adopted to determine respiratory and volume changes linked to K^+ uptake. In some experiments K^+ uptake was measured at the same time as the respiratory rates or the absorbance changes, by using suitably modified holders in order to introduce ion and reference electrodes.

Volume determinations. In order to measure the changes of mitochondrial water content induced by K^+ uptake, mitochondria (10 mg protein) were suspended in 6 ml of a medium containing 10 mM KCl, 5 mM Tris · HCl buffer (pH 7.4), 2 mM Tris/phosphate, $1 \cdot 10^{-3}$ mM valinomycin, 8 mM sucrose, trace amounts of [^{14}C]-sucrose ($2.2 \cdot 10^5$ dpm/ml; The Radiochemical Centre, Amersham, Bucks) or of [*carboxy*-^{14}C]dextran ($2.2 \cdot 10^5$ dpm/ml; M_r 50 000; The Radiochemical Centre), 217 mM mannitol, and ^3H_2O ($4 \cdot 10^6$ dpm/ml; The Radiochemical Centre). The reaction was started by addition of mitochondria. After 3 min at 23 °C, samples were rapidly centrifuged at 144 000 $g \cdot$ min. The isotope content and volumes of the pellets were determined as previously described [6]. Total mitochondrial water and sucrose-impermeable space were determined as differences between ^3H_2O and [*carboxy*-^{14}C]-dextran or ^3H_2O and [^{14}C]sucrose, respectively, in the pellets [20]. In order to determine changes in mitochondrial total water induced by hypoosmotic conditions, mitochondria were incubated for 5 min at 23 °C in the same reaction mixtures used for spectrophotometric determinations, except that ^3H_2O and [*carboxy*-^{14}C]dextran were included in the medium.

Analytical determinations. The procedures for lipid extraction, purification and determination have already been described [12]. Proteins were determined by a biuret procedure [21].

RESULTS

Functional integrity of mitochondrial preparations

Mitochondrial integrity was routinely controlled by polarographic determination of State 3 and State 4 respiratory rates and of acceptor control ratios, in either presence or absence of Mg^{2+}. The divalent cation was added to the reaction system to test whether it induced stimulation of State 4 respiration and impairment of the acceptor control ratios. These deleterious effects would indicate that Mg^{2+} penetrates the mitochondria and activates ATPase. It is known that this behaviour is characteristic of damaged mitochondria [22] and has sometimes been observed in hepatoma mitochondria [8]

As shown in Table I, State 3 and State 4 respiratory rates with glutamate and malate as substrates, and acceptor control ratios of the same order, are exhibited by mitochondria from adult rat liver, cholesterol-enriched liver and hepatoma AH-130 No effect of Mg^{2+} on respiratory rates and acceptor control ratios was observed in the organelles from these sources The rates of oxygen uptake in State 3 and State 4 and the acceptor control ratios are lower in mitochondria from fetal liver and hepatomas 3924A and 5123 compared to normal liver They are not significantly modified by Mg^{2+}. It cannot be excluded that the low respiratory rates observed in mitochondria from fetal liver or hepatomas 3924A and 5123 are related to a low degree of functional integrity. The acceptor control ratios, however, are still high in these particles and are not modified by Mg^{2+}, so the existence of gross damage to membranes might be excluded. It should be noticed that no gross morphological damage was found in mitochondrial preparations from hepatomas showing acceptor control ratios even lower than those found in this study [7, 23].

Cholesterol and phospholipid content

As shown in Table II, the content of free cholesterol is 4.7 times higher in mitochondria from cholesterol-fed rats than in control mitochondria. Since the phospholipid content is unmodified, the cholesterol to phospholipid ratio increases directly with the free cholesterol. An increase in free cholesterol content, ranging between 4 and 6 times the control value was observed in mitochondria from hepatomas. Phospholipid contents do not change The cholesterol to phospholipid ratios are 4.0, 4,6 and 7.9 times higher than the control value in mitochondria from hepatomas AH-130, 3924A and 5123, respectively. No modifications in cholesterol and phospholipid contents occur in fetal liver mitochondria. These results confirm the Ruggieri and Fallani's earlier observations on hepatoma 5123 [11]. These authors also found 1 35 times more cholesterol in hepatoma AH-130 than in liver mitochondria [10] This increase, however, was not significant and is lower than the 4 35-times increase observed in this study. Perhaps this discrepancy may be explained by differences in the purity of mitochondrial preparations, since Ruggieri and Fallani [10] used once-washed mitochondria

In order to determine to what extent mitochondrial membranes contribute to the rise in cholesterol in the organelles isolated from cholesterol-enriched liver and hepatomas, we separated the soluble and the insoluble components of mitochondria. Table III shows that strong sonic oscillation releases 29–40 % of the protein from mitochondria of different sources There also occurs a 75–80 % release of the matrix enzyme

TABLE I

RESPIRATORY RATES, ACCEPTOR CONTROL AND ADP 0 RATIOS OF MITOCHONDRIA FROM ADULT RAT LIVER, FETAL LIVER, CHOLESTEROL-ENRICHED LIVER AND HEPATOMAS

The reaction mixture contained, in 2 ml, 145 mM KCl, 20 mM Tris HCl buffer (pH 7 4), 1 mM Tris/phosphate, 3 mM L-glutamate, 1 5 mM L-malate and, when indicated, 1 mM $MgCl_2$ The reaction was started by addition of 0 2 ml of mitochondria (2 0–2 5 mg protein) State 3 was induced by 0 2 mM ADP Temperature, 23 °C The results are mean values of 4–10 experiments ±S D , expressed in natoms O_2/min per mg protein.

Source of mitochondria	Respiratory rate				Acceptor control ratio		ADP 0	
	State 3		State 4					
	$-Mg^{2+}$	$+Mg^{2+}$	$-Mg^{2+}$	$+Mg^{2+}$	$-Mg^{2+}$	$+Mg^{2+}$	$-Mg^{2+}$	$+Mg^{2+}$
Adult liver	72 0±11 0	67 6±12 1	7 3±1.3	7 1±2 6	9 9±2 6	9 5±1 2	2 74±0 8	2 82±0.0
Fetal liver	26 8± 7 7	24.1± 4 5	5 3±0 8	4 2±1 4	5 1±1.4	5 7±2 0	2 67±0.3	2 72±0 5
Cholesterol-enriched liver	63 6± 3 3	70 0±15 0	5 9±0 6	6 8±4 0	10 8±4 4	10 3±4.9	2 96±1 4	2.89±0.6
Hepatoma AH-130	64 4± 2 8	68 0±11 0	7 0±3 1	8 2±2 6	9 2±3 4	8 3±3.3	2 63±1 0	2 74±0.7
Hepatoma 3924A	24 9± 5 6	23 1± 4.9	4.3±0.9	5.6±0 8	5 8±0 9	4 1±0 6	2 76±0 4	2 69±0 3
Hepatoma 5123	23 4± 6 2	21 2± 0 6	4.4±1 7	5.0±1 2	5 3±0 8	4.2±0 2	2 69±0 4	2 80±1 0

TABLE II

CHOLESTEROL AND PHOSPHOLIPID CONTENTS OF MITOCHONDRIA FROM ADULT RAT LIVER, FETAL LIVER, CHOLESTEROL-ENRICHED LIVER AND HEPATOMAS

Data are mean values ±S D The S D of the ratio cholesterol phospholipid (μg/mg) was calculated according to Worthing and Jeffner [41] Molecular weight of cholesterol = 387, phospholipid = 700

Source of mitochondria	No of expts	Cholesterol (μg/mg protein)	Phospholipid (mg/mg protein)	Cholesterol (μg/mg phospholipid)	Molar ratio (cholesterol phospholipid)
Adult liver	20	5 2±1 5	0 158±0 012	33± 9	1 16 8
Fetal liver	4	5 1±1 1	0 130±0 022	39±11	1 14 1
Cholesterol-enriched liver	10	19 2±0 6	0 182±0 008	107±15	1 5 2
Hepatoma AH-130	5	22 6±2 4	0 168±0 026	134±24	1 4 1
Hepatoma 3924A	7	20 2±4 2	0 134±0 008	151±29	1 3 7
Hepatoma 5123	8	32 4±6 7	0 124±0 035	261±91	1 2 1

TABLE III

CHOLESTEROL CONTENT OF INSOLUBLE AND SOLUBLE FRACTIONS OF MITOCHONDRIA FROM ADULT RAT LIVER, CHOLESTEROL-ENRICHED LIVER AND HEPATOMAS

Mitochondria (1.5 mg protein/ml) were suspended in water and submitted to 3 min of sonic oscillation at 0 °C using a Biosonik III sonifier at its maximum output. Aliquots were then centrifuged at 21 10^6 *g* min The supernatants and sediments are referred as soluble and insoluble components, respectively Data (mean values ±S.D) are expressed as percent of the total contents determined in sonicated, unfractionated mitochondria.

Source of mitochondria	No. of expts	Protein		Malate dehydrogenase		Cholesterol
		Insoluble	Soluble	Insoluble	Soluble	Insoluble
Adult liver	4	60±4	40±2	16±6	80±15	82±10
Cholesterol-enriched liver	2	58	40	19	79	76
Hepatoma AH-130	4	54±3	37±1	20±4	79± 8	78± 6
Hepatoma 3924A	2	68	30	21	77	80
Hepatoma 5123	4	71±2	29±3	21±3	75±12	76± 4

malate dehydrogenase. It appears that the main portion of cholesterol is in the insoluble material of any mitochondrial preparation.

The cholesterol to phospholipid ratio in the isolated inner and outer mitochondrial membranes was determined in hepatoma AH-130, cholesterol-enriched liver and adult rat liver. Five-times-washed mitochondria were used in these experiments. The NADPH-cytochrome *c* reductase associated with these mitochondrial preparations was only 2–3 % of that found in microsomes. These figures ranged

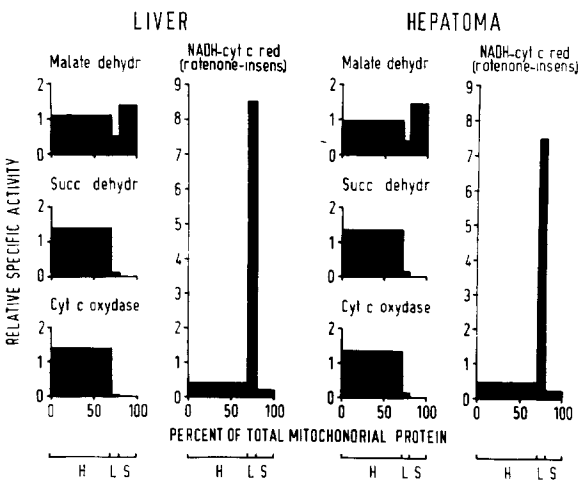

Fig 1. Distribution of some enzymatic activities in the inner membrane plus matrix, outer membrane and soluble subfractions of liver and hepatoma AH-130 mitochondria The ordinates represent relative specific activities on protein basis, taking the specific activities of the swollen-contracted and sonicated mitochondria before subfractionation as 1. The abscissas indicate the percentages of total mitochondrial proteins in inner membrane plus matrix (H), outer membrane (L) and soluble (S) subfractions.

between 0 and 0 8 % in the case of inner membrane plus matrix subfractions. The degree of microsomal contamination in the outer membrane subfractions of the three types of mitochondria was about 10 %

Fig. 1 illustrates the distribution and recovery of different enzymatic activities after subfractionation of normal liver and hepatoma AH-130 mitochondria Results obtained with cholesterol-enriched mitochondria corresponded to those with control liver mitochondria; they were not included in the figure It appears that the cytochrome *c* oxidase and succinate dehydrogenase are concentrated to the same extent in the inner membranes plus matrix subfractions of the two types of mitochondria Malate dehydrogenase has the same distribution between the inner membrane plus matrix and the soluble subfractions in both types of mitochondria. This indicates that rupture of the inner membrane proceeds to the same extent during subfractionation of liver and hepatoma mitochondria under the adopted conditions The NADH-cytochrome *c* reductase (rotenone-insensitive) is also concentrated to roughly the same extent in the outer membranes of hepatoma and liver mitochondria The relative specific activity of this enzyme is slightly higher in the inner membrane plus matrix of hepatoma mitochondria than in the same subfraction of liver mitochondria. The percent contamination of outer in inner membrane, as calculated from the specific activities of the marker enzyme in the two subfractions, was 9 3% (5 6–13 0%) for liver mitochondria and 16 % (14 2–22 0 %) for hepatoma mitochondria In digitonin-purified inner membranes the degree of contamination dropped to 2 5–4.2 % in liver and hepatoma mitochondria

As shown in Table IV, the outer membrane of liver mitochondria contains about 8 times more cholesterol and 3 times more phospholipid than the inner membrane Consequently, the cholesterol to phospholipid ratio is about 3 times higher in the outer membrane. The cholesterol to phospholipid molar ratios in our preparations of outer and inner membranes are in agreement with those found by others [24, 25] In the outer and inner membranes of cholesterol-enriched mitochondria there are 1.6 and 4 3 times increases, respectively, in endogenous levels of cholesterol. The phospholipid contents are unmodified, consequently, the cholesterol to phospholipid ratios of the two membranes increase directly with cholesterol. There is 83 % more cholesterol in the inner membrane of hepatoma AH-130 mitochondria than in the same subfraction of liver mitochondria No changes in the phospholipid content occur, so the cholesterol to phospholipid ratio is about 5 times higher in hepatoma mitochondria than in liver mitochondria inner membranes. When the purified inner membranes are considered, this figure becomes 4.1 The cholesterol content of outer membrane is only 21 % higher in hepatoma mitochondria than in liver mitochondria However, the phospholipid content of outer mitochondrial membrane is low in hepatoma. Consequently, the cholesterol to phospholipid ratio increases 3.8 times with respect to the outer membrane from liver mitochondria.

Mitochondrial swelling

Hypoosmotic and phosphate-induced swellings were studied in cholesterol-enriched and hepatoma mitochondria, to determine whether high cholesterol to phospholipid ratios influenced the swelling extent. Data in Table V show that a decrease in extent of hypoosmotic and phosphate-induced swelling occurs in cholesterol-enriched as well as in hepatoma mitochondria It appears that the decrease of

TABLE IV

CHOLESTEROL AND PHOSPHOLIPID CONTENTS OF ADULT RAT LIVER, CHOLESTEROL-ENRICHED LIVER AND HEPATOMA AH-130 MITOCHONDRIA AND ISOLATED INNER AND OUTER MITOCHONDRIAL MEMBRANES

Data are mean values ±S D The S D of the ratio cholesterol phospholipid (μg/mg) was calculated according to Worthing and Jeffner [41] Molecular weight of cholesterol = 387, phospholipid = 700.

Fractions	No. of expts	Cholesterol (μg/mg protein)	Phospholipid (mg/mg protein)	Cholesterol (μg/mg phospholipid)	Molar ratio (cholesterol · phospholipid)
Liver					
Whole mitochondria	3	3 2±1 1	0 125±0 000	25± 9	1 21 6
Inner membrane	3	4 7±1 3	0.223±0 031	21± 6	1 26 4
Purified inner membrane	2	4 1	0 185	22	1 24 0
Outer membrane	3	39 5±2 3	0 656±0.100	60±10	1 9 2
Cholesterol-enriched					
Whole mitochondria	4	17 7±2 4	0.177±0 023	100±18	1 5 5
Inner membrane	2	20 1	0 282	71	1 7 7
Outer membrane	2	63 0	0 693	92	1 6 1
Hepatoma					
Whole mitochondria	6	15 8±2 0	0 136±0 031	116±31	1 4 1
Inner membrane	4	27 3±4 3	0 265±0 134	102±54	1 5 4
Purified inner membrane	3	18 3±1 6	0 203±0.092	90±16	1 6 1
Outer membrane	6	50 0±9 0	0 221±0 044	226±61	1 · 2 4

TABLE V

LARGE-AMPLITUDE SWELLING INDUCED BY HYPOOSMOTIC CONDITIONS OR PHOSPHATE IN MITOCHONDRIA FROM ADULT RAT LIVER, FETAL LIVER, CHOLESTEROL-ENRICHED LIVER AND HEPATOMAS

Conditions as described under Methods Data are mean values ±S D

Source of mitochondria	Swelling	
	Hypoosmotic (ΔA/mg protein)	Phosphate-induced (ΔA/mg protein)
Adult liver	1 88±0 39(7)	1 31±0 20(4)
Fetal liver	1 81 (2)	1 20 (2)
Cholesterol-enriched liver	1 30±0 11(3)	0 77±0 03(3)
Hepatoma AH-130	0 82±0 15(5)	0 36±0 07(4)
Hepatoma 3924A	0 60±0 10(3)	0 11±0 02(3)
Hepatoma 5123	0 31±0 07(5)	0 06±0 01(4)

swelling extent is more marked in mitochondria with high cholesterol to phospholipid ratios (see also Table II). No changes occur in fetal liver mitochondria as compared to adult rat liver organelles

A statistical analysis of the results of swelling determinations is reported in Fig 2 Each value of swelling for mitochondrial preparations from different sources is plotted against the correspondent cholesterol to phospholipid ratio It can be seen that the correlation coefficients for hypoosmotic and phosphate-induced swelling are –0.74 and –0 78, respectively These figures indicate the existence of good correlation between swelling extent and cholesterol to phospholipid ratios

Absorbance decrease does not necessarily reflect mitochondrial volume change. However, the correspondence between these parameters has already been demonstrated for phosphate-induced swelling of hepatoma mitochondria [6] Measurements

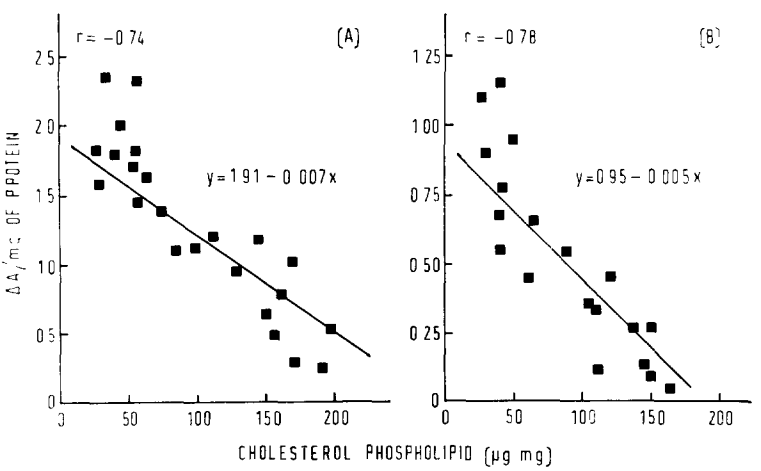

Fig 2 Scatter diagrams with a linear regression showing a negative correlation between the amplitude of mitochondrial hypoosmotic (A) or phosphate-induced (B) swelling and cholesterol to phospholipid ratio. Regression coefficients are significantly different from zero ($P < 0\ 001$)

TABLE VI

OSCILLATORY PARAMETERS IN MITOCHONDRIA FROM ADULT RAT LIVER, FETAL LIVER, CHOLESTEROL-ENRICHED LIVER AND HEPATOMAS

Conditions as described under Methods Phase I refers to the time of phosphate addition, Phase II to the absorbance minimum following this addition, Phase III to the absorbance minimum following the first ADP addition, i e after the 1st swelling-shrinkage cycle Data are mean values ±S D.

Source of mitochondria	No of expts	Amplitude		Period	
		Swelling (ΔA/mg protein)	Shrinkage (ΔA/mg protein)	Phase I to II (s)	Phase II to III (s)
Adult liver	10	0 122±0 000	0 098±0 000	69 2±13 4	123 5±34 1
Fetal liver	4	0 112±0 000	0 127±0 031	46 2±17 0	133 7±43 0
Cholesterol-enriched liver	4	0 097±0 021	0 099±0.034	40 0±12 0	90 0±27 0
Hepatoma AH-130	8	0 093±0 012	0 048±0 000	64 6±24 5	80 4±26 6
Hepatoma 3924A	2	0 081	0 042	57 2	80 9
Hepatoma 5123	8	0 067±0 000	0 035±0 000	54 9±13.2	85 2±28 9

of total mitochondrial water during hypoosmotic swelling indicated that adult rat liver mitochondria take up, after 5 min incubation in 10 mM Tris HCl buffer, 2.41 (S.D. = ±0 36) μl water/mg protein In hepatoma AH-130 mitochondria this amount is 1.26 (S D = ±0 51) μl/mg protein. Thus, water intake determinations show a 1.94 times decrease in swelling extent in AH-130 hepatoma mitochondria. This figure is in agreement with the 2.29 times decrease found by absorbance measurements

Volume oscillations

In view of the above observations, we investigated different types of mitochondria for small amplitude volume oscillations related to changes in energy states Volume oscillations probably represent a more physiological phenomenon than large-amplitude swelling. After addition of phosphate, fully reversible cycles of absorbance decrease and increase occur [26]. They can be better followed, however, if synchronized by addition of ADP [4, 9]

The parameters measuring volume oscillations in mitochondria from different sources are shown in Table VI. It appears that the amplitude of oscillations is lower in cholesterol-enriched and hepatoma mitochondria than in control mitochondria As with large amplitude swelling, the decrease is inversely proportional to cholesterol to phospholipid ratio The amplitude of volume oscillations of fetal liver mitochondria appears similar to that of organelles from adult rat liver. No alterations in the oscillatory periods are exhibited by mitochondria from any tissue examined.

Statistical analysis of the correlation between amplitude of volume oscillations and cholesterol to phospholipid ratios are shown in Fig. 3 Regression coefficients of −0.85 and −0.75 were found for the swelling and shrinkage phases, respectively These values indicate the existence of a significant correlation between cholesterol to phospholipid ratios and volume oscillations.

K^+ uptake

According to the results of recent work, transport of monovalent cations through natural membranes may be affected by cholesterol content and fatty acid composition [27, 28] The possibility of differences in K^+ transport through mitochon-

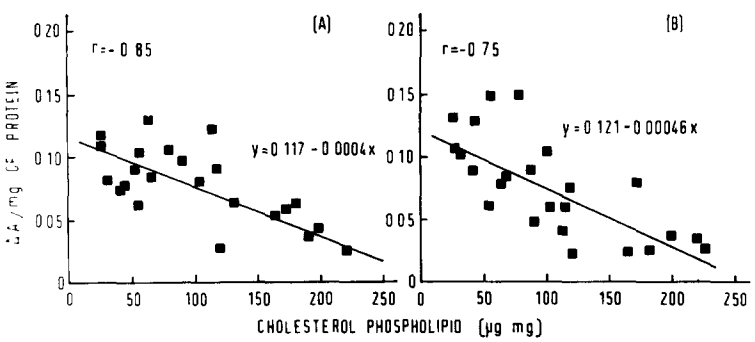

Fig 3 Scatter diagrams with a linear regression showing a negative correlation between mitochondrial small amplitude swelling (A) or shrinkage (B) and cholesterol to phospholipid ratio Regression coefficients are significantly different from zero ($P < 0\ 001$)

TABLE VII

K$^+$ UPTAKE AND INCREMENTS IN OXYGEN CONSUMPTION IN MITOCHONDRIA FROM ADULT RAT LIVER, FETAL LIVER, CHOLESTEROL-ENRICHED LIVER AND HEPATOMAS

The test system contained 10 mM KCl, 2 mM Tris HCl buffer (pH 7 4), 2 mM Tris/phosphate, 3 mM Tris/glutamate, 1,5 mM Tris/malate, 280 mM sucrose and mitochondria (1 0–1 6 mg protein). The reaction was started by addition of $1 \cdot 10^{-3}$ mM valinomycin. Data are mean values ±S D

Source of mitochondria	No of expts	K$^+$ uptake (nequiv /mg protein)	Extra oxygen (natoms O_2/min per mg protein)
Adult liver	10	250±44	31 5±5 4
Fetal liver	2	230	26 2±6 5
Cholesterol-enriched liver	3	264±41	30 2±4 2
Hepatoma AH-130	5	290±47	31 9±3 0
Hepatoma 3924A	3	240±37	14 4±1 6
Hepatoma 5123	6	210±19	13 5±0 2

drial membranes having different cholesterol contents was investigated by studying various parameters related to monovalent cation uptake. These parameters include K$^+$ concentration in the reaction medium as well as respiratory and volume changes. Table VII shows that no differences exist between mitochondria from livers of control rats, fetuses and hypercholesterolic rats, concerning K$^+$ uptake and extra oxygen consumption in the presence of valinomycin. Hepatoma AH-130 mitochondria behave exactly as the preceeding mitochondrial preparations. The accumulation of K$^+$ in mitochondria from hepatomas 3924A and 5123 is of the same order as in adult rat liver organelles. However, the extra oxygen is lower. This indicates that the cation uptake proceeds at a slower rate in the organelles from the two hepatomas compared to control mitochondria (cf. ref. 29). This was indeed demonstrated by recording the changes in K$^+$ concentration in the reaction mixture after valinomycin addition (not shown).

Data in Table VIII show that marked decrease in extent and initial rate of

TABLE VIII

VALINOMYCIN-INDUCED SWELLING IN MITOCHONDRIA FROM ADULT RAT LIVER, FETAL LIVER, CHOLESTEROL-ENRICHED LIVER AND HEPATOMAS

The same conditions as in Table VII. Data are mean values ±S D

Source of mitochondria	No. of expts	Swelling	
		Amplitude (ΔA/mg protein)	Initial rate (ΔA/s per 10^3)
Adult liver	18	0.399±0 024	29 1±3 0
Fetal liver	2	0 489	24 5
Cholesterol-enriched liver	8	0 300±0.065	20 5±4.2
Hepatoma AH-130	10	0 181±0.020	6.9±1.2
Hepatoma 3924A	3	0 148±0 030	4 4±1.6
Hepatoma 5123	7	0 120±0 020	3 2±1 3

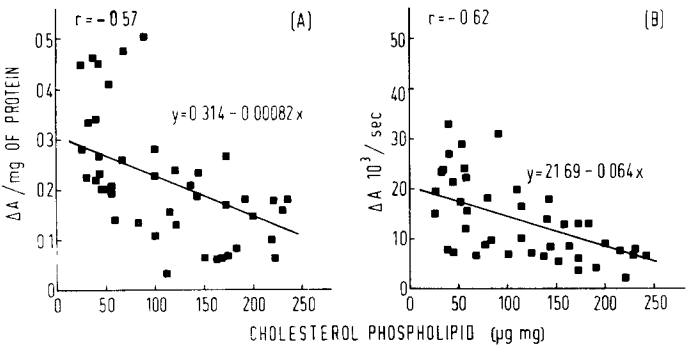

Fig 4 Scatter diagrams with a linear regression showing a negative correlation between amplitude (A) or initial rate (B) of mitochondrial swelling, induced by K^+ plus valinomycin, and cholesterol to phospholipid ratio Regression coefficients are significantly different from zero ($P < 0\ 001$)

swelling linked to K^+ uptake occurs in mitochondria from hepatomas A lower decrease takes place in cholesterol-enriched mitochondria, while no changes were observed in the organelles from fetal liver. Negative correlations of swelling extent or initial rate to cholesterol to phospholipid ratios are shown by data in Fig. 4 The correlations are acceptable, even though not so high as those observed for the other types of swelling studied in this paper

There exists an evident discrepancy between the values of K^+ uptake by different mitochondrial preparations and the swelling measurements Therefore, changes in mitochondrial water content, as a consequence of K^+ uptake, were determined in adult rat liver and hepatoma AH-130 mitochondria The results are shown in Table IX. Short-term incubation with valinomycin, in the presence of low K^+ concentration and phosphate, induces a 20 % increase in total water of both adult rat liver and hepatoma mitochondria Increases in the sucrose-impermeable spaces are 42 and 48 % for the two types of organelles, respectively. Thus, it may be calculated, by difference, that the intermembrane spaces decrease Similar results were obtained by Harris and Van Dam [30] on rat liver mitochondria. These observations are in disagreement with the results of absorbance measurements The latter show a two-fold diminution in

TABLE IX

VALINOMYCIN-INDUCED VARIATIONS IN TOTAL WATER AND SUCROSE-IMPERMEABLE WATER OF MITOCHONDRIA FROM ADULT RAT LIVER AND HEPATOMA AH-130

Conditions as described under Methods When indicated, valinomycin was added to a final concentration of 1 10^{-3} mM Data are mean values ±S D of duplicate counting vials of triplicate incubations

Source of mitochondria	Addition	Total water (μl/mg protein)	Sucrose-impermeable water (μl/mg protein)
Adult liver	None	1 31±0 03(7)	0 68±0 11(6)
	Valinomycin	1 63±0 08(7)	1 17±0 09(6)
Hepatoma	None	1 60±0 08(7)	0 53±0 07(6)
	Valinomycin	1 98±0 11(7)	1 02±0.14(6)

swelling extent of hepatoma AH-130 mitochondria compared to adult rat liver mitochondria. There is no obvious explanation for this phenomenon. Absorbance records do not appear to correspond merely to volume changes in the case of valinomycin-induced swelling

DISCUSSION

It has been demonstrated that cholesterol to phospholipid ratios of mitochondria [10–12], microsomes [10–12, 31] and plasma membranes [32] for hepatomas are higher than for liver. According to the results in this communication the increased cholesterol content of hepatoma mitochondria almost exclusively concerns the mitochondrial membranes. In the hepatoma AH-130 mitochondria, as in liver mitochondria, there is more cholesterol in the outer than in the inner membrane. Hepatoma outer mitochondrial membrane is characterized by low phospholipid content. This contrasts with the behaviour of the liver mitochondria outer membrane, whose high phospholipid content is well known [24, 25] (Table IV).

Our data indicate that increases in the cholesterol to phospholipid ratio of hepatoma mitochondria seem to be involved in the decreased ability of these organelles to undergo swelling and conformational changes. This conclusion is based on the following considerations. (1) Cholesterol to phospholipid ratios are significantly correlated to the extent of large amplitude and small amplitude swelling. This is also true with regard to both extent and initial rate of the absorbance decrease which accompanies the K^+ uptake by mitochondria The higher the cholesterol to phospholipid ratio, the lower the ability of mitochondria to perform volume and conformational changes. (2) The same pattern of alterations observed in hepatoma mitochondria may be induced, although to a lower extent, in liver mitochondria by enriching their membranes with cholesterol. (3) The suggested effects of cholesterol are in line with the actual knowledge of its influences on physical properties of artificial membranes [2].

These points deserve further comments. A statistically significant correlation between two phenomena tells nothing about the nature of the correlation. The relationship could be indirect. For instance, decreased ability to perform volume changes could depend on mechanical damage of mitochondria whose membranes are more fragile due to lower elasticity caused by elevated cholesterol content. This possibility, however, seems unlikely under our experimental conditions. In fact, impairment of volume and conformational changes was observed in hepatoma AH-130 mitochondria, which were shown to be as tightly coupled as adult rat liver mitochondria. By contrast, no alterations of swelling or conformational changes exist in fetal liver mitochondria, whose cholesterol to phospholipid ratio is the same as in adult rat liver organelles, even though the respiratory rates and acceptor control ratios are lower. In addition, the enrichment with cholesterol of liver mitochondrial membranes results in a lower ability for volume and conformational changes of mitochondria without variations in the degree of coupling (see also ref. 9).

Mitochondria isolated from the hepatomas 3924A and 5123 show lower acceptor control ratios than adult rat liver mitochondria. The possibility of a certain degree of damage to membranes cannot be excluded for these organelles. However, at least the decrease in extent of valinomycin-induced swelling, observed in the mitochondria

from the two hepatomas, does not appear to be a consequence of functional impairment In fact, these organelles take up the same amounts of K^+ as other types of mitochondria, although at a slower rate

Hepatoma AH-130 mitochondria take up, in the presence of valinomycin, the same amounts of K^+ and water as adult rat liver mitochondria This contrasts with results of spectrophotometric determinations of swelling during K^+ uptake. The optical effects of swelling are complex They may be related to variations in two volumes. the space between membranes and matrix space. The changes induced in these compartments by K^+ uptake are similar in liver and in hepatoma mitochondria Differences in refractive index between particles and medium as well as conformational changes of membranes may be also considered .The existence of a significant correlation between the cholesterol to phospholipid ratio and absorbance decrease linked to K^+ uptake indicates that at least one of the events which contribute to absorbance changes is influenced by cholesterol content of mitochondria One possibility could be that absorbance measurements record primarily changes in refractive indexes in mitochondrial suspensions. This explanation is discounted by the good agreement between absorbance decrease and water intake in liver and hepatoma mitochondria submitted to different swelling-inducing conditions in which K^+ is not involved [6] (see also Results section) It should be noted that Cockrell et al [33] presented calculations indicating that valinomycin-induced swelling is four or five times greater than that predicted from the amounts of K^+ taken up According to these authors the swelling cannot be entirely accounted for by water accompanying the uptake of K^+ and permeant counteranion. Mechanochemical changes in mitochondrial membranes were suggested to occur simultaneously with K^+ movements (cf. ref. 34). If so, one could tentatively assume that the increase in cholesterol to phospholipid ratio affects the ability of mitochondrial membranes to perform conformational changes during valinomycin-induced swelling. Relatively small volume increases, linked to K^+ uptake, could be unaffected by the sterol content of membranes Proofs that the above supposition is correct do not actually exist. Perhaps the problem will be clarified by electron microscopy

Large-amplitude swelling is characterized by marked increase in matrix volume, unfolding of inner membrane and rupture of outer membrane [35]. The changes in light absorbance observed during small amplitude volume oscillations reflect modifications of conformation of mitochondrial inner membrane plus matrix [36–39] and perhaps also of structural organization of outer membrane [39]. Evidence has been presented indicating that the physical state of lipids may influence the above phenomena [39]. Modifications in mitochondrial shape and volume should involve deformation and stretching of membranes. Phospholipids of liver mitochondria contain higher amounts of unsaturated acyl chains than saturated chains [40]. In hepatoma mitochondria phospholipid acyl chains are also largely unsaturated, even though a decrease in polyunsaturated chains occurs [10, 11] The incorporation of cholesterol between phospholipid molecules containing unsaturated paraffin chains induces lipids to be less fluid [2] and increases total hydrophobic interactions [9], thus giving greater cohesion to membranes. Membranes transformed in this way should be more resistant to deformation and stretching. This could be a simple mechanism to explain inhibition of swelling and conformational changes in mitochondria with high cholesterol to phospholipid ratio. As already reported [9], a

corollary of the above views is that metabolic changes of mitochondria do not necessarily depend on conformational changes of membranes, even though the converse may be true.

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