Metabolism of Free and Esterified Cholesterol by Leydig-Cell Tumour Mitochondria

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1. Experiments were designed to localize intracellularly the enzymes and sterol substrates required for steroidogenesis in Leydig-cell tumours. Subcellular fractions were prepared by differential centrifugation of tumour homogenates. Both free and esterified cholesterol were associated primarily with the fractions sedimenting at 1400g_{av} and the lipid layer floating on the surface of the isolation tubes; they were not found in the mitochondria, where the conversion of cholesterol into pregnenolone occurred. 2. Hydrolysis of esterified cholesterol was required before it could be oxidized to pregnenolone. 3. An enzyme capable of hydrolysing cholesterol esters was located external to the mitochondria. 4. Mitochondria were subfractionated by allowing them to swell in 0.02M-phosphate buffer (pH 7.2) and separating the inner and outer membranes by sedimentation in sucrose gradients. The outer membrane, identified by its content of monoamine oxidase, contained most of the cholesterol associated with the mitochondria. The inner membrane, identified by its content of succinate dehydrogenase, contained the cholesterol side-chain-cleaving enzyme and very little cholesterol. 5. Accumulation of sterols by the mitochondria was studied by incubating this fraction with labelled free and esterified cholesterol suspended in lipid-free bovine serum albumin. Two phases of cholesterol accumulation were observed. The first phase, requiring 10–15 min, was independent of the incubation temperature, and was inhibited by the presence of bovine serum albumin in the incubation medium. The second phase of accumulation was independent of the serum albumin content of the medium but was inhibited by low incubation temperature. 6. Esterified cholesterol was not accumulated by the mitochondria after the initial rapid binding phase. 7. The findings suggest that cholesterol was not rapidly accumulated by the mitochondrial fraction *in vitro* and that mechanisms may be required to facilitate cholesterol transport into mitochondria in intact tumour cells during the periods in which steroidogenesis is stimulated maximally.

It was shown in the preceding paper that one of the actions of LH* on Leydig cells is to promote the hydrolysis of esterified cholesterol stored within these cells (Moyle *et al.*, 1973). Some of the cholesterol so produced is in turn converted into steroids, as was also shown by Hall & Young (1968) and Menon *et al.* (1965). Very few studies of this steroidogenic process in testicular tissue have appeared. Hall *et al.* (1969) found that the cleavage of the side chain of cholesterol occurred in Leydig cells and not in the seminiferous tubules. A major difficulty in the study of testicular steroidogenesis is the fact that Leydig cells comprise less than 10% of the testes in many animals (Bascom & Osterud, 1925). We have therefore used transplantable mouse Leydig-cell tumours as a tissue source to study the metabolism of free and esterified cholesterol in relation to steroid production. Since the enzyme responsible for cleavage of the cholesterol side chain has been localized in mitochondria by Toren *et al.* (1964), we have focused attention on these organelles.

* Abbreviation: LH, luteinizing hormone.

Experimental

Growth of LH-responsive tumours

Tumours (weighing 0.2–1 g) were grown subcutaneously in C57B1/6J mice (Jackson Laboratories, Bar Harbor, Me., U.S.A.) as described previously (Moyle & Armstrong, 1970). As certain generations of tumours (a series of tumours started from the same tumour) do not respond to LH, each generation was tested for LH responsiveness (Moudgal *et al.*, 1971; Moyle *et al.*, 1971, 1973) and only responding tumours were used in these experiments.

Preparation of fractions

Subcellular fractions. Tumours were homogenized by hand in glass homogenizers in cold 0.25M-sucrose–1mM-EDTA (pH 7.4). The homogenate was centrifuged at 1400g_{av} (10 min) to remove nuclei and tissue debris. The supernatant was centrifuged at 7000g_{av} (10 min) to isolate mitochondria. Broken mitochondria and some of the microsomal fraction were

collected at 30000g_{av}. (20 min). Microsomal fractions were prepared by centrifuging the 30000g_{av}. supernatant at 140000g_{av}. (1 h). Unless noted, the mitochondrial fraction was washed twice by resuspension and centrifugation at 7000g_{av}. (10 min) in the 0.25 M-sucrose–1 mM-EDTA solution.

Submitochondrial fractions. The inner and outer mitochondrial membranes were prepared essentially by the method of Parsons *et al.* (1966). This consisted of allowing the mitochondria to swell for 20 min in 20 mM-potassium phosphate buffer (pH 7.2) containing 0.02% bovine serum albumin (buffer A) and subsequently centrifuging at 30000g_{av}. (20 min). The pellets were resuspended in buffer A and centrifuged at 4000g_{av}. (15 min). The supernatant remaining was centrifuged at 30000g_{av}. (20 min). The pellets formed at 4000g_{av}. and 30000g_{av}. were dispersed separately in 3 ml of buffer A and individually placed on discontinuous sucrose gradients produced by layering successively 3 ml of each of the following solutions (% w/v): 51.3%, 37.7% and 25.2% sucrose in buffer A; 13 ml cellulose nitrate centrifuge tubes were used. Separation of the two mitochondrial membranes was accomplished by centrifuging the gradients at 140000g_{av}. (1 h). The material collected at the bottom of the tube and at the interfaces of each of the sucrose layers was removed with a Pasteur pipette and freed from sucrose by dilution with buffer A and centrifugation at 100000g_{av}. (15 min). Material recovered in corresponding bands from the 4000g_{av}. and 30000g_{av}. pellet preparations was recombined for enzyme assay.

Inner and outer membranes were distinguished on the basis of enzyme content. The outer membrane was assumed to contain monoamine oxidase (Schnaitman *et al.*, 1967) and the inner membrane was noted by its succinate dehydrogenase activity (Schnaitman & Greenawalt, 1968). Monoamine oxidase was assayed by the method of Schnaitman *et al.* (1967), in which the oxidation of benzylamine hydrochloride to benzaldehyde is followed spectrophotometrically at 250 nm. Succinate dehydrogenase activity was measured after sonication by using cytochrome *c* or dichlorophenol-indophenol as electron acceptors. With cytochrome *c* the procedure was as follows: 10 μmol of succinate was added to 1 ml of solution containing the enzyme to be measured and 50 μmol of Tris-HCl buffer (pH 7.4), 5 μmol of NaCN and 1.5 mg of oxidized horse heart cytochrome *c* (Sigma Chemical Co., St. Louis, Mo., U.S.A.). The change in *E*₅₅₀ was recorded with time. With dichlorophenol-indophenol the procedure was as follows: 10 μmol of succinate was added to 1 ml of a solution containing the enzyme to be measured and 200 μg of phenazine methosulphate, 50 μmol of potassium phosphate buffer (pH 7.4) and 5 μmol of dichlorophenol-indophenol. The *E*₆₀₀ was recorded as a function of time. All enzyme assays were shown to be

proportional to the amount of enzyme added and were linear with time during the period of measurement.

Protein was measured by the modified biuret reaction described by Jacobs *et al.* (1956) or by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

Enzyme activities

Measurement of cholesterol side-chain cleavage activity. Cholesterol side-chain cleavage activity was measured by following the conversion of labelled cholesterol into pregnenolone and progesterone (see table legends for the label and incubation mixture used in each experiment). Labelled compounds (New England Nuclear Corp., Boston, Mass., U.S.A.) were added to the incubation flask in 50 μl of acetone-propylene glycol (1:1, v/v). At the end of the incubation period the reaction was stopped with 20 vol. of chloroform-methanol (2:1, v/v). After addition of 0.2 vol. of 0.9% NaCl to separate lipids and non-lipids, the chloroform layer was evaporated with a stream of N₂ and the residue subjected to t.l.c. on silica gel G with hexane-diethyl ether-acetic acid (15:10:1, by vol.). Silica gel from the areas of the chromatogram corresponding to pregnenolone and progesterone was counted for radioactivity in a liquid-scintillation spectrometer as described in the preceding paper (Moyle *et al.*, 1973). Recrystallization of the steroids in one experiment confirmed their radiochemical identity.

Measurement of cholesterol ester hydrolase. Labelled cholesteryl palmitate was prepared by heating [1,2-³H]cholesterol or [7α-³H]cholesterol and palmitoyl chloride (10 μl) in 0.5 ml of benzene in a stoppered tube in a boiling-water bath for 1 h. The esterified and free cholesterol were separated by chromatography on silica gel layers with hexane-diethyl ether-acetic acid (90:10:1, by vol.). With this procedure more than 90% of the initial cholesterol was esterified. The hydrolase was measured by following the conversion of the ester into free cholesterol as a function of time. The reaction conditions are described in the figure and table legends. The reaction was stopped with chloroform-methanol (2:1, v/v) and the lipids were extracted and subjected to the same chromatographic procedure as described above. Areas of the chromatogram corresponding to the location of cholesterol were subjected to liquid-scintillation counting. Recrystallization of the ester and the liberated cholesterol confirmed their identities.

Cholesterol assay and determination of transport

Measurement of cholesterol. Free and esterified cholesterol were assayed by a modified Lieberman-

Burchard reaction (Armstrong *et al.*, 1964). When the amounts of cholesterol were low, an adaptation (Pokel *et al.*, 1972) of the procedure described by Zlatkis & Zak (1969) was used.

Measurement of cholesterol transport. Mitochondria were suspended in 0.25M-sucrose solution containing 50mM-Tris-HCl at pH7.2. Fatty acid-free bovine serum albumin (type F; Sigma) was added at various concentrations together with [4-¹⁴C]cholesterol and [1,2-³H]cholesteryl palmitate or acetate. The labelled lipids were dissolved in 0.1 ml of propylene glycol-acetone (1:1, v/v) and the aqueous bovine serum albumin-containing buffers added to this solution. Study showed that all the radioactivity added was dissolved in the buffer. The palmitate esters were prepared as described above. The acetate was prepared by leaving the labelled cholesterol overnight in an evacuated desiccator containing beakers of acetic anhydride and pyridine. Purification was by t.l.c. as for the larger esters. At various times portions of the mitochondrial fraction were removed from the incubation mixture and filtered on cellulose filters (Millipore Corp., Bedford, Mass., U.S.A.; 0.3 μ m pore size). The filtrate and filter were counted for radioactivity in a liquid-scintillation counter (Packard Tri-Carb) in a fluid containing Triton X-100 (Behrman *et al.*, 1971).

In some experiments the mass of the cholesterol in the incubation buffer was increased by adding 50 μ l of a saturated cholesterol solution in acetone (50°C) to the sucrose-Tris-bovine serum albumin (25mg/ml) incubation mixture (37°C) to which [4-¹⁴C]cholesterol had already been added. The emulsion that formed immediately was incubated for 2h at 30°C to

allow equilibration of labelled and unlabelled cholesterol.

Results

Subcellular distribution of cholesterol in Leydig-cell tumours

It was noted in the preceding paper (Moyle *et al.*, 1973) that the cholesterol that serves as the precursor for testosterone synthesis in Leydig cells is derived from an intracellular source rather than from the serum in which the cells are bathed. An early step in the conversion of this cholesterol into steroids involves the cleavage of the alkyl side chain by enzymes known to be localized in the mitochondria (Toren *et al.*, 1964). Thus it was decided to determine how much of the cellular cholesterol was located in these organelles in proximity to the cleavage enzymes. Tumours were homogenized and the distribution of free and esterified cholesterol in various centrifugal fractions was determined (Table 1). The bulk of both the protein and cholesterol of the tumours sedimented at 1400g_{av.}; this fraction contained tissue debris and many erythrocytes. About one-quarter of the remaining cholesterol was recovered in the mitochondrial fraction (7000g_{av.}), whereas less than one-tenth of the remaining esterified cholesterol appeared in this fraction. The lipid layer that floats to the top of the tube contained large amounts of esterified cholesterol but only traces of free cholesterol. Since this floating esterified cholesterol is presumably of non-mitochondrial origin and yet serves as a precursor for steroid synthesis in the presence of LH (Behrman & Armstrong, 1969; Cook

Table 1. *Distribution of cholesterol and esterified cholesterol in centrifugal fractions of tumour-cell homogenates*

Fractions were prepared as described in the text. Cholesterol and cholesterol ester were extracted and separated as described and assayed by the Lieberman-Burchard reaction (Armstrong *et al.*, 1964). Proteins were determined by the method of Lowry *et al.* (1951). Values represent the mean $\pm$ range of two determinations. 'Lipid layer' is material floating after the initial 1400g_{av.} centrifugation. This material was not washed and therefore is contaminated with components from the soluble fraction.

Fraction	Distribution (% of total recovered)		
	Protein	Cholesterol	Cholesterol ester
Pellet			
1400g _{av.}	60 $\pm$ 3.0	60 $\pm$ 2.0	55 $\pm$ 5.0
7000g _{av.}	3.5 $\pm$ 0.5	10 $\pm$ 2.0	3.5 $\pm$ 1.5
30000g _{av.}	3.7 $\pm$ 0.3	15 $\pm$ 0.8	2.7 $\pm$ 1.2
140000g _{av.}	2.2 $\pm$ 0.2	7.1 $\pm$ 0.9	3.3 $\pm$ 2.7
Soluble	9.8 $\pm$ 1.8	2.6 $\pm$ 1.4	4.0 $\pm$ 2.0
Lipid layer	12 $\pm$ 5.7	5.7 $\pm$ 2.3	33 $\pm$ 2.8
Total mass	38.6mg	301 μ g	456 μ g

Table 2. *Submitochondrial localization of cholesterol side-chain-cleaving enzyme*

Mitochondrial fractions were isolated at 7000*g*_{av.} and fractionated as described in the text. Distribution of monoamine oxidase, succinate dehydrogenase, cholesterol side-chain-cleaving enzyme, cholesterol and protein were determined as described in the text. Membrane fragments were incubated in 1 ml of 0.1 M-Tris-HCl (pH 7.4), 0.005 M-MgCl₂ and 0.1 M-NaCN. NADPH (1 mg/ml) plus a mixture of [26-¹⁴C]cholesterol (0.1 μ Ci; 50 mCi/mmol) and [7 α -³H]cholesterol (0.2 μ Ci; 10 mCi/mmol) were used as the substrates. Incubation was continued for 60 min. No ¹⁴C appeared in any of the steroids isolated. Percentages are based on amounts measured in mitochondria immediately after swelling in phosphate buffer. Values are means $\pm$ s.d. The number of experiments used to calculate the means is shown in parentheses.

Membrane fraction	Protein (% of total)	Monoamine oxidase (% of total)	Succinate dehydrogenase (% of total)	Side-chain-cleaving enzyme (% of total)	Cholesterol (% of total)
Outer	15.7 $\pm$ 7.5 (3)	43.3 $\pm$ 2.2 (3)	9.1 $\pm$ 8.7 (5)	7.7 $\pm$ 5.0 (3)	34.1 $\pm$ 8.8 (3)
Inner	77.3 $\pm$ 2.0 (3)	56.1 $\pm$ 2.8 (3)	88.0 $\pm$ 7.4 (5)	85.3 $\pm$ 9.3 (3)	39.4 $\pm$ 2.0 (3)

et al., 1967), it was decided to study the process by which this precursor gains access to the mitochondrial side-chain-cleaving enzyme.

Submitochondrial location of the cholesterol side-chain-cleaving enzyme

If the cleaving enzyme is located on the outer membrane of the mitochondrion it may be relatively freely accessible to cytoplasmic cholesterol. On the other hand, if the enzyme is located in the matrix or on the inner mitochondrial membrane, there may be a membrane barrier separating it from the bulk of the cellular cholesterol stores. Table 2 summarizes the results of attempts to localize the side-chain-cleaving enzyme within the mitochondria. Complete separation of the two membranes was not achieved, as shown by the monoamine oxidase and succinate dehydrogenase activities of the fractions isolated. The outer membrane fraction contained less than 10% of the total succinate dehydrogenase and side-chain-cleaving activities but retained nearly half of the monoamine oxidase activity. The distribution of the side-chain-cleaving enzyme activity always paralleled that of the succinate dehydrogenase activity and never that of the monoamine oxidase activity. When mitochondria were not swollen in 20 mM-phosphate buffer before sucrose-gradient centrifugation, all three enzymes were recovered in the inner membrane fraction (results not shown), indicating that the phosphate buffer treatment was causing separation of membrane-bound monoamine oxidase and succinate dehydrogenase. We have concluded that the side-chain-cleaving activity is almost certainly contained in the inner membrane.

The results in Table 2 also indicate that the inner mitochondrial membrane contains relatively little of

the mitochondrial cholesterol. Thus very little substrate is localized at the site of the side-chain-cleaving enzyme. Apparently cholesterol must pass from the extramitochondrial space to the inner mitochondrial membrane before being oxidized.

Side-chain cleavage of esterified cholesterol

The conversion of cholesteryl palmitate into steroids might proceed via conversion into a pregnenolone palmitate intermediate. This would be analogous to the side-chain cleavage of cholesterol sulphate to pregnenolone sulphate (Roberts *et al.*, 1964). Preliminary experiments demonstrated that labelled steroids were synthesized when [1,2-³H]cholesteryl palmitate was incubated with Leydig-cell homogenates and NADPH. Table 3 contains the results of experiments designed to show whether cholesteryl palmitate itself is directly converted into steroids. The mitochondria used in Expt. no. 1 were contaminated with microsomal fraction (see Table 5) and in this experiment esterified cholesterol was both hydrolysed and converted into steroids. In Expt. no. 2, with cleaner mitochondria, cholesteryl palmitate was neither hydrolysed nor converted into steroids whereas steroidogenesis from free cholesterol was unaltered. This suggests that cholesteryl esters must first be converted into cholesterol before being oxidized to steroids.

As the solubility and access to the side-chain-cleaving enzyme of esterified cholesterol may differ from that of free cholesterol, the ability of broken mitochondria to oxidize these substances was investigated. The effect of adding Tween to the mitochondrial suspensions was also studied. It was found that disrupting the mitochondria by freeze-thawing or sonication inhibited cholesterol ester hydrolysis

Table 3. *Conversion of cholesteryl palmitate and cholesterol into steroids by intact mitochondria*

Expt. no. 1: a mitochondrial fraction isolated at 10000g_{av.} was incubated with 0.25 μ Ci of [7 α -³H]cholesteryl palmitate (10mCi/mmol) and 0.025 μ Ci of [4-¹⁴C]cholesterol (50mCi/mmol) for 15, 30 and 60 min (one determination at each time) in 0.25M-sucrose–0.01M-Tris–HCl–0.01M-K₂HPO₄–0.01M-isocitrate–0.01M-malate, pH7.4. Expt. no. 2: a mitochondrial fraction was isolated at 7000g_{av.} and incubated in 0.25M-sucrose–0.1M-Tris–HCl–0.01M-isocitrate, pH7.4, with 1 mg of NADPH/ml, in duplicate for 0, 5, 15, 30 and 60 min. At each time sterols and steroids were isolated as described in the text and recrystallized once. Progesterone was the major steroid found in Expt. no. 1 and pregnenolone was the principal steroid isolated in Expt. no. 2. Values are expressed as d.p.m. of product/h $\pm$ s.d. as determined by the slope of a line passing through the d.p.m. values at each time. Calculations were performed by the method of Mood & Graybill (1963).

Reaction studied	Expt. no. ...	Rate of conversion (d.p.m./h)	
		1	2
[³ H]Cholesteryl palmitate into [³ H]cholesterol		30800 $\pm$ 890	0
[³ H]Cholesteryl palmitate into ³ H-labelled steroids		2450 $\pm$ 450	0
[¹⁴ C]Cholesterol into ¹⁴ C-labelled steroids		6430 $\pm$ 960	7161 $\pm$ 500

Table 4. *Conversion of cholesteryl palmitate and cholesterol into steroids*

Mitochondria were incubated in 0.25M-sucrose–0.001M-EDTA–0.05M-Tris–HCl, pH7.4, with 0.5 mg of NADPH/ml for 0, 30, 60 and 90 min. A fraction of the mitochondria was frozen at –20°C and rapidly thawed in a water bath at 37°C and a fraction was sonicated for 2 min at 15 s intervals in an ice bath. [1,2-³H]Cholesteryl palmitate (0.05 μ Ci; 50Ci/mmol) was used to evaluate the conversion of cholesteryl palmitate into cholesterol and steroids. [26-¹⁴C]Cholesterol (0.01 μ Ci) and [1,2-³H]cholesterol (0.01 μ Ci; 50Ci/mmol) were used to evaluate the conversion of cholesterol into steroids. No ¹⁴C was isolated in any steroids. Isolation of sterols and steroids is described in the Experimental section. Values represent the slope of the line drawn through points representing the d.p.m. of product isolated at each time $\pm$ s.d. calculated by the method of Mood & Graybill (1963).

Treatment	Rate of conversion (d.p.m./h)		
	[³ H]Cholesteryl palmitate into [³ H]cholesterol	[³ H]Cholesteryl palmitate into ³ H-labelled steroids	[³ H]Cholesterol into ³ H-labelled steroids
None	3861 $\pm$ 282	157 $\pm$ 24	361 $\pm$ 10.0
Frozen–thawed	2170 $\pm$ 96	63 $\pm$ 24	552 $\pm$ 44.1
Sonication	264 $\pm$ 46	35 $\pm$ 30	938 $\pm$ 105.2
Tween (0.5 mg/ml)	–19 $\pm$ 23	13 $\pm$ 40	948 $\pm$ 97.0

and conversion into steroids, but actually increased steroidogenesis from free cholesterol (Table 4). The same result was evident in the presence of Tween, a detergent that should increase the solubility of esterified cholesterol. These results strengthen the view that hydrolysis must precede cleavage and in addition demonstrate that disrupting mitochondria unmasks latent side-chain-cleaving enzyme activity. This further suggests that the enzyme is localized on the inner mitochondrial membrane in such a way that it does not have free access to the medium cholesterol or NADPH or both.

In the experiments just described no pregnenolone palmitate could be detected in the lipid extracts of the incubation media. [³H]Pregnenolone palmitate was

also not found when mitochondria were incubated with [³H]cholesteryl palmitate in the presence of Tween and a trapping pool of unlabelled pregnenolone palmitate (results not shown). However, were pregnenolone palmitate formed, it could be converted into pregnenolone and progesterone by Leydig-cell extracts as illustrated in Fig. 1.

Hydrolysis of esterified cholesterol

The conversion of esterified cholesterol into steroids thus seems to require first the hydrolysis of the ester followed by the movement of the liberated cholesterol from the cytoplasmic compartment into the mitochondria where side-chain cleavage occurs. The

Fig. 1. Conversion of [1,2-³H]pregnenolone palmitate into pregnenolone and progesterone

[1,2-³H]Pregnenolone palmitate was synthesized by heating [1,2-³H]pregnenolone (10 μ Ci; 50 Ci/mmol) and 10 μ l of palmitoyl chloride in benzene for 1 h and separating the ester from the unchanged alcohol on silica gel G plates with hexane-ether-acetic acid (90:10:1, by vol.). The ester (0.1 μ Ci) was incubated at 37°C with the 7000 g_{av} supernatant remaining after preparation of mitochondria as described in the text. Pregnenolone (●) and progesterone (○) were isolated by the procedure described in the text for measurement of cholesterol side-chain-cleaving activity. Percentage hydrolysis was calculated by dividing the radioactivity recovered in the free steroids by the initial radioactivity of pregnenolone palmitate. Chromatography of [1,2-³H]pregnenolone palmitate before incubation indicated that it contained less than 1% of [1,2-³H]pregnenolone. Total hydrolysis (▲) is indicated by the sum of pregnenolone and progesterone.

results presented in Table 3 suggested that the ester hydrolase might be primarily located in the microsomal membranes or the soluble fraction of the Leydig cell. This possibility was examined in greater detail; the results, presented in Table 5, indicate that cholesterol esters are almost certainly hydrolysed extra-mitochondrially.

Transport of cholesterol into mitochondria

Experiments were designed to determine if Leydig-cell mitochondria possess a transport system which facilitates the entry of cholesterol into the mitochondria and thus to the site of the side-chain-cleaving enzyme. Fig. 2 shows that when mitochondria are incubated in the presence of labelled cholesterol radioactivity is removed from the medium in a complex manner. When bovine serum albumin was omitted from the medium, virtually all the cholesterol disappeared from the medium within seconds. This may reflect the low solubility of cholesterol in the aqueous medium. When albumin was included, the initial phase of cholesterol uptake was decreased in proportion to the amount of albumin added. A second phase of cholesterol uptake followed, and appears to be a first-order process unaffected by the albumin content of the medium. Moreover, this second phase was absent when the uptake of cholesteryl palmitate or cholesteryl acetate was examined under similar

Table 5. Distribution of the cholesterol ester hydrolase in subcellular fractions

Mitochondria were isolated as described in the text, in Expt. no. 1 at 10000 g_{av} . (electron-microscopic observation confirmed the presence of large amounts of microsomal contamination on isolation at 10000 g_{av}), and in Expt. no. 2 at 7000 g_{av} . Portions were incubated with 0.25 μ Ci (Expt. no. 1) or 1.0 μ Ci (Expt. no. 2) of [7 α -³H]cholesteryl palmitate in 0.25 M-sucrose-0.1 M-Tris-HCl (pH 7.4) at 37°C. Portions were removed at 15, 30 and 60 min and the radioactivity in the cholesterol was determined after extraction and purification by t.l.c. The average d.p.m./h per mg of protein $\pm$ s.d. for each fraction was calculated by the method of Mood & Graybill (1963). Values represent the % of total activity recovered $\pm$ s.d. for three measurements.

Expt. no ... Fraction	Distribution of activity (%)	
	1	2
Mitochondrial	16.8 $\pm$ 0.5	3.8 $\pm$ 0.1
Microsomal+soluble	83.2 $\pm$ 8.9	96.2 $\pm$ 18.3

Fig. 2. Accumulation of cholesterol by mitochondria as a function of time and bovine serum albumin concentration

Mitochondria were incubated at 37°C with [4-¹⁴C]-cholesterol (50mCi/mmol) as described in the text. Portions of the incubation suspension were removed and filtered at 0, 2, 4, 8, 15, 30, 45, 60, 75, 90, 105 and 120min. Results at zero time were obtained by filtering mitochondria not incubated with the tracer and then filtering the tracer through the same filter. Concentration of bovine serum albumin present (mg/ml): ●---●, 0; ○, 1; ■, 5; □, 10; ●, 50.

conditions (Fig. 3). The second phase of accumulation, in contrast to the first phase, was also greatly affected by temperature (Fig. 4).

These observations suggested that the initial phase of sterol uptake was the result of the rapid binding of cholesterol and its esters to the exposed surfaces of the mitochondria. This is followed by a second phase

Fig. 3. Accumulation of [1,2-³H]cholesteryl acetate, [1,2-³H]cholesteryl palmitate and [4-¹⁴C]cholesterol by mitochondria as a function of time

Mitochondria were incubated with either labelled cholesteryl acetate (○) and cholesterol (●) or with labelled cholesteryl palmitate (▲) and cholesterol at 37°C with bovine serum albumin at 10mg/ml as described in the Experimental section. Portions of the incubation mixture were filtered at 0, 4, 8, 15, 30, 60, 90 and 120min. The ³H (ester) or ¹⁴C (alcohol) radioactivity (d.p.m.) remaining in the medium is plotted as a function of time.

of comparatively slow and steady cholesterol accumulation, which is not shared by the cholesterol esters, and thus appears to be a specific uptake process.

The mass of the labelled cholesterol used in the previous experiments to examine accumulation was small relative to the total cholesterol content of the mitochondria. Under these conditions it seemed likely that the accumulation of radioactivity during phase two might represent an exchange reaction between labelled cholesterol in the medium and cholesterol contained within the membranes of the mitochondria. We therefore repeated the experiments, incubating mitochondria with much larger amounts of cholesterol, to test whether mitochondria would

Fig. 4. Accumulation of [4-¹⁴C]cholesterol by mitochondria as a function of time and incubation temperature

Mitochondria were incubated with [4-¹⁴C]cholesterol at 0°C (●), 12°C (○), 25°C (■) or 37°C (□) in medium containing 10mg of bovine serum albumin/ml as described in the text. Portions were removed at 0, 2, 4, 8, 15, 30, 45, 60, 75, 90, 105 and 120 min and the radioactivity (d.p.m.) remaining in the medium was plotted as a function of time. The arrow indicates the initial radioactivity.

Table 6. Distribution of cholesterol and cholesteryl palmitate after incubation *in vitro* in a cholesterol emulsion

Mitochondria, isolated at 7000g_{av.} (10min), were incubated at 30°C with a [4-¹⁴C]cholesterol emulsion prepared as described in the text. At 15 and 60 min the mitochondria from triplicate flasks were isolated by centrifugation at 7000g_{av.} (10min). Values presented are the percentages ±s.d. (three experiments) of the cholesterol found in the mitochondria and in the medium.

Location of label	Radioactivity (d.p.m.)
15 min mitochondrial cholesterol	44 600 ± 2350
60 min mitochondrial cholesterol	66 400 ± 1060
15 min medium cholesterol	173 000 ± 4470
60 min medium cholesterol	156 470 ± 2940

take up sufficient cholesterol to increase significantly their net content of cholesterol. As shown in Table 6, mitochondria incubated in an emulsion of cholesterol continued to accumulate it after 1 h. Inasmuch as the mass of cholesterol associated with the mitochondria was increased by these experiments, cholesterol must have been transported into the mitochondria. After these treatments, however, it was not possible

to localize the cholesterol to one or another mitochondrial membrane, as the mitochondria that had been incubated in large concentrations of bovine serum albumin failed to dissociate when subjected to the procedures used previously.

Discussion

The results presented in this and the preceding paper (Moyle *et al.*, 1973) provide us with the following picture of steroidogenesis in Leydig cells. In response to LH the cholesterol ester hydrolase activity of the cells is enhanced and there is an increased production of free cholesterol in the cytoplasmic compartment. This cholesterol, perhaps bound to a protein carrier (Scallen *et al.*, 1971), makes its way to the mitochondria where it passes through the outer mitochondrial membrane to reach the site of the side-chain-cleaving enzymes. Within the mitochondria the cholesterol is converted into progesterone. LH accelerates steroidogenesis by activating the ester hydrolase via a mechanism apparently involving cyclic AMP (Moyle *et al.*, 1973), by increasing the oxidation of the cholesterol side chain (Hall & Young, 1968) and perhaps by altering the activities of additional enzymes as well.

Left unclear by these studies is whether mitochondria possess a specific transport system for cholesterol or whether penetration into these organelles occurs by passive diffusion. It was not possible to demonstrate an active accumulation of cholesterol within the mitochondrial matrix by using isolated mitochondrial suspensions. The mitochondrial pool of active cholesterol may represent only a small fraction of the total mitochondrial cholesterol, and like the total cellular pool of active cholesterol it may not easily be grossly inflated by inward transport. Thus the mitochondrial uptake of cholesterol into this active pool may prove exceedingly difficult to investigate.

Cholesterol esters seem to exist primarily as a storage form of cholesterol in the tumour cells. Inasmuch as many of the cholesterol esters (particularly the oleate, which is the major ester in the tumour) are oils, esterification not only renders the cholesterol moiety less soluble but promotes the formation of lipid droplets within the cell. These lipid droplets may be expected to be metabolically rather inert. Further support for the idea that cholesteryl esters are storage forms of cholesterol was provided by the observations that the esters were not direct substrates for side-chain cleavage, that they did not accumulate in mitochondria and that they did not leave the cell during incubation in serum (Moyle *et al.*, 1973). Apparently, once cholesterol is esterified it is metabolically inactive in the tumour cells until hydrolysis occurs.

Steroid-secreting cells apparently have a need for

increased amounts of cholesterol after hormonal stimulation; addition of LH to the cells restored the concentration of free cholesterol in the cells (Moyle *et al.*, 1973). Inasmuch as the cells do not seem to store free cholesterol, the increased cholesterol requirement is satisfied by hydrolysis of the stored cholesterol esters within the cells. Perhaps it seems paradoxical that steroid-secreting cells contain much larger quantities of free cholesterol than are used to synthesize steroids, yet need the additional cholesterol supplied for steroidogenesis by hydrolysis of cholesterol esters to respond to LH. The reasons for this unexpected observation may be related to the fact that cholesterol is extremely insoluble. Most of the cellular cholesterol was associated with membranes or lipid droplets; very little appeared to be 'soluble'. Thus even though large amounts of cholesterol can be extracted from the steroidogenic cells, it is conceivable that only a very small fraction of the cellular cholesterol is available for exchange with various subcellular compartments or with the plasma. If specific means of cholesterol transport into the mitochondria are absent, the most obvious methods for increasing the supply of cholesterol to the side-chain-cleaving enzyme would be to increase the solubility of cholesterol or to increase the total mass of cholesterol in the cell after treatment with LH. The cells have the ability to do the latter. Proteins that bind liver cholesterol have been described previously (Scallen *et al.*, 1971) and have been shown to stimulate the conversion of cholesterol into pregnenolone in the adrenal (Kan *et al.*, 1972). Brownie *et al.* (1972) reported that adrenocorticotrophin may increase steroidogenesis by promoting the binding of cholesterol to the cytochrome P-450 enzyme systems responsible for cholesterol side-chain cleavage.

The location of the cholesterol side-chain-cleaving enzyme in the inner mitochondrial membrane has also been reported for adrenal and ovarian tissues (Yago & Ichii, 1969). In our hands, however, complete separation of the inner and outer membranes proved to be very difficult. Although other methods of separating the two membranes were tried (Caplan & Greenawalt, 1968; Levy *et al.*, 1966), that of Parsons *et al.* (1966) proved the most suitable. One possible reason for the failure to obtain complete separation of the membranes may be that tumour mitochondrial membranes do not dissociate well under conditions initially designed for use with liver mitochondria.

Billiar *et al.* (1971) have reported that the cholesterol side-chain-cleaving enzyme may be located on the outer mitochondrial membranes of adrenal mitochondria. The adrenal mitochondrial membranes were separated by phospholipase A digestion, a treatment which resulted in ready solubilization of side-chain-cleaving enzyme activity. Assuming that monoamine oxidase is located almost exclusively in

the outer mitochondrial membrane, as suggested by Schnaitman *et al.* (1967), the results reported for the location of adrenal enzyme are hard to reconcile with the results we have obtained with the tumours. Brdiczka *et al.* (1968) have pointed out that phospholipase A digestion of mitochondria reveals no differences between the extractability of adenylate kinase and tricarboxylic acid-cycle enzymes from liver mitochondria. They suggest that phospholipase A treatment causes opening of the intracristal space and mitochondrial matrix. In this regard phospholipase A treatment may not provide true localization of the mitochondrial enzymes. Further studies on the effects of phospholipase A on membrane isolation will have to be made to resolve this point. However, the fact that sonication or freezing and thawing increased the side-chain cleavage of cholesterol in tumour mitochondria suggests strongly that the enzyme is not located in the mitochondria in such a way as to have free access to its substrates.

The cholesterol deficiency of the inner mitochondrial membranes has been reported previously (Graham & Green, 1970). Unfortunately, it is not possible to know how much of the mitochondrial cholesterol was redistributed during the isolation procedure. Possibly some of the cholesterol from the inner mitochondrial membranes was lost. This seems unlikely, however, since the aqueous buffers used in the isolation of the membranes are not good solvents for cholesterol. Indeed, the isolation procedure may have increased the cholesterol content of both inner and outer membranes, as the normal solubility of cholesterol in the cell would be decreased by addition of the aqueous solutions required for the isolation procedure.

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