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THE ROLE OF CHOLESTEROL AND CYTOCHROME P-450 IN THE CHOLESTEROL SIDE CHAIN CLEAVAGE REACTION IN ADRENAL CORTEX AND CORPORA LUTEA

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SUMMARY

1. Rat adrenal mitochondria, and rat corpora luteal mitochondria *in vitro* convert endogenous cholesterol into pregnenolone by a cholesterol side chain cleavage mixed function oxidase.

2. These mitochondria contain a relatively small pool of cholesterol so that the decline in the observed rate of pregnenolone production over a few minutes at 37°C is due to substrate depletion.

3. It is possible to maintain the initial rate of pregnenolone formation by the inclusion of cholesterol in the incubation medium added in a small quantity of acetone or cholesterol offered in the form of a lecithin-cholesterol micelle.

4. The cytosol of the rat adrenal cortex and the cytosol of the rat corpora lutea both contain cholesterol ester hydrolases which are activated by a protein kinase in the presence of 3′5′-cyclic AMP and ATP. This activation of the cholesterol ester hydrolase is independent of protein synthesis blockers such as cycloheximide.

5. It is suggested that one method by which the trophic hormones ACTH and LH may activate their appropriate target tissues is by a 3′5′ cyclic AMP dependent activation of a protein kinase resulting in a phosphorylation and activation of the cholesterol esterase.

6. This esterase can attack the cholesterol esters in the lipid droplets bringing about a release of unesterified cholesterol.

7. The cholesterol which is in the cytoplasm moves to the mitochondria by a mechanism which is not yet clear. After the initial rapid uptake by the mitochondria, the cholesterol has to move throughout the cristae to the active site of the cholesterol side chain cleavage enzyme system.

8. The evidence suggests that the translocation or the transportation of the sterol molecule within the mitochondrion may be the rate limiting event in the overall cholesterol side chain cleavage oxygenase reaction. Labile protein(s) may be involved in this event and also in other aspects of the cholesterol side chain cleavage reaction.

9. From studies on intact mitochondria and on the isolated cytochrome P-450 involved in the cholesterol side chain cleavage reaction it seems clear that the micro-environment of the cholesterol (?phospholipid) cytochrome P-450 in the cristae may be a significant factor in the ultimate rate of the cholesterol side chain cleavage reaction.

INTRODUCTION

It has been shown that the conversion of cholesterol to pregnenolone occurs in the mitochondria of steroid hormone producing tissues [1, 2] and is catalysed by a mixed function oxidase involving cytochrome P-450 [3, 4]. Regulation of the rate of production of steroid hormones by tissues such as the adrenal cortex, corpora lutea and testes is often assumed to be through a rate limiting step in the mitochondria of these tissues namely, the cholesterol side-chain cleavage reaction [5]. It is known that oxygen interacts with the side chain of cholesterol and the suggestion has been made that the hydroxylations follow the pattern 20α, −20α, 22 and then by a desmolase event produce pregnenolone and isocaproic aldehyde [6]. Alternative proposals have been put forward that in fact the oxygen interacts with the 20 position of the sterol molecule to produce a hydroperoxide and then by a radical mechanism the side chain is cleaved [8]. There are of course further possibilities including the suggestion that a 20, 22 epoxide or radical could be involved [7]. Whatever the mechanism of this cholesterol side chain cleavage reaction, it appears that the haemoprotein cytochrome P-450 is involved and consequently, oxygen and suitable electron donors are obligatory components of this multi-enzyme reaction. One possible mechanism is shown in Fig. 1.

There is evidence that the stimulation of steroidogenesis in the adrenal cortex and the gonads is mediated by the appropriate trophic hormone and the secondary messenger 3′5′ cyclic AMP [9, 10]. In many events involving 3′5′ cyclic AMP, there appears to be a protein kinase component in this response [11, 12]. The issue is then to establish how this phosphorylation event is related to the control process under study.

In this paper we discuss some studies which have been performed on the control of steroidogenesis in rat adrenal cortex and in rat corpora lutea. This report deals with studies on the events occurring in mitochondria and the exploration of the optimal conditions for the conversion of cholesterol to pregneno-

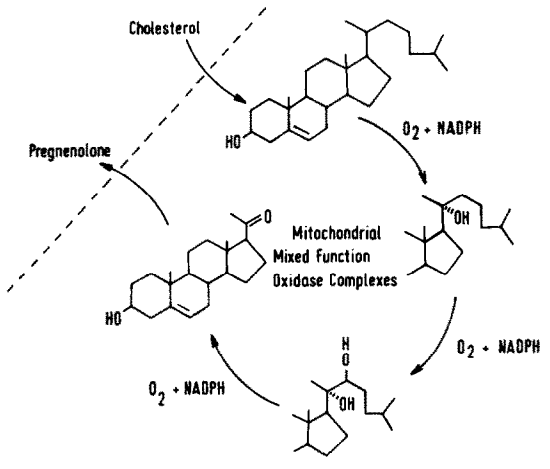

Fig. 1. Suggested scheme for the side chain cleavage of cholesterol occurring in the mitochondria of the adrenal cortex, testes, corpora lutea and placenta.

lone under *in vitro* conditions. The source of cholesterol for this mitochondrial side chain cleavage reaction has been investigated. The mechanisms which may operate in the cytosol for the delivery of this relatively hydrophobic molecule to the cristae of the mitochondrion will be considered as a factor in the overall control of steroidogenesis.

EXPERIMENTAL

Chemicals and radiochemicals

The sources and purity of the chemicals and radiochemicals used in these studies have been stated elsewhere [13–15].

Sterol and steroid assays

Cholesterol and pregnenolone were determined by gas–liquid chromatography and by enzymic-fluorimetric methods as previously published [13].

Adrenals

Adrenal mitochondria were prepared as previously described [13] from male and female rats in the weight range 150–250 g. The adrenals were trimmed and homogenized in 250 mM sucrose. The mitochondria were suspended in 250 mM sucrose to give a final protein concentration of about 3 mg/ml.

Corpora Lutea

Female rats of the Wistar strain were used in these experiments. Luteinized ovaries were obtained from animals using the pretreatment procedure devised by Parlow[16, 17]. Rats 21–24 days old were injected subcutaneously with pregnant mare serum gonadotrophin (Gestyl-Organon-50 I.U.) followed 3 days later by an injection of human chorionic gonadotrophin (pregnyl-Organon-25 I.U.) or a second injection of pregnant mare serum gonadotrophin (50 I.U.). The hormones were dissolved in 0·5 ml of 0·9% saline for injection. The rats were used 5–7 days after the second injection of hormone. The ovarian weight varied from 120–150 mg.

The ovaries were trimmed free of fat and placed in ice-cold 250 mM sucrose. The tissue was homogenized in 5 volumes of 250 mM sucrose/g tissue as described previously [18]. The homogenate was centrifuged at 650 ***g*** for 10 min to remove nuclei, red blood cells and intact cells. The supernatant was centrifuged at 8500 ***g*** for 10 min to give the mitochondrial pellet. This pellet was resuspended in one half of the original homogenate volume of 250 mM sucrose and centrifuged again at 8500 ***g*** for 10 min. This washing procedure was repeated and the final mitochondrial pellet suspended in 250 mM sucrose to give a protein concentration of 6–8 mg/ml.

Enzymic conversion of cholesterol to pregnenolone

Studies on the rate of conversion of cholesterol to pregnenolone in rat adrenal or corpora luteal mitochondrial preparations and studies on the respiration of these mitochondira were conducted in a medium consisting of 250 mM sucrose, 20 mM KCl, 15 mM triethanolamine hydrochloride, 10 mM potassium phosphate, 5 mM magnesium chloride and 0·1 mM EDTA. The buffer solution also contained 0·1% bovine serum albumin and the pH was adjusted to 7·4. The final mitochondrial protein concentration was 1–2 mg/ml. Radioactive cholesterol was added to the mitochondria in acetone.

Respiratory studies on these mitochondrial preparations were performed using a modified Clark electrode in a cell fitted with a pH electrode and a thermistor. It is possible to obtain an estimate of the cholesterol side-chain cleavage reaction in rat adrenal cortical mitochondria or in rat luteal mitochondria by the incubation of these mitochondrial preparations in the appropriate buffer in the presence of oxygen, an electron donor, and radioactive cholesterol [13].

Inhibition of the steroid 3β-hydroxy dehydrogenase

The cholesterol side chain cleavage reaction is complicated in that the product of the reaction, pregnenolone, may undergo oxidation in the presence of the steroid 3β-hydroxy dehydrogenase, present in mitochondria, resulting in the conversion of pregnenolone to progesterone. However, if the dehydrogenase inhibitor cyanoketone is added to the incubation mixture, the only radioactive products which can be detected are the substrate cholesterol and the cholesterol cleavage product, pregnenolone [13]. It has been possible to devise a simple radioactive assay for the cholesterol side chain cleavage activity and this type of assay has been applied to mitochondria from rat adrenal cortex, and rat corpora lutea.

RESULTS

When radioactive cholesterol in acetone is added to mitochondrial preparations from steroidogenic tissues the rate of uptake of this tracer dose of radioac-

Table 1. Uptake of radioactive cholesterol by bovine adrenal cortex mitochondria

Time (min)	1	2	3	5	15	20
4-^{14}C cholesterol taken up by mitochondria as a percentage of the final value	81	90	97	98	99	100

tive cholesterol into the mitochondrial preparations is extremely rapid. Even at temperatures as low as 5°C a mitochondrial preparation adsorbs most of the added cholesterol in 2–3 mins.; this is shown in Table 1. It is for this reason that in certain studies the [4-^{14}C]-cholesterol is pre-incubated with the mitochondria at a low temperature prior to initiating the enzymic oxygenase event.

By the application of standard incubation procedures, it is interesting to compare and contrast the cholesterol side chain cleavage reaction in three tissues obtained from the same species. We have compared the pregnenolone production rate in mitochondria isolated from different steroidogenic tissues in Table 2. It will be noted that in these different mitochondria, the ratio of total cholesterol in the mitochondrion to total cytochrome P-450 varies as does the calculated V_{max} for pregnenolone production.

In the radioactive assay of cholesterol side chain cleavage activity, the mitochondria are isolated, suspended in buffer solution and a tracer quantity of radioactive cholesterol is added. It is assumed that the amount of cholesterol added is insignificant in comparison with the endogenous cholesterol present in the preparation. The radioactive cholesterol is given an adequate period to equilibrate with the endogenous cholesterol. After equilibration is complete the electron donor is added to trigger the reaction. As shown in Table 1, the rate of uptake of cholesterol by rat corpora luteal mitochondria at 4°C from the aqueous environment occurs within a period of about 2 min. This initial uptake event need not be equated with complete equilibration of cholesterol within the various sub-mitochondrial cholesterol pools.

Cholesterol is distributed about all organelles, body fluids, and cell membranes and it is of importance to consider the distribution of the sterol within the cells involved in steroidogenesis. In the rat adrenal cortical cell, we find about 3 nmol cholesterol/mg protein in the cell cytosol, 20 nmol cholesterol/mg protein in the isolated washed mitochondria and about 50 nmol cholesterol/mg protein in the isolated endoplasmic reticulum or microsomal fraction. There is of course a vast reservoir of cholesterol as cholesterol esters in the lipid droplets floating in the cytosol.

In considering the events which occur in association with the addition of cholesterol to mitochondrial preparations it is important to take into account the phospholipid content of these organelles. The phospholipid content of rat adrenal mitochondria exceeds the protein content of these organelles and the analytical data suggest that there may be 3 μmol phospholipid/mg mitochondrial protein [19]. Subfractionation of the phospholipids isolated from rat adrenal mitochondria suggests that the predominant phospholipid is phosphatidyl choline or lecithin with lesser amounts of phosphatidyl ethanolamine or cephalin and then minor quantities of phosphatidyl serine, cardiolipin and some other as yet unidentified polar lipids. Thus if mitochondria from the adrenal cortex are suspended in an aqueous buffer solution and cholesterol in acetone is added to this suspension, the phospholipids undoubtedly play an important part in the uptake and subsequent organisation of this sterol within the membrane structures of the mitochondria. In considering the attack by oxygen, under the influence of the haemoprotein cytochrome P-450, on the cholesterol molecule, the unusual environment of the enzyme and the substrate must be considered. There are many examples of cytochrome P-450 dependent reactions being sensitive to the phospholipid environment [20–22] and in this particular case of the cholesterol side chain cleavage reaction in mitochondria the enormous excess of phospholipids undoubtedly influences the reaction. In these mitochondria there is about 1 nmol cytochrome P-450 per 20 nmol of cholesterol associated with about 3000 nmol of phospholipid per mg mitochondrial protein. Not all the phospholipid is phosphatidyl choline but from the analytical data there may

Table 2. The content of cholesterol and cytochrome P-450 in certain endocrine mitochondria

Species		per mg mitochondrial protein: cholesterol nmol A	cytochrome P-450 nmol B	Ratio $^{A}/_{B}$	V_{max} nmol pregnenolone nmol cytochrome P-450^{-1} min^{-1}
Human	Adrenal cortex		0·8		
	Placenta	50	0·1	500	0·08
Bovine	Adrenal cortex	30	1·0	30	0·25
	Corpora lutea	60	0·2	300	0·35
Rat	Adrenal cortex	20	0·9	22	1
	Corpora lutea	12	0·4	30	1
	Testes	70	0·01	7000	2
Pig	Testes	20	0·5	40	1

be at least 1000 nmol of phosphatidyl choline/mg of mitochondrial protein.

Although progress has been made in the resolution of the cytochrome P-450 from adrenal cortex and the other tissues [23–29] we do not have a pure sample of this haemoprotein from steroid hormone producing tissues. The inability to resolve the cytochrome P-450s present in the mitochondria from steroidogenic tissues has proved a considerable handicap to the analysis of many of the enzymic events of interest to endocrinologists. In mitochondria from tissues in which steroidogenesis is stimulated by the appropriate trophic hormone there is evidence for an increased production of pregnenolone from cholesterol.

With regard to the cholesterol side chain cleavage reaction being a rate-limiting event in steroidogenesis, one problem which arises in the interpretation of the kinetics of pregnenolone production in these mitochondria is the rate at which the mitochondrial cholesterol is depleted. It will be noted from Table 2 that the reserves of cholesterol in mitochondria from different steroid hormone producing tissues vary considerably. In the case of mitochondria isolated from the bovine adrenal cortex, it is possible to add a tracer amount of cholesterol to these mitochondria and obtain a linear production of pregnenolone over a period of 10–20 min [4]. This is because the radioactive tracer is discharged into a reactive pool of cholesterol in the mitochondrion such that subsequent enzymic events associated with the conversion of cholesterol to pregnenolone occur at a rate which does not materially affect the magnitude of the pool of cholesterol in the mitochondria. On the other hand, in the case of mitochondria isolated from the rat adrenal cortex or mitochondria isolated from rat corpora lutea, the cholesterol content of these organelles in the presence of O_2 and an electron donor at 37°C would be markedly depleted in a few minutes.

In the mitochondrion there is only a limited amount of cytochrome P-450 and if we assume that there will be a minimum of 1 molecule of substrate associated with 1 molecule of cytochrome P-450 at the appropriate reaction centre, there must be some mechanism in the mitochondrion for the movement of this non-polar material-cholesterol through the protein and phospholipid membrane structure of the cristae to the enzyme reaction centre. Spectral studies have been employed to attempt to quantitate the interaction of cholesterol with a specific cytochrome P-450. The data suggest that trophic hormone stimulation of the adrenal cortex results in an increase in the cholesterol complex of cytochrome P-450 [13, 14]. It is well known that cholesterol and phospholipids also interact to form micelles [30, 31] and that the polar end of the sterol molecule is often associated in these structures with the polar end of the phospholipid molecule [32].

Pre-treatment of rats with protein synthesis blockers, such as cycloheximide, results in a decrease in the cholesterol side chain cleavage activity in mitochondria from steroidogenic tissues such as adrenal cortex, corpora lutea and testes. Figure 2 shows the cholesterol side chain cleavage activity in mitochondria isolated from rat corpora lutea. Groups of animals were pretreated with cycloheximide for varying periods before killing. The corpora lutea were removed, homogenized and processed in an identical way and the cholesterol side chain cleavage activity of these mitochondria measured in the usual way. By operating at 29°C and by using a short observation period, an approximation can be obtained to the maximal rate of cholesterol side chain cleavage activity. It will be noted that the rate of this reaction diminishes rapidly after the administration of cycloheximide and this is an agreement with observations previously made on the rat adrenal cortex system in which the prior administration of cycloheximide resulted in a diminution of the observed cholesterol side chain cleavage activity [13]. The administration of cycloheximide to these animals does not produce any significant change in the total mitochondrial cytochrome P-450 content expressed per mg of mitochondrial protein. It has been suggested that there must be some rapidly turning over "labile protein" which is involved in the overall kinetics of the cholesterol to pregnenolone reaction and this rapidly turning over protein must be of extramitochondrial origin because as shown in Table 3 the administration of chloramphenicol to these animals has no effect on the cholesterol side chain cleavage activity. On the basis of the usual assumption that chloramphenicol is an inhibitor of mitochondrial protein synthesis, this would imply that the labile protein involved in this mitochondrial cholesterol side chain cleavage event must be of extra-mitochondrial origin.

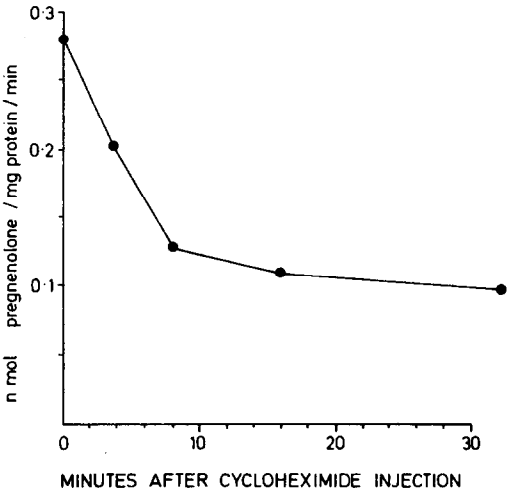

Fig. 2. Groups of immature female rats were injected with cycloheximide at various intervals before killing the animals. The cholesterol side chain cleavage reaction was measured on the mitochondria isolated from corpora lutea.

One possible explanation of these results is that in the endoplasmic reticulum of this tissue there is an active synthesis of a protein of peptide which may be involved in the translocation of the cholesterol

Table 3. The effect of chloramphenicol injection on cholesterol side chain cleavage activity in rat luteal mitochondria

Treatment		% conversion 0 min	5 min	10 min	15 min
Saline	(a)	0	17·5	24·4	29·0
	(b)	0	16·0	22·3	26·5
Chloramphenicol	(a)	0	18·0	26·2	29·5
	(b)	0	15·0	22·5	27·0

The table shows the percentage conversion of [4-^{14}C]-cholesterol into [4-^{14}C]-pregnenolone in luteal mitochondria from rats injected with saline or chloramphenicol (10 mg i.m.).

(a) Injections 3 hours before killing.

(b) Injections 3 and 10 hours before killing.

Cholesterol side chain cleavage assays were carried out as described in methods. Total mitochondrial cholesterol was unchanged by chloramphenicol treatment.

molecule through the apolar core of the mitochondrial membrane to the cytochrome P-450 reaction centre for pregnenolone formation.

Since there is only a limited amount of cholesterol in these mitochondria and the sterol is apparently in more than one pool the kinetics of the side chain cleavage reaction utilising [^{14}C]-labelled cholesterol require careful interpretation. Alterations in the pool sizes of cholesterol in the mitochondrion could greatly influence the apparent outcome of the conversion of the [^{14}C]-cholesterol into [^{14}C]-pregnenolone.

In the rat adrenal cortex, the reservoir of cholesterol appears to be in the cholesterol ester rich lipid droplets in the cytosol of the cells. The immediate precursor of the cholesterol side chain cleavage reaction appears to be non-esterified (free) cholesterol and not esterified cholesterol. Consequently, if the reservoir of cholesterol in the cells as ester cholesterol is to be utilised in the production of pregnenolone by the mitochondria the cholesterol esters must be hydrolysed to make the cholesterol available to the mitochondrion. We have studied the cytoplasmic cholesterol ester hydrolase and attempted to establish some of the characteristics of this particular sterol esterase [33].

It has been shown that 3′5′ cyclic AMP and ATP are involved in the activation of this supernatant cholesterol ester hydrolase [15]. The cholesterol ester hydrolase activity and a protein kinase activity in rat adrenal cortex 105,000 *g* supernatant fraction are significantly higher in animals subjected to stressful situations [33]. Also the injection of cycloheximide did not prevent the stress induced enhancement of the activities of either enzyme. The results of these studies are shown in Figs. 3 and 4. On the basis of these observations we conclude that in the cytosol of the cells of the adrenal cortex [33] and corpora lutea [34] in the rat and in the bovine adrenal cortex [15] there is present a cholesterol ester hydrolase which appears to be in a dephosphorylated inactive form. This inactive cholesterol ester hydrolase in the presence of ATP and an active protein kinase is phosphorylated to an active form of cholesterol ester hydrolase con-

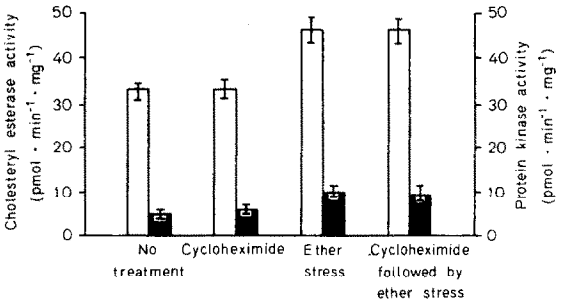

Fig. 3. The effect of stress and cycloheximide injection on the activity of rat adrenal cholesteryl esterase and protein kinase. □ esterase, ■ kinase.

taining a phosphate grouping. It seems likely that in the bovine adrenal cortex and in the rat adrenal cortex the protein kinase activity is reduced in the basal, quiescent or resting state by the presence of an inhibitory protein which has also the characteristics of a 3′5′ cyclic AMP binding protein [11]. Accordingly, when the appropriate trophic signal is administered to the plasma membrane of the cell, resulting in activation of the adenyl cyclase system, the rise in the intracellular concentration of 3′5′ cyclic AMP results in this cyclic nucleotide binding to the appropriate protein and consequently releasing the protein kinase from this inhibitor. These reactions result in a marked activation of the protein kinase with a subsequent phosphorylation of the inactive cholesterol ester hydrolase to the active form of this enzyme. Our observations so far suggest that in the rat adrenal cortex, in the bovine adrenal cortex, and in rat corpora lutea, the sequence of events outlined above appears to operate resulting in an increase of two fold in the

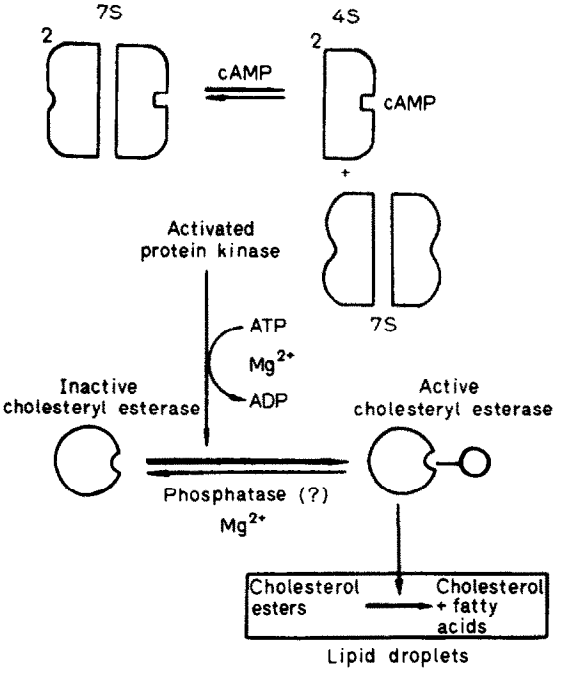

Fig. 4. Possible mechanism for the activation of cholesterol ester hydrolase enzyme system in bovine adrenal cortex or in rat adrenal cortex.

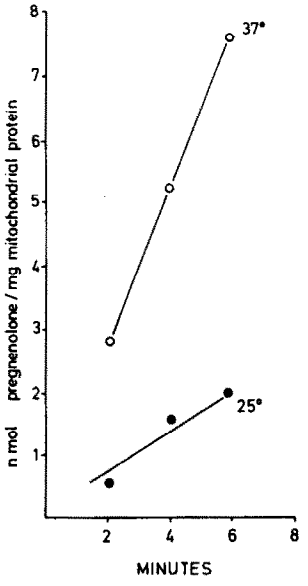

Fig. 5. The pregnenolone production rate in rat adrenal cortical mitochondria, in buffer solution as described in the text, in the presence of 10 mM isocitrate, 1 mM NADP and 0·1% BSA.

cholesterol ester hydrolase enzyme present in the cytosol of the cells [33].

This is shown diagrammatically in Fig. 4. In assays of cholesterol ester hydrolase activity, we use [4-^{14}C]-cholesterol oleate as the substrate because in lipid droplets in these cells, the predominant cholesterol ester tends to be cholesterol oleate [35]. In the cell the lipid droplets are present as spherical particles of diameter one micron or less. Lipid droplets contain varying amounts of phospholipid and in addition the droplets contain proteins.

Lipid droplets have been isolated from the adrenal cortex of rats and the corpora lutea from female rats. These lipid droplets (lipoproteins) have been washed by differential centrifugation and delipidated by methods used previously on other lipoprotein problems [36, 37]. The proteins isolated have been chromatographed by gel chromatography and electrophoresis, and there is evidence that these lipoproteins contain various proteins which appear to be disaggregated in SDS gel electrophoresis. These proteins present in the lipid droplets may have a structural role within the lipid droplet, to achieve effective interaction of the phospholipids and cholesterol esters or they may have a different role in the subsequent translocation of the sterol from the lipid droplets to the mitochondrion or to the plasma membrane of the cell.

Using methods previously described [13] a study has been made of some of the factors which influence the cholesterol side chain cleavage reaction in mitochondria obtained from rat adrenal glands and rat corpora lutea. Figure 5 shows the rate of production of pregnenolone expressed per mg mitochondrial protein in rat adrenal cortex mitochondria where the experiment has been performed at two temperatures. It will be noted that at 37°C about 8 nmol of pregnenolone per mg mitochondrial protein have been generated in 6 mins. This rate of pregnenolone production is such that a sizeable fraction of the total cholesterol present in the mitochondria has been metabolised at 37°C. Figure 5 also shows the rate of pregnenolone production at 25°C.

As a result of these experiments attempts were made to study the respiration of adrenal cortical mitochondria under certain specified conditions. As shown in Fig. 6 when adrenal mitochondria were suspended in the appropriate incubation mixture and isocitrate was added, the respiration rate increased. These mitochondria showed respiratory control. The addition of cyanide to the mitochondria blocks the respiratory chain without affecting the hydroxylating chain.

In Figure 6 these mitochondria have been respiring at 37°C for 12 min. It could be predicted from the previous results that much of the endogenous cholesterol in the mitochondria will have been oxidised to

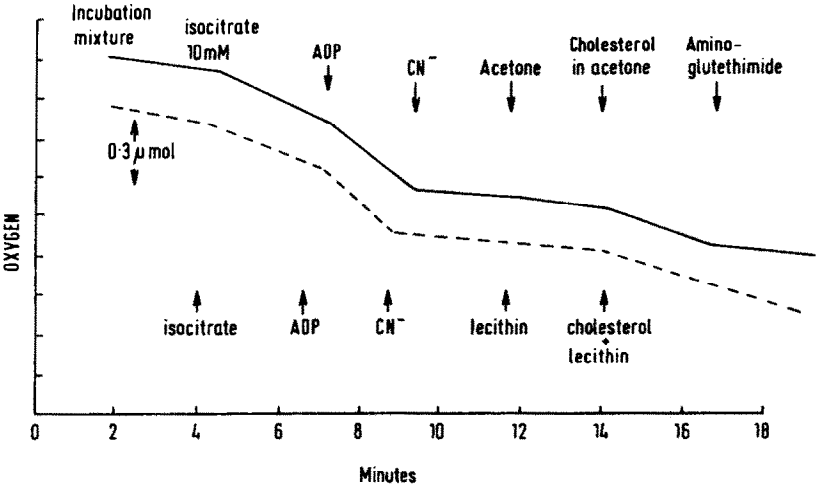

Fig. 6. Respiration of rat adrenal mitochondria in buffer solution as in Fig. 5, the experiment was conducted at 37°C. Note the increase in the rate of respiration upon the addition of cholesterol.

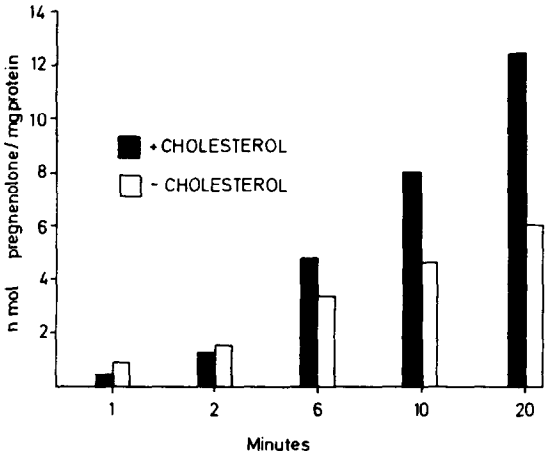

Fig. 7. Pregnenolone production in rat corpora luteal mitochondria in buffer solution in the presence of an electron donor. The solid histograms are incubations in the presence of cholesterol while the open histograms are results obtained in the absence of cholesterol [18].

pregnenolone. To test this acetone is added to the preparation and as shown in Figure 6 there is no change in the rate of respiration but when cholesterol in acetone is added there is an increase in the respiratory rate. That this increase in respiration is due to the cholesterol side chain cleavage reaction is shown by the inhibition of respiration achieved by 1 μM aminoglutethimide.

It could be argued that it is unphysiological to add cholesterol in acetone to these mitochondrial particles. Accordingly as shown in Fig. 6 the experiment was repeated with the variant that after blocking respiration with cyanide, lecithin was added producing no change in the respiratory rate but when lecithin-cholesterol micelles were added, again the rate of respiration increased. These observations suggest that during incubation in the presence of electron donors, rat adrenal mitochondria are quickly depleted of their available cholesterol. The addition of cholesterol in acetone or as lecithin-cholesterol micelles restores the respiratory rate and restores the rate of production of pregnenolone from cholesterol.

In order to test this hypothesis, mitochondria from rat corpora lutea, which behave in these respiratory experiments in a fashion comparable to mitochondria from the adrenal cortex, were incubated with an electron donor for periods from 1 to 20 min. Similar mitochondria were incubated with excess cholesterol present in the incubation medium. The results of the experiment are shown in Fig. 7 where it can be seen that the pregnenolone production by rat corpora luteal mitochondria is greatly increased in the presence of cholesterol. These observations taken collectively imply that rat adrenal and rat corpora luteal mitochondrial particles are quickly depleted of cholesterol; consequently pregnenolone production is a function of the supply of cholesterol to the mitochondria.

It is well known that all mitochondria and these mitochondria from steroid hormone producing tissues in particular are very rich in phospholipids. From evidence collected in various model systems especially from lecithin-cholesterol liposome models [38, 39] there is a suggestion that cholesterol and lecithin interact in a specific fashion to produce in these artificial membranes a situation similar to that which may prevail in the inner cristae of mitochondria.

If cholesterol and phospholipids do interact in mitochondria in this way it is likely that the interaction will be dependent upon the ratio of cholesterol to phospholipid in the membrane and also dependent upon the temperature. Since these mitochondria rapidly metabolise cholesterol we decided to test the integrity of the mitochondria, through assessment of the respiratory control of the mitochondria at 29°C and then block the respiration by cyanide at this stage. The temperature was raised to 37°C followed by the addition of cholesterol to the mitochondria

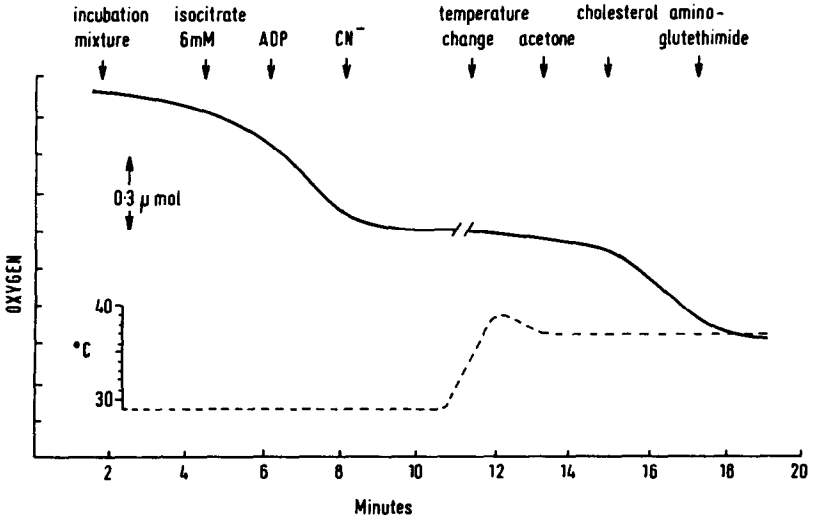

Fig. 8. Respiration of rat adrenal mitochondria at 29°C showing respiratory control. The change in temperature showed that the addition of cholesterol in acetone produced a rapid rise in the respiratory rate.

at this higher temperature. Such an experiment is shown in Fig. 8. The advantage of this approach is that it allowed assessment of the integrity of the mitochondria at a reduced temperature where the rate of cholesterol depletion would be reduced followed by a rapid transition to the physiological temperature.

It is established that calcium ions stimulate mitochondrial hydroxylation reactions *in vitro* [40–43] and it has been suggested that some of these effects could be due to changes in the permeability of the mitochondria induced by these divalent ions. It is well known that calcium ions interact with the polar heads of phospholipids in micelles and it has been shown that the permeability of cholesterol-phospholipid liposomes is affected by calcium ions [44]. It has been shown that calcium ions influence the cholesterol side chain cleavage reaction in adrenal mitochondria *in vitro* [41, 42] and it has been deduced that this effect of calcium ions is due to an increased formation of a cytochrome P-450 cholesterol complex attributed to the formation of a high spin complex [43].

The effect of calcium ions on the cholesterol side chain cleavage reaction revealed that these ions at concentrations as low as 0·1 mM produced a marked increase in the pregnenolone production rate [41, 42]. The effect of calcium ions on the respiration of rat adrenal mitochondria was studied as described previously. As shown in Fig. 9 when calcium ions at 0·1 mM concentration was applied to these mitochondrial preparations there was an immediate increase in the respiration rate which could be confirmed as due to an elevated conversion of cholesterol to pregnenolone. This effect was transient and upon the addition of cholesterol the rate of respiration was markedly elevated. It would be possible to interpret these results in the light of the other observations as due to the displacement of substrate cholesterol from some phospholipid binding site to another site—the cytochrome P-450 reaction centre [43]. Furthermore 0·1 mM calcium ions also alter the permeability of

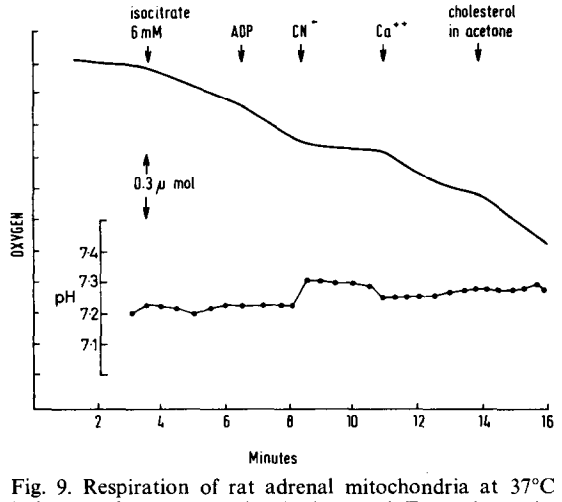

Fig. 9. Respiration of rat adrenal mitochondria at 37°C in buffer solution as previously described. Trace shows the increase in respiration as a result of the addition of 0·1 mM calcium followed by a further increase upon the addition of cholesterol in acetone.

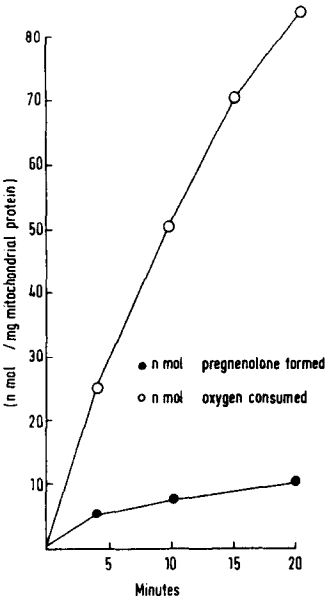

Fig. 10. Oxygen consumption and pregnenolone production rate of rat adrenal mitochondria respiring in buffer (as previously described) at 37°C in the presence of cyanide.

the mitochondria and so facilitate ingress of sterol to the cytochrome P-450 oxygenase. It will be noted in Fig. 9 that these alterations in the rate of respiration of the mitochondria due to low concentrations of calcium ions is accomplished without substantial change in the pH of the environment so that the change in the cholesterol side chain cleavage reaction cannot be due to alterations in the cholesterol-cytochrome P-450 spin state as a consequence of an alteration in the hydrogen ion concentration of the medium [14].

Employing these *in vitro* respiration studies on rat adrenal cortical mitochondria coupled to pregnenolone assays it has been possible to explore the stoichiometry of the cholesterol side chain cleavage reaction in these organelles. As shown in Fig. 10 the oxygen uptake of rat adrenal cortical mitochondria exceeds the pregnenolone production rate by a factor of about nine. If the cholesterol side chain cleavage event followed the reaction sequence shown in Figure 1 the stoichiometry would be three molecules oxygen consumed for each cholesterol molecule converted to pregnenolone. This three to one molar ratio for the cholesterol side chain cleavage mixed function oxidase has been confirmed in a cytochrome P-450 preparation from bovine adrenal cortex [45]. It appears therefore that in the intact mitochondrial preparation in which the respiratory chain is inhibited by cyanide that there must be other hydroxylation events or peroxidation events occurring or there must be certain "non-productive" cytochrome P-450 cycles which result in the consumption of oxygen without the production of a hydroxylated intermediate—other than perhaps H_2O_2. This situation would be comparable to the uncoupling of hydroxylation events seen in

liver microsomal preparations under certain circumstances [47].

During maximum pregnenolone production in mitochondria from rat corpora lutea in the presence of exogenous cholesterol, EPR spectroscopy has shown that the iron sulphur protein in mitochondria is about 70% reduced. By contrast in the absence of exogenous cholesterol, where the cholesterol content of these mitochondria is declining, the iron sulphur protein is about 90% reduced. These observations support the concept that the supply of cholesterol to these mitochondria may influence the electron flux through the hydroxylation chain [48].

DISCUSSION

The work reported in this paper on cholesterol metabolism in mitochondria is in general agreement with recent studies of several other laboratories [42, 46, 50] in that one of the key events in the adrenal cortex, under stimulation with ACTH, or in the corpus luteum under stimulation with LH, is the transportation of cholesterol to the cytochrome P-450 reaction centre located in the inner cristae of the mitochondria. While the overall production of pregnenolone remains the rate limiting event in steroidogenesis in these tissues, the translocation, or transportation component in intact mitochondria appears to impart the actual slow step to this process. Once the cholesterol has been delivered to the cytochrome P-450 enzyme concerned with the side chain cleavage of this sterol the subsequent oxygenase reaction is rapid.

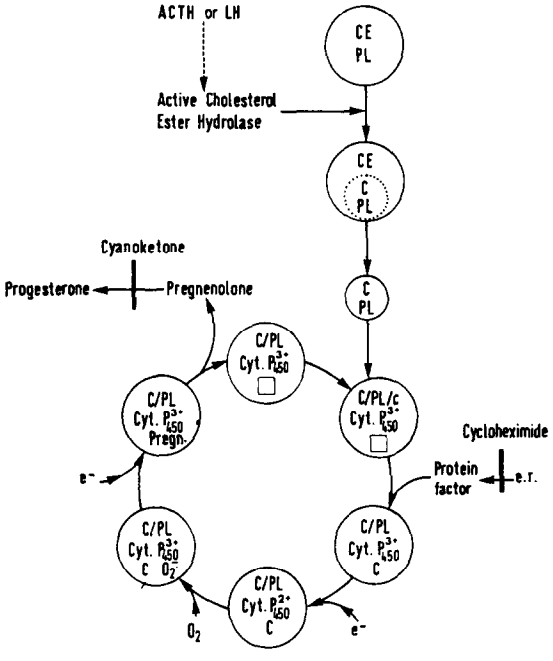

Fig. 11. Diagrammatic representation of the events which may operate in the adrenal cortex and in corpora lutea. It is suggested that the cholesterol esterase is activated by a protein kinase reaction followed by the release of cholesterol to the cytosol. The cholesterol is transferred to the mitochondria where it is taken up into a reserve of available cholesterol. This cholesterol can then move through the cristae of the mitochondrion to the cytochrome P-450 reaction centre. The cholesterol movement may be controlled by a protein or peptide of extramitochondrial origin. The cytochrome P-450-cholesterol complex may be a high spin species which is then subjected to the usual events resulting finally in the production of pregnenolone and a change in the spin state of the cytochrome P-450.

In these mitochondria, the available cholesterol is quite limited (Table 2) and hence there must be a mechanism for the supply of sterol to the oxygenase system as and when required. Some of the results reviewed in this paper show that there is present in the cytosol of the adrenal cortex and corpora lutea a cholesterol ester hydrolase whose activity may be modified under the influence of 3′5′ cyclic AMP and a protein kinase. This hydrolase is capable of attacking the cholesterol ester stores in the lipid droplet storage granules in the cytoplasm to release unesterified or free cholesterol. The lipid droplets are also rich in phospholipids and it is possible that cholesterol and the phospholipids could form micelles of a liposome type for transportation of the sterol to the mitochondria, as shown in Fig. 11.

The mitochondria of these steroidogenic tissues have been shown to be capable of utilising for pregnenolone formation, exogenous cholesterol whether supplied in an acetone solution or in phospholipid micelles [18, 46]. The interaction of a relatively small amount of cholesterol with an excess of phospholipids in the cristae of the native mitochondria is obviously an area for exploration in the future. The cholesterol side chain cleavage reaction occurring in this essentially hydrophobic environment presents a challenge to enzymologists. The substrate cholesterol may be delivered to the mitochondrial cristae in the form of a micelle, and presumably this micelle may fuse with the micelle like, or liposome like, cristae and achieve a translocation of the cholesterol contents to the cristae. It is known that even in artificial liposomes of cholesterol and phospholipids the structure is not uniform and there are areas of solidity and areas of fluidity in the membrane [30]. As the ratio of cholesterol molecules to cytochrome P-450 molecules in mitochondria is quite low the problem is to discover how the cholesterol molecules get to the cytochrome P-450 enzyme involved in the cholesterol side chain cleavage reaction. There is therefore a requirement for the cholesterol molecule to be removed through the hydrophobic core of the cristae. On the basis of other evidence on the interaction of substrates with cytochrome P-450 it is deduced that the cholesterol-cytochrome P-450 ligand forms a high spin complex. Acetone extracted bovine adrenal mitochondria which have been depleted of cholesterol and phospholipids do in fact produce optical and EPR spectral evidence of the formation of a cytochrome P-450 high spin complex on treatment with lecithin-cholesterol micelles [49]. It is interesting that intact mitochondria which have been depleted of cholesterol by the addition of an electron donor in the presence of oxygen, followed by gentle washing, do not produce a type I characteristic spectrum when treated with choles-

terol. Presumably there is some barrier to the access of the substrate to the haemoprotein in these intact cholesterol depleted mitochondria.

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