

Cholesterol increase in mitochondria: its effect on inner-membrane functions, submitochondrial localization and ultrastructural morphology

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The effect of cholesterol incorporation on some functions of the mitochondrial inner membrane and on the morphology of rat liver mitochondria was studied. Basal ATPase and succinate dehydrogenase activities remained unchanged after cholesterol was incorporated into the mitochondria; however, uncoupled ATPase activity was partially inhibited. The presence of several substrates and inhibitors did not change the amount of chol-

esterol incorporated, which was localized mostly in the outer membrane. Electron-microscope observations revealed the presence of vesicles between the outer and inner membranes; these vesicles increased in number with the amount of cholesterol incorporated. The data suggest that cholesterol induces the formation of vesicles from the outer membrane, and modifies the activity of stimulated ATPase.

INTRODUCTION

Cholesterol is required for normal growth and cell functions. The cell maintains a specific distribution of this sterol among its different membranes (Kumar-Jain, 1975; Yeagle, 1985). The plasma membrane has the highest cholesterol concentration, whereas mitochondria have the lowest (Colbeau et al., 1971; Kumar-Jain, 1975; Lange and Ramos, 1983). At present, the mechanisms used by the cell to keep this distribution are unknown. However, several hypotheses have been proposed: (1) desorption of cholesterol from membranes (i.e. entry of cholesterol into the aqueous phase: McLean and Phillips, 1981, 1982; Bellini et al., 1984; Bruckdorfer and Sherry, 1984; Cooper and Strauss, 1984; Fugler et al., 1984); (2) vectorial vesicular transport between membranes (Lange and Matties, 1984; Steck et al., 1988); (3) cholesterol transport by protein carriers (Chanderbhan et al., 1982; Vahouny et al., 1983; Scallen et al., 1985); (4) thermodynamic equilibrium, in which the composition of the membrane (i.e. phosphatidylcholine and sphingomyelin) determines its cholesterol content (Wattemberg and Silbert, 1983); and (5) cholesterol transport through a contact between membranes (Stevens et al., 1985). Further, in some pathological states, such as myocardial ischaemia (Rouslin et al., 1982) or some tumoral processes (Feo et al., 1973; Sabine, 1975; Brosa et al., 1980; Coleman and Parlo, 1984; Coleman, 1986), mitochondria acquire a high level of cholesterol. The mechanism that leads to such high accumulation of cholesterol is unknown, but this modifies some properties and functions of mitochondria (Daum, 1985).

Recently, a method that allows incorporation of cholesterol into mitochondria in a short time, i.e. 1 min, was described. Using this method (Martínez et al., 1988a), we studied ATPase and succinate dehydrogenase activities, and the morphology of rat liver mitochondria enriched with different amounts of cholesterol, as well as the distribution of the cholesterol accumulated in the inner and outer membranes.

MATERIALS AND METHODS

All reagents used were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Rat liver mitochondria were isolated by differential centrifugation, as previously described (Martínez et al., 1988b). Incorporation of cholesterol into mitochondria was performed by using a cholesterol-BSA mixture as reported by Martínez et al. (1988a). Different amounts of the cholesterol-BSA complex were incubated at 4 °C with 50 mg of mitochondrial protein for 1 min; mitochondria were recovered by centrifugation and washed three times with 0.25 M sucrose/1 mM EDTA, pH 7.4, to eliminate excess cholesterol. These cholesterol-enriched mitochondria were used in all the experiments described in this paper. Parallel experiments were performed with mitochondria incubated with BSA, but without cholesterol, to evaluate the possible role of BSA on the results. Lysophosphatidylcholine (40 µg/mg of protein) was used as control to determine the possible detergent-like effect of cholesterol.

Submitochondrial particles (SMP) were isolated after sonic disruption, as reported by Chávez and Bravo (1982). SMP (10 mg) were incubated at 4 °C with different concentrations of the mixture of cholesterol-BSA. After 2–5 min, the mixture was centrifuged at 15000 g for 15 min and the supernatant was centrifuged at 105000 g for 45 min. SMP were suspended in 0.25 M sucrose/1 mM EDTA, pH 7.2. Protein was quantified by the method of Lowry et al. (1951) with BSA as standard.

ATPase activity was assayed in a medium containing 100 mM KCl and 10 mM Tris/HCl, pH 7.3, in a final volume of 2 ml at 30 °C with 1 mg of mitochondrial protein/ml (Martínez et al., 1988b). The ATPase activity in SMP was assayed in a medium containing 100 mM KCl, 10 mM Tris/HCl, pH 8, 10 mM MgCl₂ and 0.5 mg of protein/0.5 ml. After incubation for 5 min at 30 °C, the reaction was started by addition of 2.5 or 4 mM ATP, pH 7.2, to the mixture, and the hydrolysis of ATP was stopped after 10 min with 6% trichloroacetic acid. The mixture was

centrifuged at 2500 *g* for 10 min in a Sorvall RT600 refrigerated centrifuge, and 0.5 ml of the supernatant was used to determine the amount of P_i released from ATP, by the method described by Sumner (1944). Stimulated ATPase activity in mitochondria was determined in the presence of 75 μ M 2,4-dinitrophenol (DNP).

Succinate dehydrogenase was assessed by the dichlorophenol-indophenol (DCPIP) method in disrupted mitochondria (Baginsky and Hatefi, 1969), in a medium containing 5 μ M rotenone, 100 μ M KCN, 1.1 mM phenazine methosulphate, 10 mM succinate, pH 7.2, and 0.5 mg of protein/ml. The activity was initiated with 80 μ M DCPIP, and its reduction was monitored at 600 nm.

Monoamine oxidase (MAO) activity was assayed as reported by Cárabez and Sandoval (1974). Briefly, 60–1000 μ g of protein from each membrane fraction was incubated in 10 mM phosphate buffer, pH 7.6, and 2% Lubrol in a final volume of 2 ml at 37 °C. After incubation for 2–5 min the reaction was started with 3.3 mM benzylamine, and its oxidation was monitored at 250 nm in a Beckman spectrophotometer. The specific activity was calculated by using an absorption coefficient of $11.4 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

To determine cholesterol content in outer and inner membranes, three different preparations of mitochondria were pooled. Outer and inner membranes were obtained as described by Parsons et al. (1966). Cholesterol concentration was determined by the colorimetric method or by high-pressure g.l.c. using a model 433 Packard instrument as described by Martínez et al. (1988a). Other experimental conditions are described in each Figure legend.

For electron-microscope observations, mitochondria were fixed for 20 min in buffered 3% glutaraldehyde in ice, pH 7.2. Mitochondria were centrifuged and fixed for 60 min in osmium tetroxide; the samples were dehydrated with increasing concentrations of ethanol. Mitochondria were finally included in epoxy resin. Thin sections of samples were contrasted with uranyl acetate and observed through a JEOL 100-B or Zeiss M-10 electron microscope.

The experiments were performed in duplicate at least three times.

RESULTS

Effect of cholesterol incorporation on some mitochondrial functions

It has been described that rat liver mitochondria increase their cholesterol content during the first 1 min of incubation with a cholesterol-BSA complex (Martínez et al., 1988a) and maintain this level constant over 30 min. Figure 1 shows the effect of increasing concentrations of ATP on stimulated ATPase activity from mitochondria enriched with $45.8 \pm 3.5 \mu\text{g}$ of cholesterol/mg of protein. It is observed that above 0.5 mM ATP, cholesterol-rich mitochondria have a lower stimulated ATPase activity than do untreated mitochondria, suggesting an inhibitory effect of cholesterol on the stimulated ATPase activity. The differences are statistically significant at concentrations of ATP above 0.5 mM. The activity of BSA-treated mitochondria was similar to that of the controls (results not shown).

In order to determine whether cholesterol-enriched mitochondria could be depleted of cholesterol, mitochondria were incubated with an equal volume of a solution containing BSA essentially free of fatty acids (5 mg/ml) and asolectin (50 μg /ml) for 17 h at 37 °C, as reported previously (Bartholow and Geyer, 1982). The results showed that cholesterol-rich mitochondria ($21.7 \pm 1.05 \mu\text{g}$ of cholesterol/mg) did not release cholesterol after this treatment ($24.5 \pm 2.49 \mu\text{g}$ of cholesterol/mg).

Figure 2 shows the ATPase activity of mitochondria enriched with different amounts of cholesterol and the cholesterol content per mg of mitochondrial protein. The increase of cholesterol in mitochondria was related to the amount of cholesterol-BSA present in the incubation medium (Figure 2, upper abscissa). Basal ATPase activity showed a similar activity in both untreated and cholesterol-treated mitochondria, whatever the amount of cholesterol incorporated (Figure 2); however, addition of 75 μ M DNP increased ATPase activity, although in cholesterol-enriched mitochondria stimulation by the uncoupler was lower.

The following experiment was performed to determine whether the effect of cholesterol on ATPase was related to the incubation

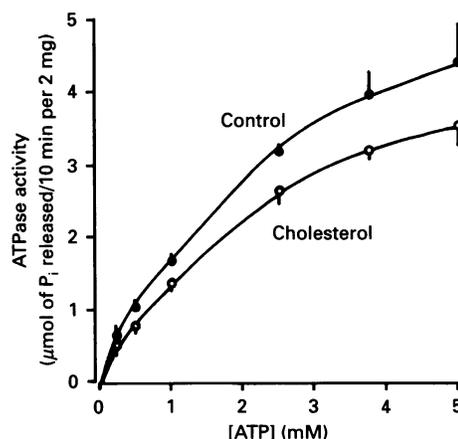


Figure 1 Effect of cholesterol incorporation on ATPase activity in rat liver mitochondria

Mitochondria were incubated in presence of 3.49 μmol of cholesterol for 1 min at 4 °C. The experimental conditions were described in the Materials and methods section. The effect of increasing ATP concentration on ATPase activity is shown. ●, Untreated mitochondria ($n = 3$); ○, mitochondria treated with cholesterol ($n = 4$). Bars indicate S.D.

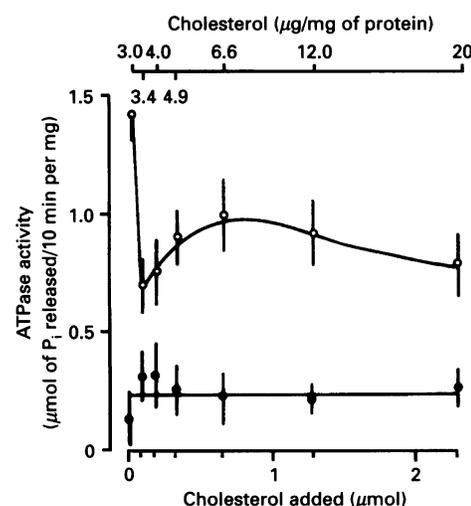


Figure 2 Effect of increase in cholesterol on ATPase activity in mitochondria

Cholesterol enrichment of mitochondria was achieved by incubating mitochondrial protein (50 mg) with different amounts of cholesterol-BSA complex. The lower abscissa shows the total amount of cholesterol-BSA complex present in the mixture, and the upper abscissa shows the amount of cholesterol per mg of protein. ATPase activity was assayed as described in the Materials and methods section, supplemented with 2.5 mM ATP: ●, basal ATPase activity; ○, ATPase activity plus 75 μ M DNP ($n = 3$). ATPase activity without cholesterol added is the control value (untreated mitochondria).

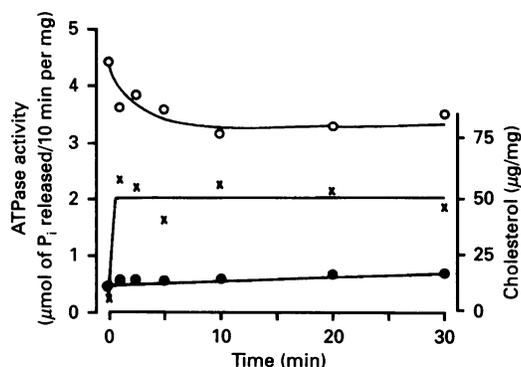


Figure 3 Time course of cholesterol incorporation into mitochondria and its effect on ATPase activity

Mitochondria were incubated with 3.49 μmol of cholesterol-BSA complex. At the times indicated on each point, mitochondria were washed three times with 0.25 M sucrose/1 mM EDTA, adjusted to pH 7.3 with Tris/HCl. After washes, ATPase activity was determined as described in the Materials and methods section. Symbols: X, concn. of cholesterol per mg of protein; ●, basal ATPase activity; ○, ATPase activity in the presence of 50 μM DNP. This is a representative experiment. Zero time is the control value (untreated mitochondria).

Table 1 ATPase activity in SMP which were incubated with different amounts of cholesterol and recovered by ultracentrifugation

SMP were incubated for 3–5 min with the amount of cholesterol shown, and then recovered by ultracentrifugation. ATPase activity was assayed without extra addition of cholesterol. The values are means \pm S.D. ($n = 3$).

Cholesterol (μmol)	ATPase activity (%)
0	100
0.66	91.80 \pm 13.13
1.28	96.90 \pm 18.15
2.30	104.45 \pm 25.37

Table 2 Effect of the presence of cholesterol on ATPase activity in SMP

SMP were incubated in the medium described in the Materials and methods section in the presence of the amounts of cholesterol shown (as cholesterol-BSA complex). The results are means \pm S.D. of three experiments.

Cholesterol (μmol)	ATPase activity (%)
0	100
0.35	98.8 \pm 6.5
0.66	96.7 \pm 6.2
1.28	93.9 \pm 5.2
2.30	95.0 \pm 9.8

time. As previously reported (Martínez et al., 1988a), the concentration of cholesterol remained unchanged throughout the incubation time (Figure 3, X symbol), as did the ATPase activity in the absence of DNP (basal activity). In treated mitochondria, ATPase activity in the presence of DNP showed a lower activity compared with controls (○ symbol, Figure 3), suggesting that cholesterol inhibits the stimulated ATPase.

Table 3 Cholesterol quantification in inner and outer membranes from rat liver mitochondria

MAO is expressed as μmol of benzaldehyde produced/min per mg of protein. The number of determinations (n) represents the pooled membranes. The total cholesterol concentration for control mitochondria was 3.3 $\mu\text{g}/\text{mg}$, whereas that of mitochondria loaded with cholesterol was 19.85 $\mu\text{g}/\text{mg}$.

Cholesterol ($\mu\text{g}/\text{mg}$ of whole mitochondria)	Inner membrane		Outer membrane	
	(μg of cholesterol/mg)	MAO	(μg of cholesterol/mg)	MAO
3.3	4.36 \pm 0.60 ($n = 3$)	3.8 ($n = 2$)	15.38 \pm 1.97 ($n = 2$)	17.1 ($n = 2$)
19.85	4.93 \pm 0.79 ($n = 3$)	4.6 ($n = 2$)	392.00 \pm 21.17 ($n = 3$)	16.4 ($n = 2$)

A control experiment was done with lysophosphatidylcholine to determine whether cholesterol was working as a detergent. Mitochondria incubated with 40 μg of lysophosphatidylcholine showed an uncoupling of respiration and an increase in ATPase activity, similar to the effect observed with DNP on untreated mitochondria (results not shown).

To ascertain the effect of cholesterol on ATPase, the experiments described in Tables 1 and 2 were performed. Table 1 shows that ATPase activity remains unchanged in SMP recuperated after their incubation with different amounts of cholesterol, although the protein/cholesterol ratio was 5 times that used with whole mitochondria. In this case, SMP incubated with the cholesterol-BSA complex showed a 60–75% decrease in cholesterol content with respect to the control after the treatment.

Table 2 demonstrates that cholesterol added to the reaction medium did not modify ATP hydrolysis in SMP, suggesting that the effect of cholesterol in the whole mitochondria was on a different level from the ATPase.

Another inner-membrane enzyme evaluated was succinate dehydrogenase. The results (not shown) indicated that, regardless of the amount of cholesterol incorporated or the time of incubation with cholesterol, this activity was not modified.

The following experiment was designed to determine whether the presence of substrates could produce an increase in cholesterol incorporation. The results showed that the presence of succinate or malate/glutamate did not modify the incorporation of cholesterol into mitochondria (values around 20 μg of cholesterol/mg of protein). Furthermore, cholesterol uptake was not affected by ADP, oligomycin or cyanide.

Cholesterol quantification in mitochondrial membranes

The inner and outer membranes of mitochondria loaded with cholesterol were isolated and the amount of cholesterol was quantified. MAO was the marker enzyme used to determine the purity of the inner and outer membranes. The ratio of MAO in the outer and inner membranes is close to 10 (Schnaitman and Greenawalt, 1968; Hovius et al., 1990). In the inner membrane of our untreated mitochondria, the activity of MAO was 25–30% of that in the outer membrane, with an outer/inner ratio around 4.0 (Table 3). However, in the cholesterol-enriched mitochondria the outer membrane had 80 times more cholesterol than the inner membrane, suggesting that cholesterol was mainly incorporated into the outer membrane. The slightly higher values of cholesterol in the inner membrane of cholesterol-treated mitochondria could be due to contamination with the outer membrane.

Morphology of mitochondria enriched with cholesterol

The structural changes produced by cholesterol were determined. In mitochondria enriched with 20 μg of cholesterol/mg, vesicles lying between the inner and outer space were clearly apparent (Figure 4), although some vesicles were also observed in control and BSA-treated mitochondria, probably due to mitochondrial damage; however, the number of vesicles observed was related to the amount of cholesterol incorporated, i.e. a high cholesterol incorporation gave a larger amount of intermembrane vesicles, whereas at low cholesterol uptake a smaller amount of vesicles was observed.

The amount of vesicles was quantitatively evaluated by electron microscopy (magnification $\times 3000$). A clear grid with 48 squares of $1.5 \times 1.5 \text{ cm}$ was overlaid on the picture, and the number of vesicles in each square was recorded in at least four different samples. Each square contained 10–13 mitochondria. The results showed that the number of vesicles in untreated mitochondria was 11 ± 2.9 ($n = 5$), 11.75 ± 4.2 ($n = 4$) in mitochondria treated

with BSA alone, and 30 ± 3.6 ($n = 4$) in mitochondria treated with 2.3 μmol of cholesterol.

DISCUSSION

In accordance with previous data (Martínez et al., 1988a), it was found that rat liver mitochondria incorporate cholesterol and produce inhibition of ATPase activity stimulated by DNP. Although addition of DNP increased ATPase activity in cholesterol-rich mitochondria, the phosphate release did not reach the values of the stimulated ATPase in untreated or BSA-treated mitochondria. However, the response to DNP indicates that mitochondria were not disrupted by the cholesterol incorporation.

The described ATPase activity and the existence of numerous vesicles in cholesterol-enriched mitochondria are not apparently related to mitochondrial damage. This is inferred from the results (not shown) that indicated that excess of incorporated

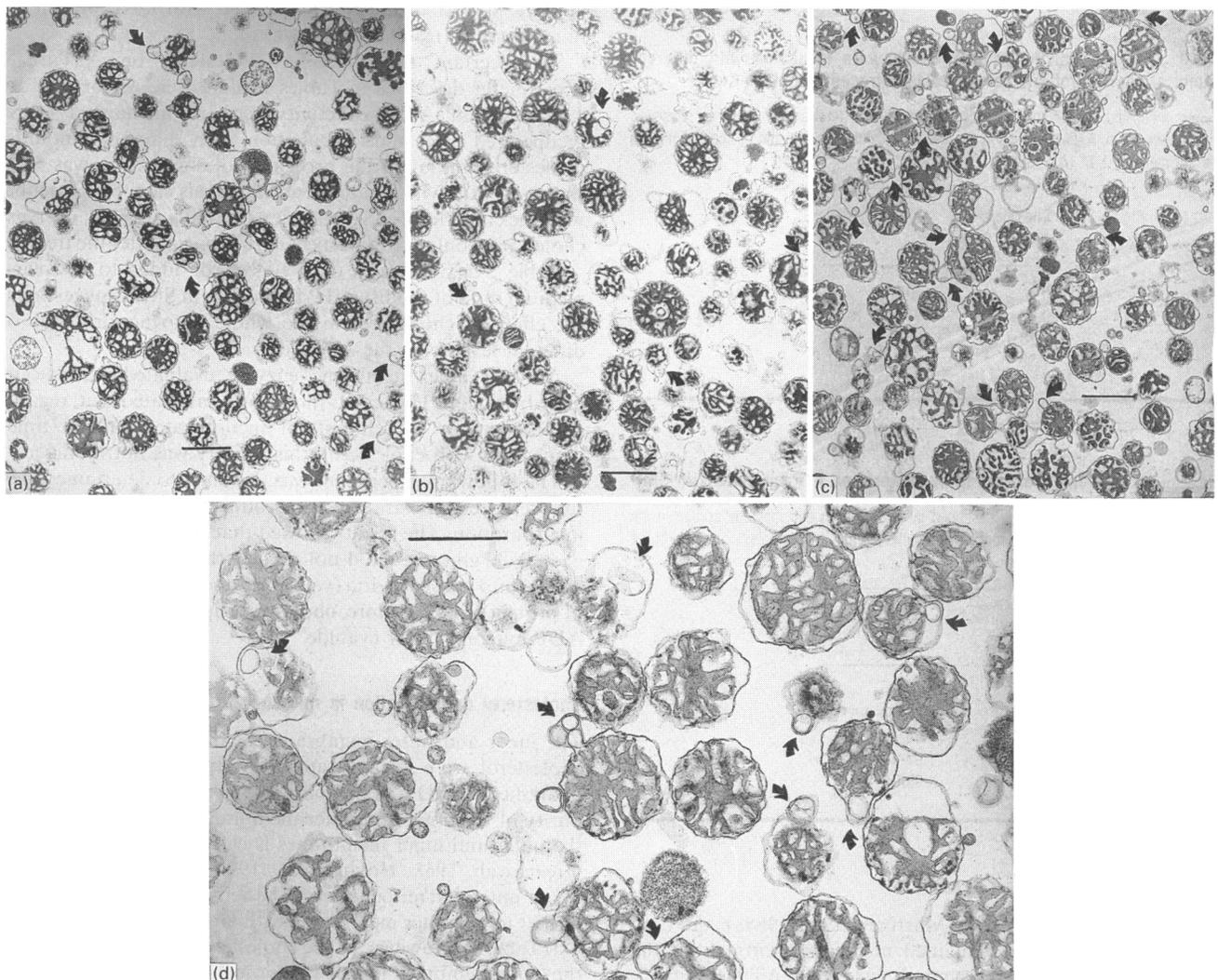


Figure 4 Electron micrography of mitochondria enriched with cholesterol

Mitochondria were treated with cholesterol or BSA, and after three washes were fixed and processed for electron-microscope observation. (a) Untreated mitochondria; (b) mitochondria incubated with BSA; (c) and (d), mitochondria incubated with 2.3 μmol of cholesterol-BSA complex. Arrows indicate the sites of vesicles or vesiculation. Bars indicate 1 μm .

cholesterol did not uncouple respiration, and that the basal ATPase activity was not stimulated. Furthermore, electron-microscope observations did not show any gross structural damage, such as broken mitochondria. Thus the overall data suggest that cholesterol leads to vesicle formation and modification of ATPase activity. In this regard, accumulation of cholesterol in mitochondria up to values of 37 $\mu\text{g}/\text{mg}$ (8 times that of controls) has been reported (Graham and Green, 1970). Mitochondria with 4–46 μg of cholesterol/mg were used in this study without effect on respiratory activity; cholesterol was localized mainly in the outer membrane, affecting stimulated ATPase activity, a function associated exclusively with the inner membrane.

In contrast with our results, it has been reported that mitochondria with high cholesterol content from hepatoma cells (25.7 μg of cholesterol/mg) exhibit a low state-3 respiration (Brosa et al., 1980), probably due to an increase in citrate export from mitochondria (Coleman, 1986). Madden et al. (1980) and Schneider et al. (1982) have reported that incorporation of cholesterol in the mitochondrial inner membrane produces an increase in electron-transport activity. Coleman et al. (1978) loaded rat liver mitochondria with cholesterol by using Sephadex G-10 beads coated with cholesterol. In their studies, the respiratory control decreased and ATPase activity increased as a function of the cholesterol content, but cholesterol was not quantified in the isolated membranes. The above reports agree with those by Spector and Yorek (1985) and Daum (1985), where an increase in cholesterol in mitochondria modifies the activity of several membrane enzymes. The differences between these results and ours could be due to differences in the methodology employed.

However, Rouslin et al. (1982) demonstrated an increase in cholesterol in mitochondria (from 73 to 130 $\mu\text{g}/\text{mg}$ of protein) from pig heart during myocardial ischaemia with a concomitant decrease in the specific activity of ATPase. Graham and Green (1979) and Feo et al. (1975) reported an increase in cholesterol in whole mitochondria without modification of the respiratory control or basal ATPase activity, supporting our results.

Two sets of experiments suggest that the mechanism of the modification of ATPase activity by cholesterol could be at the level of the outer membrane. First, cholesterol was mainly incorporated into the outer membrane, and second, the ATPase activity in SMP was not modified by the presence of cholesterol in the reaction medium (Table 2), or by previous incubation of SMP with cholesterol (Table 1). These data suggest that the effect of cholesterol is through the outer membrane, or alternatively is due to the presence of vesicles associated with the cholesterol incorporation. On the other hand, the inner membrane was unable to incorporate cholesterol, demonstrating that the inner membrane has a restricted access to the cytoplasmic cholesterol pool. With respect to this point, it is known that cholesterol plays an important role in some membrane processes, such as membrane adhesion (Ohki and Leonards, 1984) and mitochondrial import of proteins (Hartl et al., 1989). Our results show that, associated with the accumulation of cholesterol in mitochondria, there is an increase in vesicles in the intermembrane space, as reported by Ohlendieck et al. (1986). At present the composition of these vesicles is unknown, but they might interact with the inner membrane like attachment points or membrane contacts, and this could be related to the decrease in ATPase activity observed in this work. This assumption is supported by the previous reports (Kimura, 1986; Cornelius, 1990), where cholesterol induced changes at the membrane level (reverse hexagonal phase), producing attachment points between membranes. In fact, Levrat and Louisot (1992) have demonstrated that outer-

membrane contact sites are dolichol- and cholesterol-rich, whereas inner-membrane contact sites are not. Also, these contact sites have been proposed as a mechanism for the regulation of mitochondrial functions (Werner and Neupert, 1972; Ohlendieck et al., 1986; Dorbani et al., 1987).

In our experimental conditions, the cholesterol content of mitochondria increased about 7–15-fold, increasing the cholesterol/phospholipid ratio from 0.042 in the control to 0.256 in the cholesterol-rich mitochondria. These results are similar to the values reported previously (Graham and Green, 1970; Brosa et al., 1980); but, interestingly, the incorporated cholesterol was associated mostly with the outer membrane and was not released from mitochondria. This unequal distribution could be explained on the basis of a highly regulated mechanism of cholesterol transport. As noted previously, it has been suggested that cholesterol transfer could take place by vesiculation (Lange and Matties, 1984; Steck et al., 1988). This mechanism could account for our results, since the morphology of mitochondria showed that vesiculation was related to the amount of cholesterol incorporated. However, further research is needed to elucidate the mechanism responsible for the formation of these vesicles and their role in the modification of the mitochondrial functions.

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