Cholesterol increase in mitochondria: a new method of cholesterol incorporation

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Abstract In this study, we report a new method for cholesterol incorporation in mitochondria. This method is based on the coupling of cholesterol to bovine serum albumin (BSA) to form a structure with a spherical shape (designated C-BSA-C). The results show that C-BSA-C induces a specific cholesterol incorporation in mitochondria without interference from BSA. The cholesterol increase is complete in 1 minute, and is dose-related and pH-dependent. Temperature also has an effect. In mitochondria from dog adrenal glands, pregnenolone biosynthesis induced by C-BSA-C is increased, indicating that only cholesterol is transferred to the membrane.— **Martínez, F., S. Eschegoyen, R. Briones, and A. Cuellar.** Cholesterol increase in mitochondria: a new method of cholesterol incorporation. *J. Lipid Res.* 1988. **29:** 1005–1011.

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Cholesterol is a steroidogenic compound required for cell growth and development, with a specific distribution in different kinds of membranes (1). At present, the cellular mechanism that maintains such a specific cholesterol distribution is unknown. In this regard, it has been suggested that microtubules and microfilaments are involved in cholesterol transfer into the cell (2, 3), or that it might be accomplished by vesiculation (4).

On the other hand, it has been proposed that cholesterol distribution can be maintained by a thermodynamic equilibrium (1, 5) in such a way that the membrane composition (proteins and lipids) determines the relative cholesterol content, or, as is the case for sphingomyelin (6, 7), only the membrane lipids can determine cholesterol concentration at this level.

The mechanism involved in cholesterol transport to the different cell membranes is still unknown (1, 8–11). This fact is relevant in mitochondria, since their cholesterol concentration is very low. Several experiments have been performed to study the interaction between cholesterol and mitochondria, as well as to explore how this compound changes the properties of the membrane (12, 13).

Studies in this area have resulted in artificial methods to verify the relevant role of cholesterol in membranes, as well as its transport (12, 14–15). At present, the method to study the interaction of cholesterol and mitochondria (actually mitoplasts) employs liposomes (16–18). The use of liposomes to increase the amount of cholesterol in mitoplasts produces some unwanted effects, because the liposomes fuse to the membrane and increase the volume of mitoplasts, thus diluting the concentration of metabolites or ions, or both, in the system. Also, fusion between mitoplasts and liposomes produces incorporation of other phospholipids into the membrane, which could have stimulatory or inhibitory effects on the catalytic activities of some of the proteins embedded in the membranes (19).

Taking this into consideration, we decided to design a new specific method to incorporate cholesterol into mitochondria by using bovine serum albumin (BSA) as a carrier. In this sense, the BSA associated with phospholipids has been used to induce a decrease of cholesterol levels in the membrane, but it must be kept in mind that BSA in the serum is able to bind steroids without changing the cholesterol concentration in the membrane. Also, some methods employ cholesterol combined with human serum, inactivated at 60°C, to enrich the membrane with cholesterol, but the disadvantage of this method lies in the long incubation time required to obtain the best results (16 hr); thus, this method does not work in mitochondria.

The present results show that cholesterol incorporation is maximal at 1 minute of incubation, and is dose-related and pH-dependent. Temperature changes the maximal increase, and BSA does not interfere with the system, functioning just like a carrier.

Abbreviation: C-BSA-C, cholesterol-bovine serum albumin complex.

MATERIALS AND METHODS

Bovine serum albumin, essentially fatty acid-free, acrylamide, sodium dodecylsulfate, and cholesterol were obtained from Sigma Co., St. Louis, MO. Cholesterol from Matheson Coleman and Bell, Norword, OH, was also used. Sucrose was purchased from J. T. Baker. [^{14}C]Cholesterol and ^{125}I were purchased from Amersham International Plc., England.

Isolation of mitochondria

Rat liver mitochondria and dog adrenal mitochondria were isolated using 0.25 M sucrose, 1 mM EDTA, adjusted to pH 7.3 with Tris, employing the method described by Chávez et al. (20). The purity of the mitochondrial preparation was assayed by measuring ATPase activity (reported as nmol of Pi liberated per min per mg of protein) and the respiratory control (as the ratio of oxygen consumption of state 3/state 4) as reported (20).

Preparation of cholesterol-BSA-complex (C-BSA-C)

A flow chart of the preparation of C-BSA-C is shown in **Fig. 1**. A 10-ml aliquot of 1% cholesterol in ethanol was added to the same volume of double-distilled water under continuous magnetic stirring at room temperature. The milk-like solution was then centrifuged at 2,000 g for 10 min in a refrigerated Sorvall RC-5B centrifuge. The supernatant was discarded, and the pellet was resuspended in 10 ml of 0.25 M sucrose, 1 mM EDTA, pH 7.3. The white solution was stirred gently and 4 g of BSA was slowly added at room temperature. Once the BSA was completely dissolved, the pH of the solution was adjusted to 7.3 with Tris, and then centrifuged in the cold at 12,000 g for 10 min. The supernatant was collected and used for cholesterol incorporation.

Electron microscopy

The C-BSA-C was observed through a Jeol 100-B electron microscope operated at 60 KV, using phosphotungstic acid negative staining.

Increase of cholesterol in mitochondria

Cholesterol increase in rat liver mitochondria was attained using mitochondria equivalent to 50 mg of protein; the final volume was always 1 ml. To this amount of protein, an aliquot of C-BSA-C was added. After the incubation, the mitochondria were diluted 40 times with cold 0.25 M sucrose, 1 mM EDTA, pH 7.3, and spun down immediately at 12,000 g for 10 min. The supernatant was discarded and the pellet was washed twice with the same solution. The final pellet was resuspended and the protein was quantified by the method of Lowry et al. (21). The yield of recovered mitochondrial protein was always between 90 and 95%

of the original 50 mg used in the experiments. Parallel control experiments were performed using only BSA in incubations at 30°C; the amount of added BSA was the same used in the C-BSA-C. In addition, ^{125}I-labeled BSA was employed to quantify the total BSA bound to mitochondria. The BSA was iodinated by the chloramine T method, using 5 µg of BSA and 1 mCi of ^{125}I. After 30 sec iodination, the reaction was stopped by the addition of sodium metabisulfite, and immediately passed through a Shepadex G-100 column. The fractions corresponding to BSA were collected and used the day after iodination. The specific activity of iodinated-BSA was 61.4 µCi/µg of BSA. In this experiment, 2.30 µmol of cholesterol and about 500,000 cpm of ^{125}I-labeled BSA were employed.

Gel electrophoresis

Electrophoresis of mitochondrial proteins was performed according to Weber and Osborn (22) in cylindrical gels in
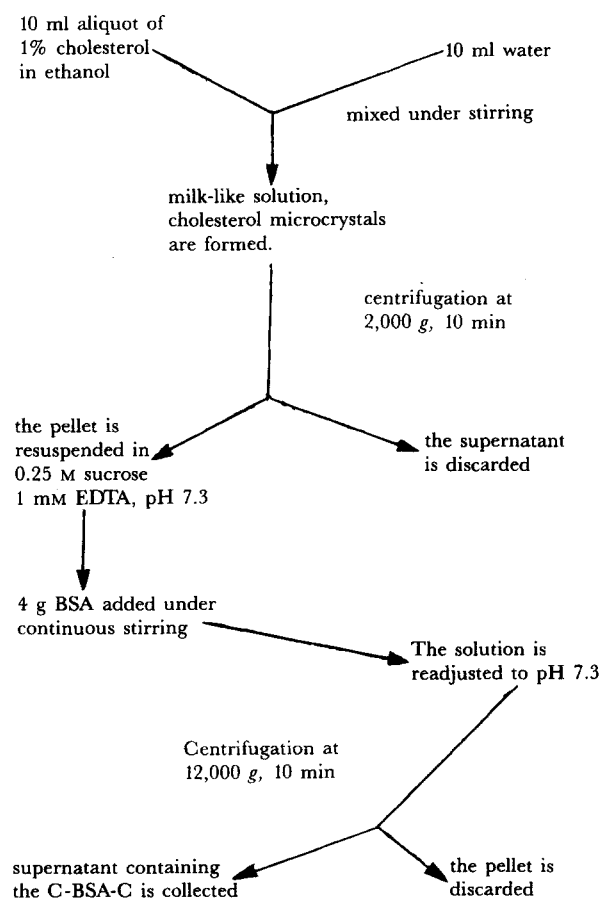

Fig. 1. Flow chart for the preparation of C-BSA-C. This is a step-by-step schematic representation. Centrifugation was done in the cold; the other steps were done at room temperature. See text for details.

10% polyacrylamide, 0.1% sodium dodecylsulfate. The C-BSA-C-treated mitochondria were processed as mentioned before. Controls were mitochondria without treatment.

Cholesterol transformation into pregnenolone

Dog adrenal mitochondria were isolated in the same manner as rat liver mitochondria. The adrenal mitochondria were incubated in a medium containing 0.25 M sucrose; 20 mM KCl; 10 mM phosphate, pH 7.4; 5 mM triethanolamine, pH 7.4; 5 μg of rotenone; 1 mM KCN, and 0.8 mg of mitochondrial protein. Cholesterol (176 μg) from C-BSA-C was added to this medium. After 10 min of preincubation, 10 mM malate was added (time 0) to start the reaction. At indicated times 25-μl aliquots with 20 μg of mitochondrial protein were collected for pregnenolone assay. Pregnenolone determination was performed by Dr. Vicente Díaz from the "Instituto Nacional de la Nutrición, Salvador Zubirán" by an RIA method, with less than 1% cross-reaction for other steroids.

The same experiment was repeated using [4-^{14}C]cholesterol. The sterols were separated by thin-layer chromatography (TLC) in a system containing ethyl acetate–hexane 2:8 (v/v), and the TLC plate was redeveloped in chloroform–ethyl acetate–ethanol–hexane 20:40:2:60 (v/v). The area corresponding to sterols was scraped from the plate after comparison with the standard, put into vials, and counted in a Packard scintillation spectrometer.

Cholesterol determination

The amount of cholesterol contained in C-BSA-C and in mitochondria was determined using a colorimetric method (23). Rat liver mitochondria (10 mg of protein) were treated three times by shaking with 5 ml of ether for 1 min. After this extraction, no cholesterol was detectable in the remnant protein. For cholesterol determination in C-BSA-C, an appropriate dilution was made and the same ether extraction was carried out. For improved accuracy, in some experiments the cholesterol incorporated into the mitochondria was analyzed using a Packard model 433 gas chromatography apparatus.

Determination of phosphorus

The total amount of mitochondrial phospholipids was determined by measuring the phosphorus as previously reported (24).

RESULTS

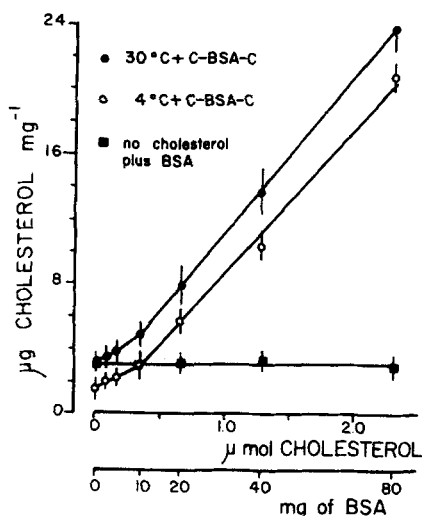Fig. 2 shows an electron micrograph of the C-BSA-C; the spherical shape of this complex with two populations
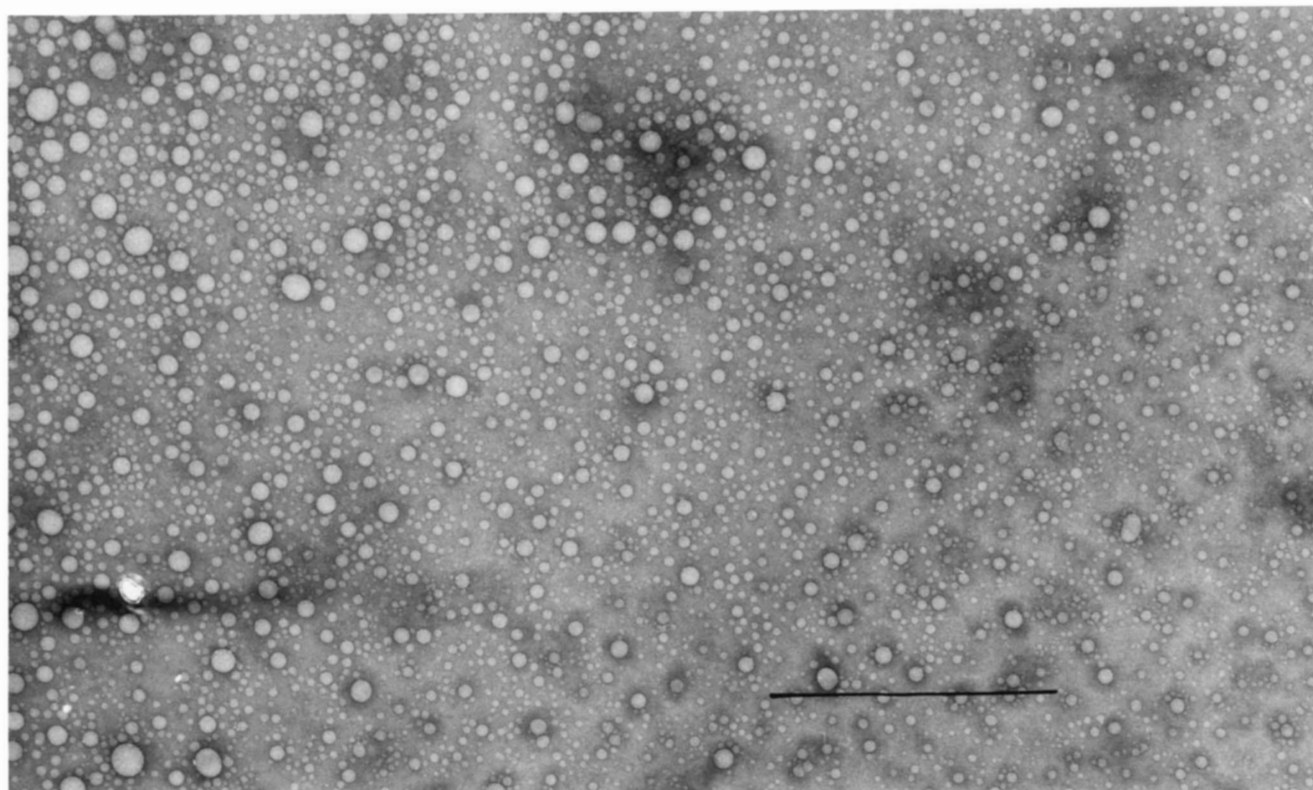
Fig. 2. Electron micrograph of the C-BSA-C. The cholesterol-BSA was diluted 8–10 times in phosphate buffer, and then plated on a grid covered with a carbon film. The negative staining shows the spherical shape of the C-BSA-C. Two different particle sizes can be observed; the average size is ca. 0.045 μm. The bar represents 0.5 μm.
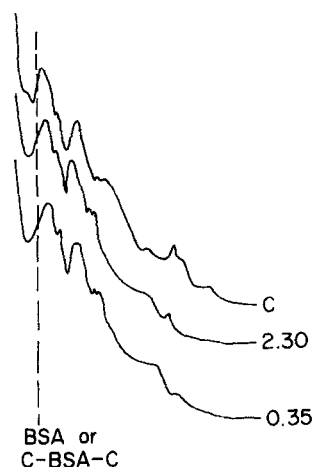
of different particle size can be observed. The average size was ca. 0.042 μm. It is not known how cholesterol is distributed in this C-BSA-C. The cholesterol concentration in these structures was very constant from batch to batch, with values around 4.4 mg/ml; the BSA content was 300–320 mg per ml. The C-BSA-C was stable under refrigeration for 6 months without changes in structure and function. It was not possible to determine whether the structures shown were microcrystals.

The ATP hydrolysis produced by the specific mitochondrial enzyme ATPase was 120 nmol of Pi released per min per mg of mitochondrial protein. The respiratory control was obtained as the ratio of ng at oxygen consumed per min per mg of mitochondrial protein between state 3 and state 4 of the mitochondrial respiration; the respiratory control value was 6 to 8. These results suggest the enrichment of mitochondrial preparation.

The experiment depicted in **Fig. 3** was carried out to determine the ability of the C-BSA-C to deliver cholesterol into the mitochondria. It is noteworthy that the amount of mitochondrial protein recovered after washing the mitochondrial-C-BSA-C suspension was consistent from experiment to experiment, ca. 45 mg with a loss of nearly 5 mg. This suggests that there was no retention of BSA even though the amount of added BSA was between 2 and 80 mg for each 50 mg of mitochondrial protein. Similar results in protein recovery were obtained when equivalent amounts of BSA were used. After each experiment, ATPase activity was measured to insure that no BSA was attached to mito-

Fig. 4. Densitometry of SDS gel electrophoresis of mitochondria treated with C-BSA-C. The mitochondria were incubated with C-BSA-C as in Fig. 3 using 2.30 or 0.35 μmol of cholesterol. An aliquot of 50 μg of protein was used in each gel. Controls were mitochondria without any treatment. The value indicates the amount of cholesterol from C-BSA-C. The dotted line shows the peak corresponding to BSA or C-BSA-C.

chondria. The results show that the specific activity of this enzyme did not change with any of the cholesterol concentrations tested, suggesting strongly that there was no BSA retention in the mitochondria. Fig. 3 shows a net cholesterol transfer from C-BSA-C to the mitochondria. Cholesterol incorporation was linear from 0.35 to 2.3 μmol. It can also be observed that this release of cholesterol from C-BSA-C was affected when the reaction temperature was 4°C.

Fig. 4 shows densitometric tracings of the gel electrophoresis obtained when mitochondria were incubated with 2.30 or 0.35 μmol of cholesterol from C-BSA-C, or with BSA alone (C). Mitochondria treated with C-BSA-C showed an electrophoretic pattern similar to that of the control. This result suggests no BSA retention and reinforces the statement that BSA is functioning just as a carrier.

An experiment was performed using ^{125}I-labeled BSA. **Table 1** shows that iodinated BSA is released totally in the first mitochondrial wash. All the radioactivity was recovered in the washes, and mitochondria had no radioactivity. The amount of cholesterol incorporated was ca. 23 μg/mg of mitochondrial protein. This is conclusive evidence that there is no BSA retention in mitochondria, but a true incorporation of cholesterol in this organelle.

Table 2 shows the percent of cholesterol incorporation, and the total amount of phosphorus from mitochondrial phospholipids. The results suggest that only cholesterol, and no other lipids, is incorporated in the mitochondria. This finding was also supported by the data obtained with gas–liquid chromatography (not shown).

Considering the possibility that cholesterol could have adhered to the mitochondria, the same experiment was per-

Fig. 3. Cholesterol incorporation into mitochondrial membrane induced by C-BSA-C. Mitochondria (50 mg) from rat liver were incubated with different amounts of cholesterol from C-BSA-C. After 30 min incubation at 30°C (-●-), or 4°C (-○-), the mixture was diluted with 0.25 M sucrose, 1 mM EDTA, pH 7.3. The same procedure was followed for the control substituting the BSA C-BSA-C by the corresponding amount of BSA (■). Final volume, 1 ml. The bars indicate SD; n = 5.

TABLE 1.

	^{125}I-Labeled BSA	Cholesterol Content
	total cpm	µg per mg of protein
Mitochondria-C-BSA-C	480 000	
First supernatant	490 000	
Second supernatant	344	
Third supernatant	0	
Mitochondria	0	22.7

An aliquot of rat liver mitochondria (50 mg of protein) was incubated with 2.3 µmol cholesterol and about 500,000 cpm of ^{125}I-labeled BSA from the C-BSA-C. After 30 min incubation, the C-BSA-C-treated mitochondria were diluted 40 times with a cold solution of 0.25 M sucrose, 1 mM EDTA, pH 7.3, and immediately centrifuged at 12,000 g for 10 min. The mitochondrial fraction was washed by suspending it in the same above-mentioned solution. In each step, an aliquot of the sample was used to record the radioactivity.

formed adding 0.1% BSA to the sucrose used in the mitochondrial wash. The results demonstrated that the addition of BSA to the washing medium did not change the increase in cholesterol.

Fig. 5 shows cholesterol enrichment induced by C-BSA-C as a function of time. It is clear that C-BSA-C reaches its maximal cholesterol delivery in a short time. It has been shown that pH affects the liposome-induced incorporation of cholesterol in membranes (16). In this regard, we decided to study whether the pH had the same effect in this system. The results in **Fig. 6** indicate that at pH 6.6, C-BSA-C induced the maximal increase of cholesterol incorporation into the mitochondrial membrane with a nonlinear decrease with increasing pH to 8.2.

To test the efficiency of the method, incorporation of cholesterol was studied in another biological system. Mitochondria from adrenals have the capacity to use free cholesterol, which migrates rapidly through the outer and inner membrane to bind to cytochrome P-450, and is then transformed to pregnenolone (25). **Fig. 7** shows the time course of cholesterol transformation in incubations of dog

TABLE 2.

Cholesterol	Cholesterol	Increased	Total Phospholipids
µmol	µg per mg of protein	%	µg P per mg of protein
0.00	3.31 ± 0.46	0.0	0.203 ± 0.035
0.09	3.42 ± 0.87	3.3	0.206 ± 0.023
0.17	3.96 ± 0.65	19.6	0.193 ± 0.026
0.35	4.94 ± 0.86	49.2	0.195 ± 0.024
0.66	7.99 ± 1.49	141.3	0.210 ± 0.028
1.28	13.69 ± 2.48	316.6	0.200 ± 0.025
2.30	23.94 ± 1.62	623.2	0.190 ± 0.031

Rat liver mitochondria (50 mg of protein) were incubated (1-ml volume) in the presence of C-BSA-C for 30 min at 30°C. After the incubation, the mixture of C-BSA-C and mitochondria was washed with 0.25 M sucrose, 1 mM EDTA, pH 7.3, three times; and cholesterol and protein were measured. Results are means ± SD (n = 4).
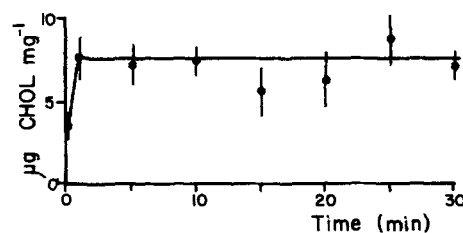

Fig. 5. Effect of time on incorporation of cholesterol into mitochondria. The experimental conditions were similar to those of Fig. 3. The amount of cholesterol was 0.66 µmol. The reaction was stopped by 40 times dilution as in Fig. 3, followed by centrifugation. CHOL, cholesterol; n = 3.

adrenal mitochondria with C-BSA-C. The C-BSA-C induced enhanced pregnenolone synthesis, which attained values 200% higher than control. This experiment was repeated by using [14C]cholesterol. The results (not shown) indicate that about 0.6 to 1% of the cholesterol added is transformed to pregnenolone after 1 min of incubation. This degree of conversion is in agreement with the results obtained by radioimmunoassay.

DISCUSSION

The present study describes a new method to increase the amount of cholesterol in mitochondria with a BSA-cholesterol mixture, using BSA as carrier.

The C-BSA-C is prepared by producing a microcrystal structure of cholesterol in an ethanol–water mixture; this is then associated with BSA. The spherical shape of the complex closely resembles that of liposomes; several hypothetical assumptions could be suggested to explain the rearrangement of cholesterol and BSA in the complex. Considering the cholesterol concentration used and the conditions of preparation of the complex, it is most probable that cholesterol attains a microcrystalline structure (26), although at this time it is difficult to provide conclusive evidence.
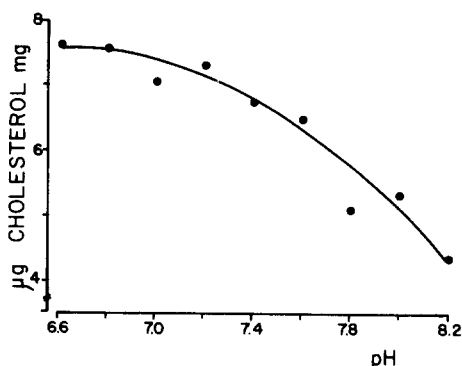
The results support the fact that the C-BSA-C induces an increase in cholesterol incorporation into mitochondria, since in mitochondria washed with 0.1% BSA the results were the same as those found in mitochondria washed without BSA. This must be taken into account, since reports exist (27) in which incubations with serum albumin combined with phospholipids are used to remove cholesterol from the cell membrane. However, no combination of BSA-phospholipids was used in our experiments; therefore no cholesterol could be released from the mitochondrial membrane. Also, it has been reported (28) that serum albumin is able to take up phospholipids added to the incubation medium and then this combination releases cholesterol from the cell membrane. Table 2 clearly shows that membrane phospholipids remained unchanged under the experimental conditions used. On the other hand, under the con-

ditions shown, a high concentration of cholesterol existed outside the mitochondria, producing a chemical gradient favoring the transfer of this sterol to the mitochondria.

The fact that only cholesterol is incorporated into the mitochondria is established by the following findings. *1)* When 2.3 μmol of cholesterol and 80 mg of BSA were added from the C-BSA-C to the mitochondria (50 mg of protein), the amount of mitochondrial cholesterol determined under these conditions was 22–24 μg cholesterol per mg of protein. If the C-BSA-C remained bound to mitochondria to provide that amount of cholesterol, then about 60–70 mg of BSA should also have adhered to the organelle. The amount of protein recovered from mitochondria was always around 45 mg, and the protein in the supernatant always matched the amount of BSA added from the C-BSA-C. *2)* If BSA were retained by mitochondria, a decrease in the specific activity of ATPase (a specific mitochondrial enzyme) would be expected. However, the ATPase activity remained unchanged (120 nmol of Pi released per min per mg of mitochondrial protein) whatever amount of C-BSA-C was tested. *3)* In the electrophoresis gels using 2.3 μmol of cholesterol and 80 mg of BSA from C-BSA-C, there was no observable peak corresponding to BSA. *4)* The results obtained with iodinated BSA gave conclusive evidence that BSA is not bound to mitochondria and that it functions just as a carrier.

As mentioned before, the arrangement of cholesterol in the C-BSA-C is unknown, but it is clear that cholesterol is easily released from the spherical particles and rapidly transferred to the membrane in only 1-min of incubation. The possibility of a single adsorption process of microcrystals could explain this fast transference process.

The fact that mitochondria from dog adrenals are able to take cholesterol from the C-BSA-C and synthesize pregnenolone suggests not a single adsorption phenomenon, but an incorporation of cholesterol in such a way that preg-
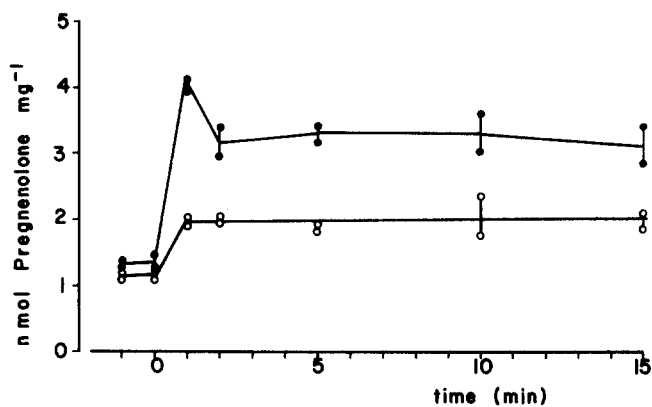
Fig. 7. Pregnenolone biosynthesis by mitochondria from dog adrenals induced by C-BSA-C. Mitochondria from dog adrenals were incubated in the presence of 176 μg of cholesterol from C-BSA-C in the medium as described under Material and Methods. At the time shown, the reaction was stopped by addition of an aliquot of these mitochondria to 1 ml of benzene, followed by ether extraction. Pregnenolone was measured by radioimmunoassay. The SD is shown by the spread of the symbols. The experiment was carried out in triplicate. Control (-O-); mitochondria plus C-BSA-C (-●-).

nenolone synthesis is promoted, maintaining a higher synthesis for a longer time than in the control studies. It should be noted that there is a little increase in pregnenolone synthesis when isolated mitochondria are incubated in the absence of substrate. However, this could be explained if we consider that mitochondria contain some endogenous substrates able to support an increase of pregnenolone as was observed. On the other hand, the diminution in the synthesis of pregnenolone, after 1 min of incubation in the presence of malate, could be explained by its transformation to progesterone. This assumption is based on the fact that the incubation was carried out in the absence of inhibitors of 3β-hydroxy-steroid dehydrogenase, an enzyme that is responsible for the conversion of pregnenolone. This enzyme has been reported to be present in isolated mitochondria from adrenals (29, 30).

When [^{14}C]cholesterol was used, about 1% of the label was recovered as pregnenolone. The total amount of cholesterol added was 455.2 nmol (176 μg) from C-BSA-C. The percent of increase, observed by RIA, was about 1.7%. These results support the fact of free cholesterol incorporation and, at the same time, this sterol remains available to be used for pregnenolone synthesis.

These data, taken altogether, suggest that the C-BSA-C behaves in a manner similar to that shown by liposomes. In the same way, the C-BSA-C also shows a better incorporation of cholesterol at pH 6.6, supporting the idea that some ionizable groups are important in cholesterol transfer. Using this method, it is possible to obtain a 3 to 600% higher cholesterol incorporation than in the controls, as reported by Schneider, Höchli, and Hackenbrock (18), but with the difference that no other lipids are incorporated into the

Fig. 6. pH effect on cholesterol incorporation. The mitochondria were incubated in the presence of 10 mM Tris-HCl at the pH shown. During the experiment, the pH was monitored and after 15 min of incubation, the reaction was stopped as mentioned above. The amount of cholesterol was 0.66 μmol. Final volume was 1 ml; temperature, 30°C.

membrane, and with the advantage of using the whole mitochondria.

Based on the data presented, it is possible that this C-BSA-C could function with other biological systems; and, on the other hand, the method more nearly resembles a physiological movement of cholesterol, and probably could be a great asset to study the mechanisms of cholesterol transfer into the cell. ■

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