THE CONTROL OF STEROID METABOLISM IN THE RAT CORPUS LUTEUM

by

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This thesis was composed by myself and the results described therein are the product of my own work.

ABSTRACT OF THESIS

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(a) Methods for the investigation of cholesterol side chain cleavage activity in rat luteal mitochondria are described. The activity can be measured by the total amount of pregnenolone produced in incubations of luteal mitochondria or by the appearance of [4 - ^{14}C] pregnenolone from [4 - ^{14}C] cholesterol, which has been preincubated with the mitochondria for ten minutes.

(b) The general properties of the luteal mitochondrial cholesterol side chain cleavage system are described. The enzymic activity is supported by Krebs Cycle intermediates and NADPH in the presence of calcium ions. Alterations in the composition of the mitochondrial incubation medium have been shown to have a large effect on cholesterol side chain cleavage activity.

(c) The luteal mitochondria were shown to rapidly metabolise their endogeneous cholesterol in the presence of reducing equivalents. Cholesterol, added to the mitochondria in an acetone solution could be converted to pregnenolone.

(d) The cytochrome P450 of luteal mitochondria was found to bind pregnenolone to give an inverted type I difference spectrum. A similar spectrum was induced by the addition of malate to the luteal

(e) An iron sulphur protein (ovarian ferredoxin) was found to be almost fully reduced during cholesterol side chain cleavage in luteal mitochondria. It is concluded that supply of electrons to cytochrome P450 is not rate limiting in luteal steroidogenesis.

(f) Treatment of rats with LH was found to increase the activity of the luteal mitochondrial cholesterol side chain cleavage enzymes. This increase in activity could only be observed on the provision of cholesterol to the enzymes.

(g) Treatment of rats with cycloheximide prevented LH induced changes in luteal mitochondrial cholesterol metabolism. These results are discussed in terms of a rapidly turning over protein which influences the binding of cholesterol to cytochrome P450 and thus the rate of steroidogenesis in the corpus luteum.

(h) LH treatment of rats is also shown to cause the release of cholesterol from cholesterol esters, stored in the lipid droplets of the luteal cell cytoplasm. This cholesterol may then be used for steroidogenesis.

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ABBREVIATIONS

The following abbreviations will be used throughout the text:-

ACTH - Adrenocorticotrophic hormone

ADP - Adenosine 5'- diphosphate

ATP - Adenosine 5'- triphosphate

BSA - Bovine serum albumin

Cholesterol - 5 cholesten - 3 β - ol

Cyanoketone - 2 α-cyano - 4,4 - 17 αΔ- trimethyl - 17 β -hydroxy - 5-androsten - 3 - one

Cyclic AMP - Cyclic adenosine 3'5' - monophosphate

" CMP - " cytidine " "

" GMP - " guanosine " "

" IMP - " inosine " "

" UMP - " uridine " "

Dexamethasone - 1,4,Pregnandien-9-fluoro-16 α -methyl-11 β ,17 α ,21-triol- ℨ ,20-dione

Dihydrotestosterone - 5 α Androstan - 17 β -ol - 3 - one

EDTA - Ethylenediamine tetraacetic acid

EGTA - Ethyleneglycol - bis (β -aminoethyl ether)-N,N'- tetra-acetic acid

EPR - Electron paramagnetic resonance

FSH - Follicle stimulating hormone

GLC - Gas liquid chromatography

G-1-P - D Glucose - 1 - phosphate

G-6-P - " " - 6 - "

G-6-PDH - " " " " dehydrogenase

HCG - Human chorionic gonadotrophin

LH - Luteotrophic hormone, Luteinizing hormone

NAD(H) - Nicotinamide adenine dinucleotide (reduced)

NADP(H) - " " " phosphate (reduced)

pregnenolone - 3 β - Hydroxypregn - 5en - 20 - one

progesterone - Pregn - 4ene - 3, 20 - dione

Enzymes

Cholesterol esterase or sterol ester hydrolase (EC.3.1.1.13).

Protein kinase or ATP protein phosphotransferase, (EC.2.7.1.37).

Steroid-3 β -ol dehydrogenase or 3 β hydroxy.steroid; NAD(P) oxido-reductase, (EC.1.1.1.51).

Steroid-11$_4$β-hydroxylase (EC.1.14.1.6).
Steroid Δ^4 - Δ^5- isomerase or 3.ketosteroid Δ^4 - Δ^5isomerase,(EC.5.3.3.1.).

SUMMARY

A brief summary of the work described in this thesis is given below:-

(a) Methods for the investigation of cholesterol side chain cleavage activity in rat luteal mitochondria are described. The activity can be measured by the total amount of pregnenolone produced in incubations of luteal mitochondria or by the appearance of /4 -^{14}C/ pregnenolone from /4 -^{14}C/cholesterol, which has been preincubated with the mitochondria for ten minutes.

(b) The general properties of the luteal mitochondrial cholesterol side chain cleavage system are described. The enzymic activity is supported by Krebs Cycle intermediates and NADPH in the presence of calcium ions. Alterations in the composition of the mitochondrial incubation medium have been shown to have a large effect on cholesterol side chain cleavage activity.

(c) The luteal mitochondria were shown to rapidly metabolise their endogenous cholesterol in the presence of reducing equivalents. Cholesterol, added to the mitochondria in an acetone solution could be converted to pregnenolone.

(d) The cytochrome P450 of luteal mitochondria was found to bind pregnenolone to give an inverted type I difference spectrum. A similar spectrum was induced by the addition of malate to the luteal mitochondria. The luteal mitochondrial cytochrome P450 appeared to have similar spectral properties to the cholesterol side chain cleavage cytochrome P450 of adrenal cortex mitochondria.

(e) An iron sulphur protein (ovarian ferredoxin) was found to

be almost fully reduced during cholesterol side chain cleavage in luteal mitochondria. It is concluded that supply of electrons to cytochrome P450 is not rate limiting in luteal steroidogenesis.

(f) Treatment of rats with LH was found to increase the activity of the luteal mitochondrial cholesterol side chain cleavage enzymes. This increase in activity could only be observed on the provision of cholesterol to the enzymes.

(g) Treatment of rats with cycloheximide prevented LH induced changes in luteal mitochondrial cholesterol metabolism. These results are discussed in terms of a rapidly turning over protein which influences the binding of cholesterol to cytochrome P450 and thus the rate of steroidogenesis in the corpus luteum.

(h) LH treatment of rats is also shown to cause the release of cholesterol from cholesterol esters, stored in the lipid droplets of the luteal cell cytoplasm. This cholesterol may then be used for steroidogenesis.

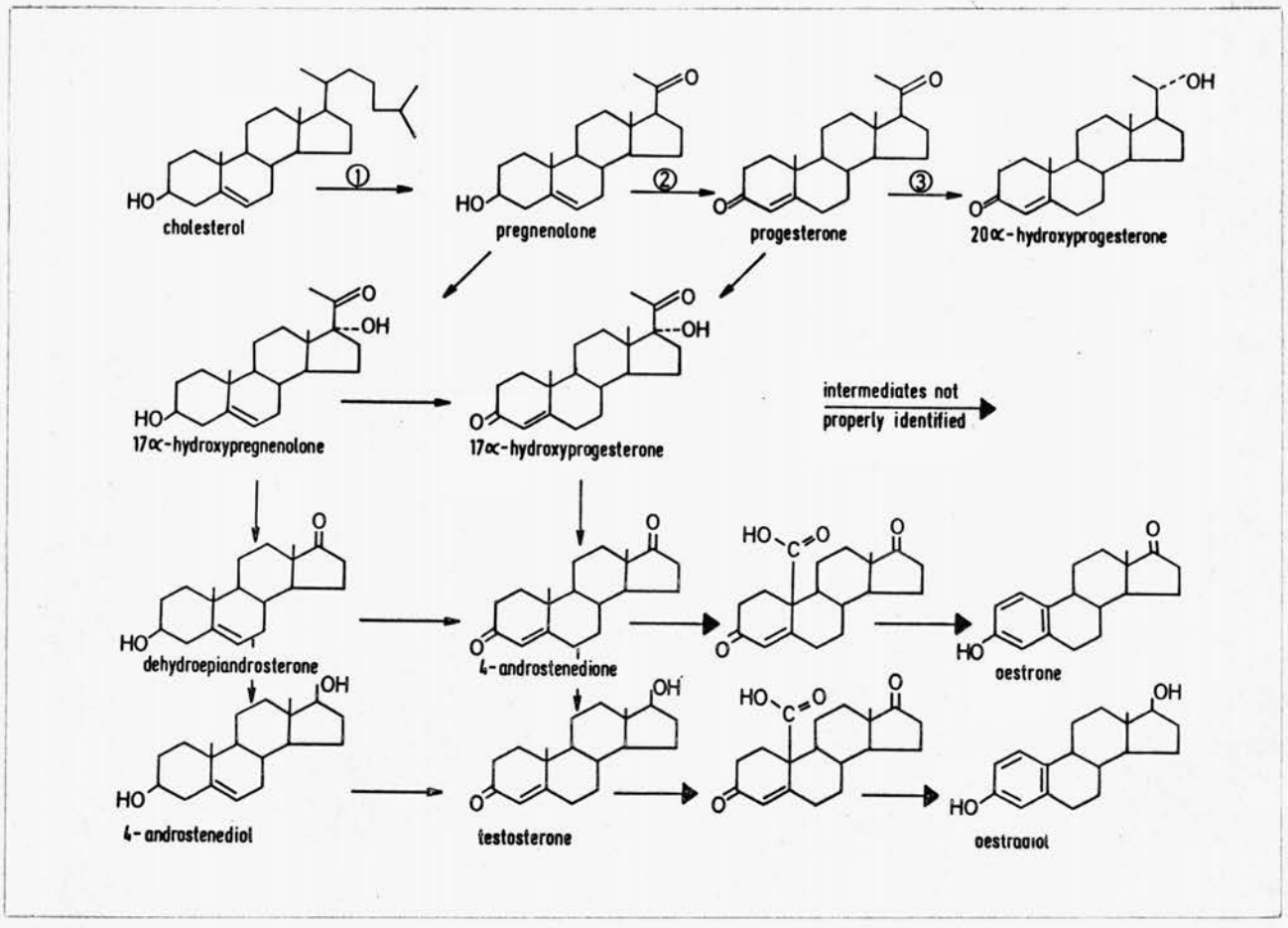


Figure 1.1. The pathways of steroid biosynthesis in gonadal tissues (After Savard (1973)). Steps 1 ⟶ 3 are those which occur in rat luteal tissue. They are catalysed by (1) cholesterol side chain cleavage cytochrome P450, (2) a 3 β-hydroxysteroid dehydrogenase and (3) a 20 α-hydroxysteroid dehydrogenase.

CHAPTER 1

GENERAL INTRODUCTION

Steroidogenesis in Gonadal Tissues

Cholesterol is the most abundant sterol in animal tissues. One of its many functions is as an intermediate in the formation of steroid hormones in certain endocrine tissues of the body (see Hayano et al (1956); Sulimovici (1968)).

The enzyme systems involved in steroidogenesis appear to be fairly similar in ovarian, testicular, adrenal cortical and placental tissue. The corpus luteum of the ovary is involved in the formation of progesterone which is needed for the maintenance of pregnancy and other functions in the animal. Progesterone is the major product of steroidogenesis in the corpus luteum in most mammals although the human corpus luteum can also produce oestrogens and those of the rabbit, sow and sheep can produce androgens (review Savard (1973)).

The pathways of biosynthesis of gonadal steroid hormones from cholesterol are shown in figure 1.1 (adapted from Savard (1973)). Cholesterol is known to be an obligatory intermediate in the biosynthesis of steroids (Krum et al (1964); Flint and Armstrong (1972)). It is converted to pregnenolone by the cholesterol side chain cleavage enzymes in adrenal cortex, testis, corpus luteum and placenta (review Sulimovici and Boyd (1969b)). Pregnenolone is also an important intermediate of steroidogenesis in the adrenal cortex and gonadal tissues (Hayano et al (1956); Savard et al (1965); Nissim and Robson (1952); Pearlman et al (1954); Tamaoki et al (1969)). Figure 1.1 shows the accepted route for the conversion of pregnenolone to C_{21}

progestagens, C_{19} androgens and C_{18} oestrogens in gonadal tissues (reviewed by Sulimovici (1968); Savard (1973)).

In the rat the corpus luteum produces progesterone with smaller amounts of pregnenolone and 20 ∝ -hydroxyprogesterone (Huang and Pearlman (1962)). 20 ∝ -Hydroxyprogesterone is less biologically active than progesterone in the rat (Zander et al (1958)). Production of 20 ∝ -hydroxyprogesterone in relation to progesterone rises when the corpus luteum begins to age in the rat (Hashimoto et al (1968)).

Cholesterol Side-Chain Cleavage

Cholesterol side chain cleavage is a step in steroidogenesis which is common to adrenal and gonadal tissues (step 1, figure 1.1). It is thought to be the rate limiting step in steroidogenesis and its activity may be influenced by trophic hormones (Stone and Hechter (1954); Hall and Young (1968); Channing and Villee (1966)). Cholesterol is converted by the enzyme complex to pregnenolone and a six carbon fragment isolated as either isocaproaldehyde or isocaproic acid. The cholesterol side chain cleavage reaction also requires oxygen and NADPH for activity (Halkerston et al (1961)). This type of reaction has been termed a mixed function oxidase by Mason (1957). The stoichiometry of mixed function oxidases is $NADPH + H^+ + RH + O_2 \longrightarrow NADP^+ + ROH + H_2O$, where RH is the substrate to be hydroxylated. The mechanism of the conversion of cholesterol to pregnenolone is the subject of some controversy. Originally it was postulated that cholesterol was hydroxylated to give 20 ∝ -hydroxycholesterol. The 20 ∝ -hydroxycholesterol was then hydroxylated at the 22 position

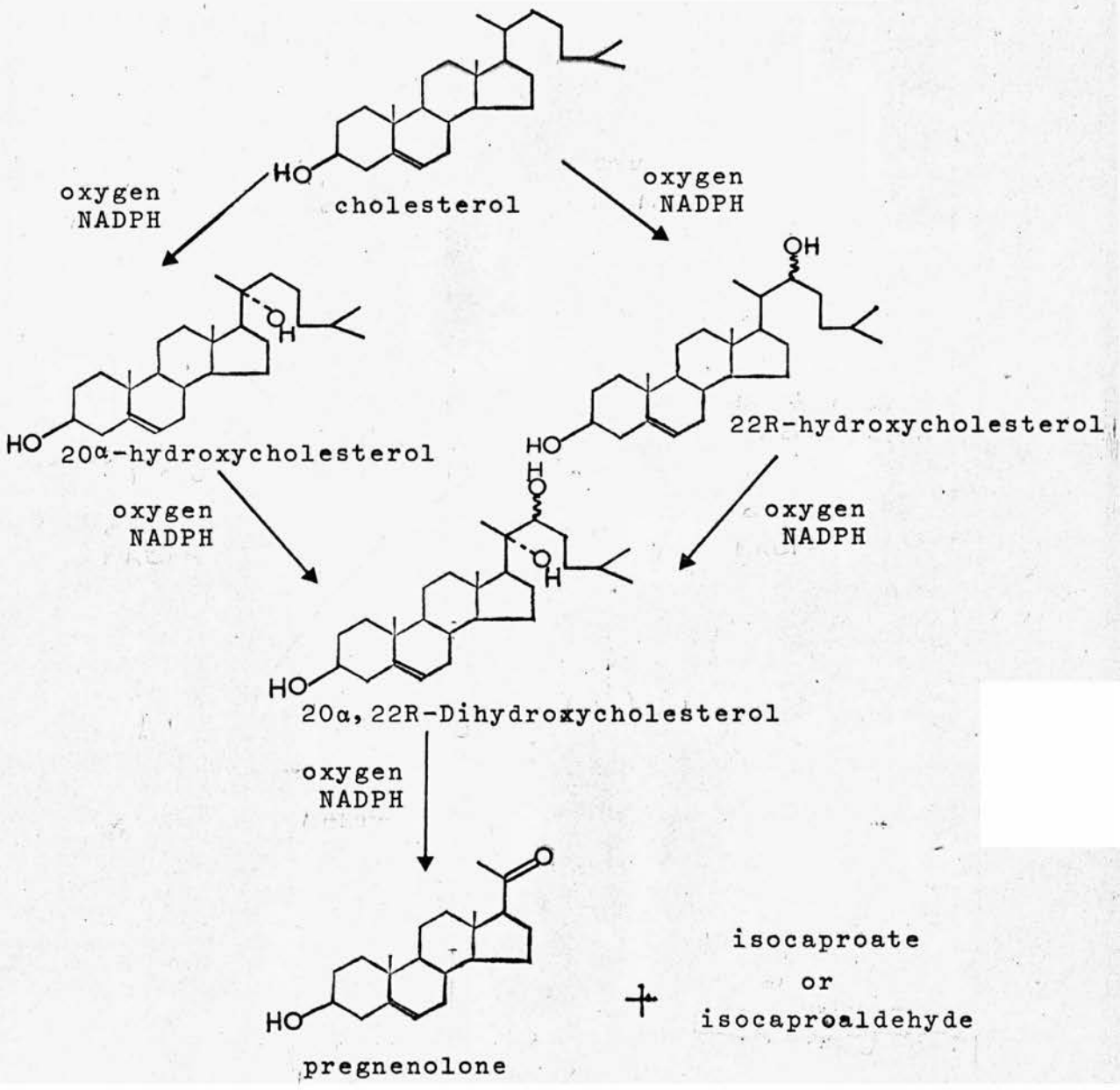


Figure 1.2. Suggested schemes for the side chain cleavage of cholesterol occurring in the mitochondria of adrenal cortex, corpora lutea, testes and placenta.

to give 20∝ , 22 R-dihydroxycholesterol. The side chain was then thought to be cleaved by a C 20-22 desmolase to give pregnenolone and the six carbon atom compound, isocaproaldehyde (Figure 1.2, Constantopoulos and Tchen (1961); Shimizu et al (1961), (1962)). This scheme was based on several pieces of evidence. Isotope trapping experiments showed that 20 ∝-hydroxycholesterol became labelled on incubation of adrenal cortex homogenates with [4 -^{14}C] cholesterol (Solomon et al (1956)). 20∝-hydroxycholesterol and 20∝ , 22 R-dihydroxycholesterol were converted to pregnenolone at faster rates than cholesterol (Shimizu et al (1961), (1962)). Cholesterol is cleaved between carbon atoms 20 and 22, giving rise to a six carbon fragment and pregnenolone (Staple et al (1956); Constantopoulos et al (1962)). More recently, the proposed intermediates of cholesterol side chain cleavage, 20 ∝ -hydroxycholesterol, 22 R-hydroxycholesterol and 20∝ , 22 R-dihydroxycholesterol have been isolated from large quantities of adrenal cortex tissue (Roberts et al (1969); Dixon et al (1970)). This has been cited as further evidence in favour of the mechanism of side chain cleavage shown in figure 1.2. It is also possible that 22 R-hydroxycholesterol could be formed from cholesterol as the first step in cholesterol side chain cleavage, followed by 20∝ -hydroxylation of 22 R-hydroxycholesterol (review Burstein and Gut (1971)). This was based on evidence that radioactive cholesterol was converted more readily to the radioactive 22 R-hydroxylated derivative than the 20∝ -hydroxylated derivative by acetone powders of bovine adrenal cortex. Other investigations by Koritz and Hall (1964b), Hall and Koritz (1964a), and Simpson and Boyd (1967b) failed to show hydroxylated intermediates in the

formation of pregnenolone from cholesterol. Experiments by Shikita and Hall (1974), on the stoichiometry of cholesterol side chain cleavage with respect to NADPH, oxygen and H^+, have shown that the conversion of cholesterol to pregnenolone can be described by the following equation:-

Cholesterol + 3NADPH + $3H^+$ + $3O_2$ ⟶ Pregnenolone + isocaproaldehyde + $3NADP^+$ + $3H_2O$

With 20∝-hydroxycholesterol two molecules of NADPH and two O_2 were required for the formation of pregnenolone. 20∝, 22R-dihydroxy cholesterol required one NADPH and one O_2 for the formation of pregnenolone. This data, they suggest, is in keeping with the scheme shown in figure 1.2 for cholesterol side chain cleavage. However, they did not present data for the stoichiometry of side chain cleavage of other postulated intermediates in the reaction such as 22R-hydroxy cholesterol. Thus mechanisms other than that shown in figure 1.2 are not excluded.

Alternative mechanisms for cholesterol side chain cleavage have been suggested. Lieberman et al (1969), Luttrel et al (1972) and Hochberg et al (1974) have suggested that pregnenolone production from cholesterol could occur via radical or ionic mechanisms. Such mechanisms were proposed to explain the side chain cleavage of an analogue of 20∝-hydroxycholesterol, (20R) 20-tbutyl-5-pregnene-3-20-diol, which is completely substituted at C 22; thus the position is not available for hydroxylation.

Van Lier and Smith (1970a,b) have put forward the theory that the 20∝-hydroperoxide of cholesterol is an important intermediate in cholesterol side chain cleavage. The 20∝-hydroperoxycholesterol

was converted to 20α, 22R-dihydroxycholesterol under anaerobic conditions in the presence of a bovine adrenal cortex mitochondrial enzyme system. In the presence of oxygen and NADPH this was then converted to pregnenolone. These authors demonstrated that /1 - 2^{3}H/ cholesterol could be incorporated into /1 - 2^{3}H/ 20α-hydroperoxy-cholesterol and /1 - 2^{3}H/ 20α, 22R-dihydroxycholesterol in adrenal cortex mitochondrial preparations. Both these compounds, when unlabelled, could decrease the formation of /1 - 2^{3}H/ pregnenolone from /1 - 2^{3}H/ cholesterol (Van Lier and Smith (1970b)). This was taken as evidence as to their intermediacy in the side chain cleavage of cholesterol. Similar observations with 20α-hydroxycholesterol have been used to suggest it is an intermediate in cholesterol side chain cleavage (see Simpson (1967)). Simpson and Boyd (1967b) have shown that 24-hydroxycholesterol,25-hydroxycholesterol and 26-hydroxy-cholesterol can inhibit conversion of /4 -^{14}C/ cholesterol to /4 -^{14}C/ pregnenolone in a similar fashion to 20α-hydroxycholesterol. It is therefore difficult to use this type of evidence to show a compound is an intermediate in cholesterol side chain cleavage. These hydroxy derivatives of cholesterol were shown to inhibit the conversion of /4 -^{14}C/ cholesterol to products in an enzyme preparation of bovine adrenal cortex mitochondria (Simpson and Boyd (1967b)). It is possible that they were in fact better substrates for the cholesterol side chain cleavage enzymes than the added /4 -^{14}C/ cholesterol.

Kraaipoel et al (1974) have proposed that a 20-22 cyclic peroxide of cholesterol is an intermediate in cholesterol side chain cleavage. This compound has not been isolated and its role in cholesterol side chain cleavage is therefore very speculative.

It is unclear which of the suggested intermediates, if any, are actually involved in cholesterol side chain cleavage. Some of the hydroxy compounds isolated could be byproducts of peroxide or radical mechanisms. It remains for intermediates formed during the conversion of cholesterol to pregnenolone to be isolated and well characterised. If these intermediates are enzyme-bound and transient this may prove to be very difficult.

The involvement of cytochrome P450 in the cholesterol side chain cleavage system

Klingenberg (1958) and Garfinkel (1958) independently reported the presence of a carbon monoxide binding pigment in liver microsomes. The carbon monoxide complex of reduced microsomes gave a peak at 450 nm. This was called cytochrome P450 by Omura and Sato (1964a,b). Ryan and Engel (1957) showed that the adrenal cortex microsomal 21-hydroxylation was inhibited by carbon monoxide, an inhibition which was reversed by light. Estabrook et al (1963) showed that cytochrome P450 was involved in the 21-hydroxylation of 17α -hydroxyprogesterone. Further evidence for this was obtained by Omura et al (1965) who measured reversal of carbon monoxide inhibition of 21-hydroxylation, using light of different wavelengths but of similar intensity. The photochemical action spectrum obtained for this reversal corresponded to the light absorption spectrum of the carbon monoxide complex of cytochrome P450. It was suggested that the cytochrome P450 was the oxygen activating enzyme and substrate binding site for the hydroxylation reaction. Photochemical action spectra have been used by Simpson and Boyd (1967b) and Mason (1970) to show that cytochrome

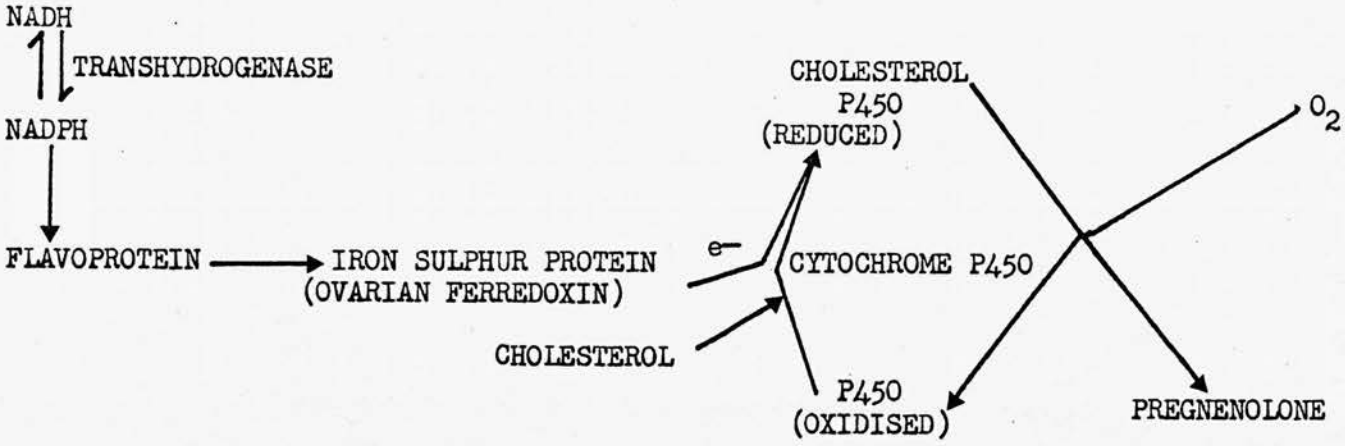


Figure 1.3. The cholesterol side chain cleavage enzyme system of luteal mitochondria.

P450 is involved in the cholesterol side chain cleavage reaction of bovine adrenal cortex mitochondria and human placental mitochondria. Sulimovici and Boyd (1968b) and McIntosh et al (1973) have shown that in rat and bovine luteal tissue a mitochondrial fraction containing cytochrome P450 is essential in the reconstitution of cholesterol side chain cleavage activity from its component parts. Cytochrome P450 is also involved in many other steroid hydroxylation reactions and in drug metabolising systems (see Simpson et al (1969); Gillette et al (1972)).

The components of the cholesterol side chain cleavage system and purification of cholesterol side chain cleavage cytochrome P450

The adrenal cortex mitochondrial steroid 11 β -hydroxylase system was fractionated into three components; a flavoprotein, a non-haem-iron protein and cytochrome P450 (Omura et al (1966)). The system for cholesterol side chain cleavage in bovine adrenal cortex mitochondria has been resolved into similar components as the 11 β -hydroxylase (Simpson and Boyd (1967a); Bryson and Sweat (1968)). Similar findings have been reported by Sulimovici and Boyd (1968b) and Mason and Boyd (1971) for the rat luteal and human placental mitochondrial cholesterol side chain cleavage systems. The non-haem-iron protein and the flavoprotein components of the system are involved in the supply of reducing equivalents from NADPH to cytochrome P450 (Omura et al (1966), see figure 1.3). Numerous attempts have been made to purify and solubilise the cholesterol side chain cleavage cytochrome P450 from endocrine mitochondria. This is complicated in adrenal mitochondrial preparations by the presence of other forms of cytochrome P450 involved

in 11 β -hydroxylation and 18-hydroxylation (Wilson and Harding (1970); Greengard et al (1967)). There appears to be only cholesterol side chain cleavage cytochrome P450 in luteal mitochondria which makes it a better starting point for purification studies. Jefcoate et al (1970) have separated two forms of cytochrome P450 from bovine adrenal cortex mitochondria using lyophilisation, iso-octane extraction and ammonium sulphate fractionation. One fraction exhibited high 11 β -hydroxylase activity and low cholesterol side chain cleavage activity. The other fraction showed high cholesterol side chain cleavage activity and low 11 β-hydroxylase activity. The absolute spectrum of this fraction had a maximum absorbance at 395 nm characteristic of a high spin haemoprotein. The 11 β-hydroxylase fraction had a maximum absorbance at 416 nm (Simpson et al (1971)) characteristic of a low spin haemoprotein. Several other preparations of adrenal cortex mitochondrial cytochrome P450, with cholesterol side chain cleavage and 11 β -hydroxylase activity, have been made (Mitani and Horie (1969a); Schleyer et al (1972); Isaka and Hall (1971); Ramseyer and Harding (1973); Shikita and Hall (1973a,b)). In these preparations oxidised cytochrome P450 appears to exist in two distinct spectral forms. These have Soret absorption maxima at 390 to 395 nm and 415 to 420 nm. The absorption at about 390 nm is probably due to a high spin complex of cholesterol with cholesterol side chain cleavage cytochrome P450 preparations (Ramseyer and Harding (1973)) or deoxycorticosterone with 11 β -hydroxylase cytochrome P450 preparations (Mitani and Horie (1969a)). The high spin form of cytochrome P450, a ferric haem containing five unpaired electrons, is a general feature of the cytochrome P450 substrate complex (Tsai et al

(1970); Peterson (1971)). The preparations with absorption maxima at 420 nm are characteristic of low spin cytochrome P450 with one unpaired electron. This is characteristic of the oxidised form of the substrate free,cytochrome (Tsai et al (1970)). Cholesterol side chain cleavage cytochrome P450 has been isolated from bovine corpus luteum mitochondria using sodium cholate for solubilisation (McIntosh et al (1973)). This preparation is not complicated by the presence of 11 β-hydroxylase cytochrome P450 and 18-hydroxylase cytochrome P450. The luteal cholesterol side chain cleavage cytochrome P450 has a Soret absorption maximum at 396 nm suggesting it is isolated as the cholesterol complex (McIntosh et al (1973)). Figure 1.3 shows the components of the luteal mitochondrial cholesterol side chain cleavage system and how they are thought to be arranged in vivo.

The binding of steroids and nitrogenous bases to cytochrome P450 involved in cholesterol side chain cleavage and 11 β-hydroxylation of corticosteroids

The binding of substrates and other molecules to P450 cytochromes produces changes which can be identified spectrally. For the investigation of these changes, difference spectroscopy is generally used. The sample containing cytochrome P450 is divided between two cuvettes of a spectrophotometer and a baseline is recorded. The compound under test is added to one cuvette and the difference spectrum then recorded. This allows very small changes in absorbance to be measured against a very turbid background such as that of a mitochondrial suspension. The absorbance due to the mitochondria is cancelled out as they are in both reference and sample cuvettes.

A type I spectral change is characterised by a difference spectrum with λ max 385 and λ min 420 nm. Such a change can be obtained by adding deoxycorticosterone to adrenal cortical 11 β - hydroxylase cytochrome P450 (Cooper et al (1965)). Whysner et al (1970) have shown, using E.P.R. spectroscopy, that this is in fact due to the steroid converting cytochrome P450 from a low spin form to a high spin form. Similar type I changes are observed when cholesterol is added to acetone powders of bovine adrenal cortex (Cheng and Harding (1973)).

An inverted type I spectral change results when pregnenolone is added to cholesterol side chain cleavage cytochrome P450. This difference spectrum has a λ min at 390 nm and a λ max at 420 nm (Mitani and Horie (1969a); Jefcoate and Boyd (1971)). E.P.R. spectroscopy has shown this change is probably due to a high to low spin transition of the cytochrome P450 (Whysner et al (1970)). Inverted type I changes have also been termed type II spectral changes (see Jefcoate et al (1974)).

The term type II spectral change is usually applied to the binding of lipophilic amines, such as aminoglutethimide, to cytochrome P450. These bind both high and low spin P450 to give difference spectra which have λ max 425 nm, λ min 390 nm and λ max 432 nm, λ min 410 nm respectively in the Soret region of the spectrum (Jefcoate and Gaylor (1970); Jefcoate and Boyd (1971); Jefcoate et al (1974)). The magnitudes of the difference spectra produced by steroids and amines are proportional to the amounts of high and low spin cytochrome P450 originally present. This has been used to quantitate the different spin forms of the cytochrome (Jefcoate and Gaylor (1970); Jefcoate

et al (1973); (1974)). The inverted type I spectrum produced on the interaction of pregnenolone with cholesterol side chain cleavage cytochrome P450 is thought to give a measure of the cholesterol bound to the enzyme (Jefcoate and Boyd (1971)). The pregnenolone is thought to alter the interaction of the cholesterol with the cytochrome P450 (Harding et al (1970)) possibly by displacing it from its binding site. The amount of cholesterol bound to the adrenal mitochondrial side chain cleavage cytochrome P450 in rats is altered by ether anaesthesia stress which raises blood levels of ACTH (Simpson et al (1972); Matsuyama et al (1971)). This is accompanied by an increase in the rate of cholesterol side chain cleavage in the mitochondria. The rise in the amount of cholesterol bound to the cytochrome P450 measured by pregnenolone binding spectra (Simpson et al (1972)) has been confirmed using E.P.R. spectroscopy (Brownie et al (1972), (1973)). Difference spectra have also been used to demonstrate spin state changes in cytochrome P450 induced by calcium ions (Simpson et al (1974)).

The control of steroidogenesis in endocrine tissues

Studies on the acute control of steroidogenesis by trophic hormones has centred on the mechanism of the effects of ACTH on the adrenal cortex, while similar observations have been made on the mechanism of action of LH on the corpus luteum. These studies have indicated that it is probable that the mechanisms of actions of LH and ACTH are very similar (Hall and Young (1968); Savard (1973); Hirshfield and Koritz (1966)).

LH has been shown to stimulate the synthesis of progestins in

the bovine corpus luteum and rat corpus luteum *in vitro* (Mason *et al* (1962); Mason and Savard (1964a); Armstrong *et al* (1964)). *In vivo* studies with the rat, rabbit and sheep have shown that ovarian and luteal progestin synthesis is stimulated by LH (Major *et al* (1967); Hilliard *et al* (1963); Armstrong (1968); Collet *et al* (1973)). Prolactin and FSH have been shown to be inert in the short term stimulation of steroidogenesis in the corpus luteum and ovary (Dorrington and Kilpatrick (1966); Cook *et al* (1967); Kaltenbach *et al* (1967); Rice *et al* (1964); Rice and Savard (1966); Huang and Pearlman (1962)). Use of an anti-LH serum has shown in the rat and mouse that LH is necessary for the production of progesterone in corpora lutea (Madhwa-Raj and Moudgal (1970); Behrman *et al* (1972)). Administration of the antiserum to female rats and mice caused a decrease in the blood levels of progesterone. Thus it is well documented that LH has an acute effect on steroidogenesis in the corpus luteum.

The proposed mechanisms of the effects of LH and ACTH on steroidogenesis

The initial eventin the stimulation of steroidogenesis by a trophic hormone is probably binding to a receptor on the cell surface. This may be the primary and sole event in which these hormones need to take part to stimulate steroidogenesis. When ACTH is bound to a macromolecule made from a cellulose derivative it cannot cross a plasma membrane. However it can still stimulate steroidogenesis in adrenal cells (Selinger and Civen (1971); Schimmer *et al* (1968)). No such experiments have been performed with LH. As it is a much larger molecule than ACTH it is likely that it does not enter the cell.

The receptors for LH in luteal cells have been well characterised (reviewed, Channing and Kammerman (1974)). When the hormone interacts with these receptors it initiates a sequence of events, the first of which is the production of cyclic AMP (see Channing and Kammerman (1974)). Cyclic AMP is probably involved in the steroidogenic action of ACTH and LH and can mimic their trophic effects (Marsh and Savard (1964); Hermier and Jutisz (1969); Grahame Smith et al (1967): reviews Robison et al (1968); Jost and Rickenberg (1971); Major and Kilpatrick (1972); Menon and Gunaga (1974); see also page 73). The ACTH sensitive cholesterol esterase of the adrenal cortex has been shown to be stimulated by cyclic AMP (Trzeciak and Boyd (1973); Boyd and Trzeciak (1973)). Thus the cyclic nucleotide could be involved in releasing cholesterol for steroidogenesis from stores of cholesterol esters. Although the cholesterol esterase of the corpus luteum has been shown to be stimulated by LH it is uncertain whether it is sensitive to cyclic AMP (Behrman and Armstrong (1969); Flint et al (1973)). The activation of the cholesterol esterase of the adrenal cortex may be by phosphorylation of the enzyme catalysed by a cyclic AMP dependent protein kinase (Trzeciak and Boyd (1974)). A protein kinase has been isolated from bovine corpus luteum. The kinase activity is stimulated by cyclic AMP and LH (Menon (1973)) but its exact role, if any, in steroidogenesis has not been established. The role of protein synthesis in the acute response of cells to LH and ACTH has been reported (Ferguson (1962); Farese (1964); Garren et al (1965); Gorski and Padnos (1966); Hermier et al (1971)). A labile protein whose synthesis is stimulated by LH or ACTH was postulated to take part in the steroidogenic response to the hormones. The

production of this labile protein is probably stimulated by cyclic AMP in the cell. The stimulatory effects of LH and ACTH on steroid production which are mimicked by cyclic AMP are both blocked by inhibitors of protein synthesis (Hermier et al (1971); Garren et al (1971)). LH and ACTH therefore may stimulate steroidogenesis via cyclic AMP and a "labile protein". The exact effect of this labile protein on steroidogenic enzymes has yet to be established.

Calcium ions and prostaglandins may also be important in mediating the effects of LH and ACTH in steroidogenic cells. Calcium ions have been shown to be necessary for the steroidogenic response of adrenal tissue to ACTH stimulation (Birmingham et al (1953); Peron and Koritz (1958); Hermier and Jutisz (1969); Carchman et al (1971)). The formation of cyclic AMP under the influence of ACTH appears to have a requirement for calcium ions (Sayers et al (1972); Rubin et al (1972)). Apart from the involvement in the activation of adenyl cyclase, calcium ions also appear to have a stimulatory role in the effects of cyclic AMP on steroiodgenesis in the adrenal cortex and corpus luteum (Rubin et al (1972); Haksar and Peron (1972); Hermier and Jutisz (1969)). Simpson et al (1974) have shown that in adrenal cortex mitochondria, in vitro addition of calcium ions can cause an increase of cholesterol bound to side chain cleavage cytochrome P450. This also causes an increase in pregnenolone production in incubations of these mitochondria with a source of reducing equivalents.

Prostaglandins have been reported to have both stimulatory and inhibitory effects on steroidogenesis in the corpus luteum. Prostaglandins of the E series have been reported to stimulate progestin synthesis in vitro when added to luteal slices from the rat or cow (Behrman et al (1971a); Marsh (1970); Speroff and Ramwell

(1970)). Kuehl et al (1970) had earlier shown in mouse ovarian tissue that a prostaglandin analogue prevented LH and prostaglandins causing a rise in the cellular levels of cyclic AMP. Thus it has been suggested that prostaglandins play a role in the activation of adenyl cyclase during trophic hormone stimulation. Recent investigations by Wilks et al (1973) have been unable to confirm that the production of progesterone in vitro in rat luteal tissue is stimulated by the E series of prostaglandins. At the present moment the exact stimulatory role, if any, of prostaglandins on luteal steroidogenesis is uncertain.

Prostaglandin $F_{2\alpha}$ can cause the regression of the corpus luteum in the rat and sheep (Behrman et al 1971b; McCracken et al (1972)); with a resultant decrease in the production of progesterone. There may therefore be a dual role for prostaglandins in luteal function; the E series being involved in positive effects on steroidogenesis, while the F series are involved in luteolysis. Recently, however, Santos et al (1973) have shown prostaglandin $F_{2\alpha}$, added in vitro, can stimulate progesterone synthesis in human corpus luteum.

The site of action of trophic hormones on steroidogenesis

The work of Stone and Hechter (1954), using perfused bovine adrenal glands, suggested that ACTH controlled steroidogenesis by stimulating the conversion of cholesterol to pregnenolone. Other studies have given rise to the conclusion that cholesterol side chain cleavage is the rate limiting step in steroidogenesis, under the control of trophic hormones, in the testis and corpus luteum as well the adrenal cortex (Hall and Eik-Nes (1964); Hall and Koritz (1965);

Karaboyas and Koritz (1965); Channing and Villee (1966); Hall and Young (1968)). In the studies mentioned above no direct measurement of the activity of the cholesterol side chain cleavage enzymes was made. Only recently has prior ACTH treatment been shown to influence cholesterol side chain cleavage activity, in isolated rat adrenal cortex mitochondria (Simpson et al (1972)). There are several ways in which trophic hormone stimulation could affect the metabolism of cholesterol in mitochondria.

The availability of the various substrates, (a) NADPH, (b) cholesterol and (c) oxygen for cholesterol side chain cleavage, could limit the rate of pregnenolone production while (d) the accumulation of pregnenolone could result in product inhibition of cholesterol metabolism. The trophic hormone stimulation could also have a direct effect on the activity of the cholesterol metabolising enzyme (e), through an activator or an inhibitor.

(a) Availability of NADPH for the cholesterol side chain cleavage reaction

The cholesterol side chain cleavage enzymes of endocrine mitochondria are mixed function oxidases (Mason (1957) and see figure 1.3) requiring NADPH and oxygen for activity. Thus any limit on the supply of NADPH could restrict the activity of the cholesterol-metabolising enzymes and other cytochrome P450 dependent reactions. Haynes and Berthet (1957) proposed that ACTH caused a rise in cellular levels of cyclic AMP which activated glycogen phosphorylase which was then thought to cause a rise in glucose-1-phosphate and glucose-6-phosphate levels in the cell. The glucose-6-phosphate could then be metabolised by the pentose phosphate pathway to produce

rises in cellular levels of NADPH. However it is uncertain how the NADPH could get into the mitochondria in the adrenal cortex or corpus luteum. Luteal mitochondria are thought to be impermeable to NADPH (Yago et al (1967a)). Hirshfield and Koritz (1964), (1965) have shown that ACTH administration to rats may alter the permeability properties of the mitochondria prepared from their adrenal glands. This was thought to allow NADPH to enter the mitochondria.

ACTH and LH have also been shown to cause a stimulation of cellular glucose-6-phosphate dehydrogenase activity. This could then cause the generation of more NADPH for steroid hormone production (McKerns (1965), (1969)). Inhibition of the activity of the pentose phosphate pathway and thus NADPH supply, by oxythiamine injection, has been shown in rat ovaries to reduce the rate of steroidogenesis (Deb et al (1973)). This work relied on histochemical measurement of enzyme activities and no absolute measurements of steroid production were made. Again this work suffers from the criticism that NADPH generated in the cell cytoplasm may not enter the mitochondrion.

Other criticisms of the hypothesis that NADPH levels could control the rate of steroidogenesis have been made. The levels of $^{NADPH}/_{NADP^+}$ in luteal and adrenal cells have been shown to remain constant when these cells have been stimulated with LH (see page 72 for discussion and references).

Work with bovine corpus luteum tissue slices has shown that, in the presence of saturating amounts of NADPH, in vitro addition of LH could still stimulate steroid production (Mason and Savard (1964b); Savard and Casey (1964); Savard et al (1963), (1965)). Thus any stimulatory effect of LH on progesterone production was thought to

be independent of NADPH production. This work may be criticised because it was performed on tissue slice preparations and as NADPH may not get into intact cells any stimulation of steroidogenesis caused by it may have been on enzymes from broken cells.

(b) The control of cholesterol availability for the cholesterol side chain cleavage reaction

The cholesterol for steroidogenesis could be (i) synthesised in the steroidogenic cell, (ii) obtained from the blood or (iii) stored in the cell.

(i) It has been postulated that the LH stimulated increase in progesterone production in the corpus luteum may be due to an increase in the de novo synthesis of cholesterol (Savard and Casey (1964)). An inhibitor of cholesterol synthesis, AY9944, has been shown not to prevent the acute effects of LH in stimulating in vitro synthesis of progesterone by bovine corpus luteum or rabbit interstitial tissue (Armstrong (1967); Armstrong et al (1970)). These investigators concluded that, in the corpus luteum, cholesterol synthesis is unimportant in the acute effects of LH on steroidogenesis.

(ii) Dietary and plasma cholesterol have been shown to be the source of over 90% of the corticoids and 40% of the androgen carbon in the rat (Morris and Chaikoff (1959)). In rabbit interstitial tissue and rat luteal tissue it has also been shown that plasma cholesterol is converted to steroids (Solod et al (1966); Major et al (1967); Flint and Armstrong (1971b)). When radioactive cholesterol was injected into rats with luteinized ovaries, progesterone and 20 α -hydroxyprogesterone in the plasma reached the same specific activity as the cholesterol within half an hour (Flint and Armstrong

(1971b)). It was eight hours before the esterified cholesterol of the corpora lutea reached the same specific activity as the free cholesterol. The free cholesterol of the cell and plasma appear to be freely exchangeable, possibly providing the precursor cholesterol for steroidogenesis. Armstrong (1968) has shown that LH can increase the uptake of plasma ^{3}H cholesterol by luteal cells and reverse the fall in this parameter produced by hypophysectomy. There is little other evidence that LH could increase the production of steroids through increasing the uptake of cholesterol in luteal cells. Dexter et al (1970) have shown that ACTH can stimulate the uptake of ^{3}H cholesterol by adrenal cells.

There is a considerable amount of evidence that cells lose free cholesterol on trophic hormone stimulation. Solod et al (1966) have shown that stimulation of rabbit ovarian interstitial tissue with LH results in a far greater loss of cholesterol from the cholesterol ester fraction in the cells than there is steroid hormone produced. In luteinized rat ovaries, cholesterol is lost from the cellular cholesterol ester fraction, on LH stimulation. Only a small portion of the cholesterol lost is converted to steroids (Flint and Denton (1970)). A similar phenomenon has been reported during LH stimulation of leydig tumour cells. The free cholesterol lost has been detected in the incubation medium of the cells (Moyle et al (1973a)). This may explain the results of investigators who have found large depletions (up to 60%) of total luteal cholesterol within three hours of treatment of rats with LH (Bell et al (1964); Clark and Zarrow (1968)). Free cholesterol could escape into the plasma, but the reason for this is unclear. It does however suggest there

is a rapid turnover of non esterified cholesterol between luteal tissue and the blood.

(iii) The stored cholesterol in the cell can account for up to one fifth of the dry weight of some ovarian tissues (Flint and Armstrong (1972)). Most of the cholesterol is stored as 3 β-esters of long chain fatty acids. In the ovary these cholesterol esters are stored in the lipid droplets within the cell cytoplasm (Claesson (1954)).

These lipid droplets have been isolated and characterised in luteinized rat ovaries (Armstrong and Flint (1973)) and rat adrenal cortex (Boyd and Trzeciak (1973)). The droplets in luteal cells decrease in size and number on trophic hormone stimulation of the tissue (Deane et al (1948); Barker (1951); Claesson (1954); Ashworth et al (1959); Giacomelli et al (1965)). These lipid droplets were thought to be stored precursors for luteal steroidogenesis (Everett (1945)). The cholesterol esters of the adrenal gland also act as precursors for steroidogenesis and appear to be depleted under the influence of ACTH (review Garren et al (1971)).

The mobilisation of the cholesterol esters, to provide cholesterol for steroidogenesis, depends on the enzyme cholesterol esterase. This enzyme has been found in the adrenal cortex and corpus luteum (Dailey et al (1963); Coutts and Stansfield (1968); Behrman and Armstrong (1969)). It has been demonstrated that ACTH and LH can cause the stimulation of the cholesterol esterase enzymes in the adrenal cortex and the corpus luteum (Behrman and Armstrong (1969); Behrman and Greep (1972); Shima et al (1972); Trzeciak and Boyd (1973)). These enzymes can liberate free cholesterol for pregnenolone

production. Thus it is clear that at least one action of trophic hormones on steroidogenesis is to increase the levels of free cholesterol in the cell. The stimulation of cholesterol ester hydrolysis appears to be unaffected by inhibitors of protein synthesis which can prevent trophic hormone stimulation of steroidogenesis (Davis and Garren (1966); see Armstrong and Flint (1973)). As discussed previously (pages 19 and 20) it seems that the amount of cholesterol released from cholesterol esters is far in excess of the needs of the luteal cell for steroidogenesis.

Accumulation of free cholesterol in the luteal cell under the influence of LH may also be due to an inhibitory effect of the gonadotrophin stimulation on the enzyme cholesterol ester synthetase (Flint et al (1973)). This enzyme in conjunction with the cholesterol esterase is important in the regulation of the levels of cholesterol esters in the cell (Flint and Armstrong (1973)). When rats are treated with aminoglutethimide phosphate, to inhibit cholesterol side chain cleavage and hence steroidogenesis, LH treatment is still able to cause the de-esterification of cholesterol (Behrman et al (1970)). Thus LH can bring about an increase in cellular levels of free cholesterol, independent of a stimulation of steroidogenesis. In normal circumstances this increased supply of cholesterol could be used for steroidogenesis and may cause the stimulation of steroid production. This work does not exclude an effect of LH on the luteal mitochondrial cholesterol side chain cleavage enzymes.

(c) Availability of oxygen for cholesterol side chain cleavage

Molecular oxygen is required for cholesterol side chain cleavage (Halkerston et al (1961)) therefore it could be rate limiting in the

reaction. The Km for oxygen of another mixed function oxidase, cholesterol 7 α -hydroxylase, has been found to be 20 μM (Boyd et al (1973)). The concentration of oxygen in the cell is however unknown. In the adrenal gland and the corpus luteum, ACTH and LH are known to increase the blood flow (Sapirstein and Goldman (1959); Ellis (1961); Wurtman (1964)). This could increase the supply of oxygen to the tissues to meet increased demands during steroidogenesis.

(d) Control of cholesterol side chain cleavage by the accumulation of pregnenolone.

The product of cholesterol side chain cleavage, pregnenolone, has been postulated to be important in the control of steroidogenesis. Pregnenolone has a direct inhibitory effect on cholesterol side chain cleavage activity in adrenal cortical and luteal mitochondria (Koritz and Hall (1964a,b); Ichii et al (1963)). Formation of corticosterone has been shown to be biphasic in incubation of mitochondria and microsomes from rat adrenal glands. This has been explained on the basis of a build up of pregnenolone inhibiting cholesterol side chain cleavage and lowering the rate of steroidogenesis (Koritz and Kumar (1970)). ACTH was postulated to increase the flux of pregnenolone from the mitochondria and thus increase the overall rate of steroidogenesis. This hypothesis has been challenged by Simpson et al (1972) and Farese (1970) who have found that ACTH can stimulate steroidogenesis even under conditions when elevated levels of pregnenolone are present.

(e) Direct effects on the cholesterol side chain cleavage enzymes.

ACTH and LH may have a direct effect on the cholesterol side chain cleavage enzymes through cyclic AMP and a rapidly turning over protein. Cyclic AMP is now thought to have no direct effect on cholesterol metabolism in mitochondria from the adrenal cortex and corpus luteum (Kahnt et al (1974); Mahaffee et al (1974); Sulimovici and Boyd (1968a); Jackanicz and Armstrong (1968)). The amount of cholesterol bound to adrenal mitochondrial cytochrome P450 in the rat can be raised by ether stress which raises plasma ACTH levels (Simpson et al (1972); Jefcoate et al (1973), (1974); Boyd et al (1972)). At the same time cholesterol side chain cleavage activity is raised in these mitochondria. The effect of stress treatment can be prevented by prior treatment of the rats with cycloheximide. From these results it was suggested that a rapidly turning over protein (Garren et al (1965)) facilitated the binding of cholesterol or transport of the steroid to the mitochondrial cytochrome P450 (Simpson et al (1972)). It is difficult to tell whether this effect on side chain cleavage is a direct stimulation of the enzyme or just an increase in the supply of substrate cholesterol. Mahaffee et al (1974) have shown that in rats treated with ACTH and cycloheximide, cholesterol accumulates in the adrenal mitochondria. Thus the "protein factor" is probably important in the mitochondrial metabolism of cholesterol rather than its transport into the mitochondria. Bell et al (1973) and Bell and Harding (1974) have also concluded that supply of cholesterol to the cholesterol side chain cleavage enzymes is the rate limiting step in adrenal steroidogenesis. They, however,

do not conclude whether the cholesterol is supplied from other sites in the mitochondria or from the cell cytoplasm. Simpson et al (1971) and Kahnt et al (1974) have concluded that in bovine adrenal cortex mitochondria the rate of metabolism of cholesterol is limited by the rate of supply of the steroid to cytochrome P450.

In experiments, using slices of human or rat corpora lutea, Hermier et al (1971) and (1972a) have shown that cycloheximide can prevent LH and cyclic AMP induced stimulation of progesterone production. They concluded that a protein factor, whose synthesis was prevented by cycloheximide, was responsible for the transfer of extra mitochondrial cholesterol into the mitochondrion. The synthesis of this factor was thought to be under the control of LH and cyclic AMP. No measurements of the rate of cholesterol side chain cleavage were made in these studies.

Stevenson et al (1973) and Robinson and Stevenson (1973) have shown HCG (HCG has LH like activity) treatment of superovulated rats, one hour before killing, caused a stimulation of the cholesterol side chain activity in isolated luteal mitochondria, compared to saline injected controls. The cholesterol side chain cleavage activity was measured as the percentage conversion of $[4\text{-}^{14}C]$ cholesterol to $[4\text{-}^{14}C]$ pregnenolone and $[4\text{-}^{14}C]$ progesterone. This does not take into account that the treatment with HCG may have altered the size of the mitochondrial pool of cholesterol. If the amount of cholesterol in the luteal mitochondria was changed by LH treatment comparison of percentage conversions of $[4\text{-}^{14}C]$ cholesterol to products may be misleading. Thus there is no conclusive evidence that LH can alter the absolute rate of cholesterol side chain cleavage in luteal mitochondria.

Ascorbic acid has been shown to inhibit cholesterol side chain cleavage in luteal and adrenal cortex mitochondria. Adrenal cortex microsomal 21 hydroxylase and mitochondrial 11 β-hydroxylase also appear to be inhibited by ascorbate (Hayano et al (1956); Cooper and Rosenthal (1962); Kitabachi (1967a,b); Sulimovici and Boyd (1968b)). Trophic hormone stimulation has been shown to deplete the stores of ascorbic acid in adrenal and gonadal tissues (Sayers et al (1944); (1948); Claesson et al (1949); Hokfelt (1950); Parlow (1958); Bell et al (1965)). Kitabachi (1967b) has therefore postulated that trophic hormones could stimulate steroidogenesis by causing the cellular levels of ascorbic acid in the cell to fall thus releasing its inhibitory effect on steroidogenic enzymes. Depletion of ascorbic acid in the adrenal gland, on ACTH stimulation, can be detected after one half to one hour. This may not be fast enough to account for the acute effects of ACTH on steroidogenesis which can be recorded after ten minutes (Garren et al (1971)).

There are many hypotheses on how trophic hormones may exert their effect on luteal and adrenal steroidogenesis. It appears that the cholesterol esterase and cholesterol side chain cleavage enzymes of the steroid producing pathways are under the control of ACTH and LH. The trophic actions of ACTH and LH may be mediated by cyclic AMP and a rapidly turning over protein. This protein could affect the activity of the adrenal cortex mitochondrial cholesterol side chain cleavage enzymes possibly by increasing the supply of substrate cholesterol. There is no direct conclusive evidence that trophic hormone stimulation can cause a stimulation of cholesterol side chain cleavage activity in luteal mitochondria.

Aims of the present study

The role of LH in controlling the output of progestins by the corpora lutea of several mammals is well established. The mechanism of this control is however unclear, several hypotheses having been proposed. The importance of the cholesterol side chain cleavage reaction in the steroidogenic response to LH has been suggested from many studies.

The object of this study was to investigate and characterise the properties of the cholesterol side chain cleavage system of the rat corpus luteum. First of all an assay system for the luteal cholesterol side chain cleavage activity had to be established. It was intended to find out how this activity could be controlled *in vitro* by various compounds which have been implicated in the control of steroidogenesis by LH. At the same time, the spectral properties of the luteal mitochondrial cholesterol side chain cleavage cytochrome P450 were to be investigated.

The effects of treatment of rats, with LH and inhibitors of protein synthesis, on the cholesterol metabolising and spectral properties of the luteal mitochondrial cholesterol side chain cleavage system could then be determined.

The supply of cholesterol to the cholesterol side chain cleavage system was also to be investigated. It was hoped these studies would give some information which would show how LH could control luteal steroidogenesis.

The superovulated "Parlow rat" was used in these studies (Parlow (1958)). These animals, due to pre-treatment with gonadotrophins, have large numbers of metabolically active corpora lutea which are useful for biochemical investigations (e.g. Sulimovici and Boyd (1968a); Armstrong (1968); Sherwood *et al* (1963); Armstrong *et al* (1964); Kidwell *et al* (1966)).

CHAPTER 2

EXPERIMENTAL PROCEDURES

Introduction

As discussed in the introductory chapter, the object of this work was to study the control of cholesterol side chain cleavage in rat luteal mitochondria. The immature rat was chosen as the experimental animal because this animal pretreated by the method of Parlow (1958) is a convenient source of metabolically active corpora lutea.

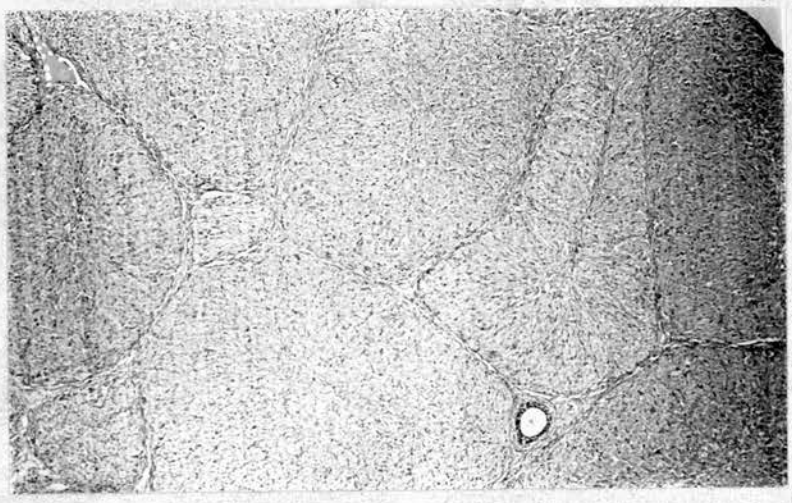
This animal is easy to inject and manipulate prior to the removal of the corpora lutea for experiments. Study of cholesterol side chain cleavage cytochrome P450 in corpus luteum mitochondria is very desirable as it is not complicated by the presence of other forms of cytochrome P450 as it is in the adrenal gland (McIntosh *et al* (1971)). This chapter describes the pretreatment of rats, before the removal of their luteinized ovaries. Preparation of subcellular fractions from these ovaries and assay of components and enzyme activities of these fractions is described later in the chapter. Chemicals and materials used in these investigations are listed.

Materials and Methods

(1) Animals and pretreatment

Female rats of the Wistar strain, obtained from The Small Animal Breeding Station, University of Edinburgh, were used in all experiments. Luteinized ovaries were obtained from these animals using the

(a)



(b)

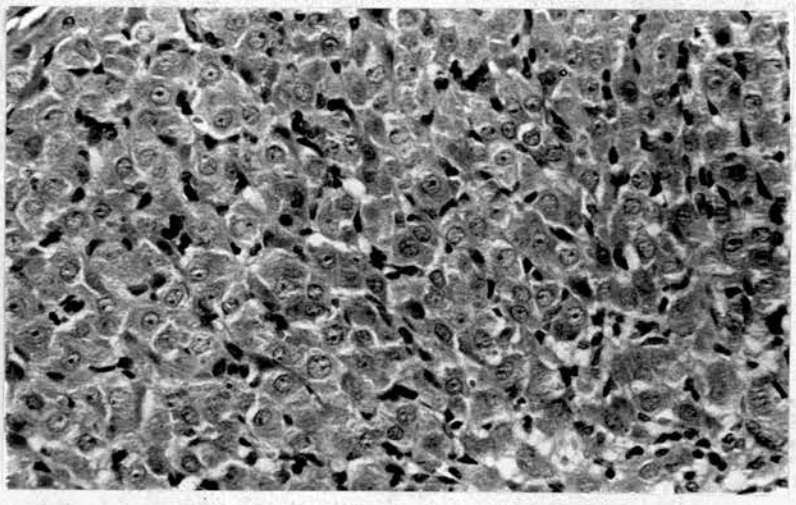


Figure 2.1. Section of a superovulated rat ovary, fixed in formaldehyde and stained with haematoxylin and eosin. Magnifications are (a) x 30(b) x 200. Note the large amount of red blood cells which appear as black dots between the cells in (b).

pretreatment procedure devised by Parlow (1958).

Rats 21 to 24 days old weighing 30 to 50 grams were injected subcutaneously with 50 international units (iu) of pregnant mare-serum gonadotrophin (Gestyl-Organon) dissolved in 0.5 ml of 0.9% saline. This preparation contains FSH like activity and induces heavy follicular growth in the ovaries of the rats. Three days later the rats were given a second injection of 50 iu pregnant mare serum gonadotrophin (Sulimovici and Boyd (1968a)) or 25 (iu) Human Chorionic Gonadotrophin (Pregnyl-Organon). Both these preparations contain luteotrophic activity and cause ovulation in the rat ovaries (Schmidt-Elmendorf et al (1962)). The granulosa and thecal cells of the ruptured ovarian follicles then develop into corpora lutea. The rats were then used 5-7 days after the second injection of human chorionic gonadotrophin; ovarian weight varied from 120 —— 150 mg compared to $<$ 10 mg in the untreated animals. Fig. 2.1 shows a section on ovary after the rat had received its pretreatment.

(2) Treatment of animals, with pretreated ovaries, prior to killing

(a) Injections of puromycin and cycloheximide

Puromycin and cycloheximide were administered to the rats by intra-peritoneal injection dissolved in 0.2 mls of 0.9% saline. The animals were generally killed twenty minutes after these injections. The doses of cycloheximide and puromycin used are shown in chapter 7. Control animals in these experiments received intra-peritoneal injections of 0.2 mls of 0.9% saline.

(b) Injections of Chloramphenicol

Chloramphenicol (5 mg/rat) was administered to the rats by intra-muscular injection suspended in 0.2 mls of 0.9% saline. The animals were killed 3 hours later, by which time the inhibitor had reached the maximum blood level (De Jonge and Hulsmann (1973)). Control animals received an intra-muscular injection of 0.9% saline before killing.

(c) Dihydrotestosterone

5 α-Dihydrotestosterone (2 mg/rat) was administered by sub-cutaneous injection suspended in 0.2 ml olive oil. Control animals received similar injections without steroid.

(d) Dexamethasone

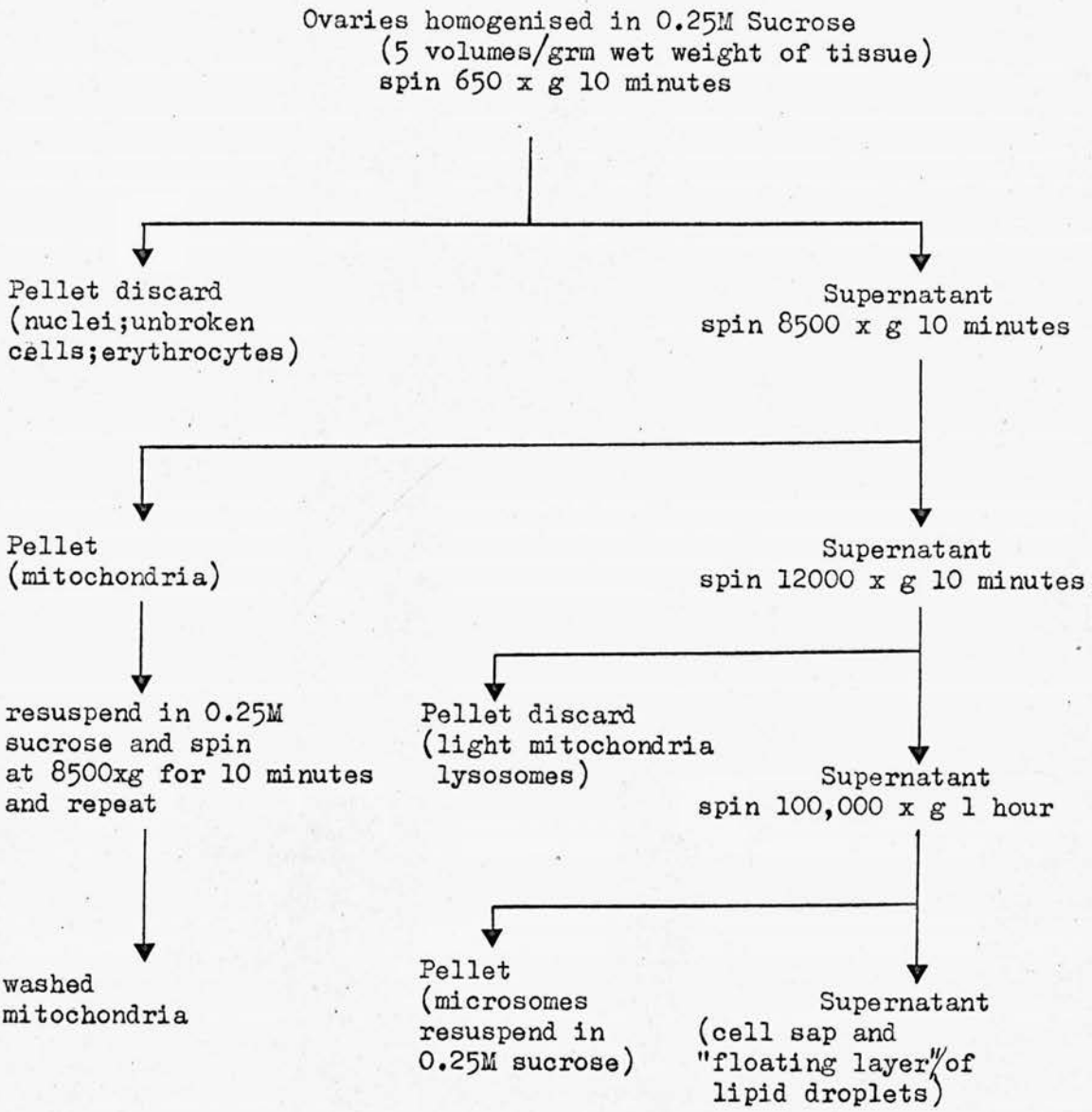
Dexamethasone (sodium salt) (20 μg/ml) was administered to the rats in their drinking water, with 1% glucose dissolved in the water to disguise the taste of the steroid. Control animals received the 1% glucose in their water without the steroid. The duration of the dexamethasone treatment is given in the results section.

(e) LH and HCG

Rats were injected with 10 μg LH (N.I.H. LH-S-18) or 25 iu Human Chorionic Gonadotrophin dissolved in 0.2 mls 0.9% saline. Two methods of injection were used; hormone was administered, either intravenously into the tail vein or subcutaneously behind the neck of the rat. The rats were killed after the injections at times indicated in the text.

Table 2.1

Fractionation Procedure for rat ovarian homogenate



(All procedures carried out at 0 to 4°C)

(3) Preparation of subcellular fractions

After pretreatment the animals were killed by cervical dislocation, their ovaries quickly removed by a dorsal approach and trimmed free of fat and connective tissue. The ovaries were kept in ice cold 0.25 M sucrose. All subsequent operations were carried out at 4°C. The trimmed ovaries were cut into smaller pieces with scissors and homogenised in 5 volumes 0.25 M sucrose/gram of tissue using a loose fitting teflon pestle, Potter Elvehjem homogeniser. No more than three passes were made with the homogeniser to avoid disrupting the luteal mitochondria. The homogenate was centrifuged at 650 x g for 10 minutes in an M.S.E. 18 High Speed Centrifuge, to remove nuclei, erythrocytes and unbroken cells, which were discarded. The supernatant was centrifuged at 8500 x g for 10 minutes to give the mitochondrial pellet. This pellet was resuspended in one half the original homogenate volume of 0.25 M sucrose and centrifuged again at 8500 x g for 10 minutes. This washing was repeated and the final mitochondrial pellet suspended in 0.25 M sucrose to give a protein concentration of 6-8 mg/ml. The post mitochondrial supernatant was spun at 12,000 x g for 10 minutes to remove any remaining mitochondria and lysosomes. The supernatant was then spun at 100,000 x g for 60 minutes in a Beckman L2 - 65B ultra centrifuge to give the microsomal pellet. The 100,000 x g supernatant which was used for some experiments had a layer of floating lipid (Claesson (1954)) which was carefully removed. This lipid layer was resuspended in 0.25 M sucrose and recentrifuged at 100,000 x g for 60 minutes. These operations are summarised in Table 2.1.

Table 2.2

Contents of incubations used for cholesterol side chain cleavage assays

Constituent	concentration	
Sucrose	250 mM	Medium A
Potassium Chloride	20 mM	
Triethanolamine Hydrochloride	15 mM	
Potassium Phosphate	10 mM	
Magnesium Chloride	5 mM	
EDTA	0.2 mM	
Bovine Serum Albumin (Defatted)	0.1%	
Luteal Mitochondria	1 - 2 mg protein/ml	
Cyanoketone	6 - 12 μM	
Cholesterol	(60,000cpm, 50 μC/μMol) in 5 μl acetone	

Incubations 1, 2 or 3 mls in volume

Cholesterol metabolism was initiated by addition of suitable electron donor and incubations carried out at 29°C.

(4) Assay of cholesterol side chain cleavage

(i) Introduction

Cholesterol side chain cleavage was assayed in luteal mitochondria after the reaction had been initiated by the addition of a suitable electron donor. Two different methods were used to assay the reaction - a radioactive assay and an assay which involved the measurement of total pregnenolone formation.

In incubations of luteal mitochondria with NADPH or Krebs cycle intermediates, cholesterol is converted to pregnenolone, progesterone and trace amounts of 20 α-hydroxy progesterone (Sulimovici and Boyd (1968a); Robinson and Stevenson (1971a)). It is obviously inconvenient to measure two or three products to assess the absolute rate of cholesterol side chain cleavage, therefore cyanoketone was included in incubations to prevent further metabolism of pregnenolone by mitochondrial 3 β-hydroxysteriod dehydrogenase (Goldman et al (1965)). Thus pregnenolone is the only steroid product of cholesterol side chain cleavage in incubations and as will be shown later (see fig. 3.5) this does not affect the overall rate of the reaction. The addition of cyanoketone to incubation therefore allows the convenient measurement of the rate of cholesterol side chain cleavage by the production of pregnenolone.

(ii) Incubation medium

Cholesterol side chain cleavage assays were carried out in the medium shown in table 2.2. As will be shown in Chapter 3 the constituents of this incubation medium are important in determining

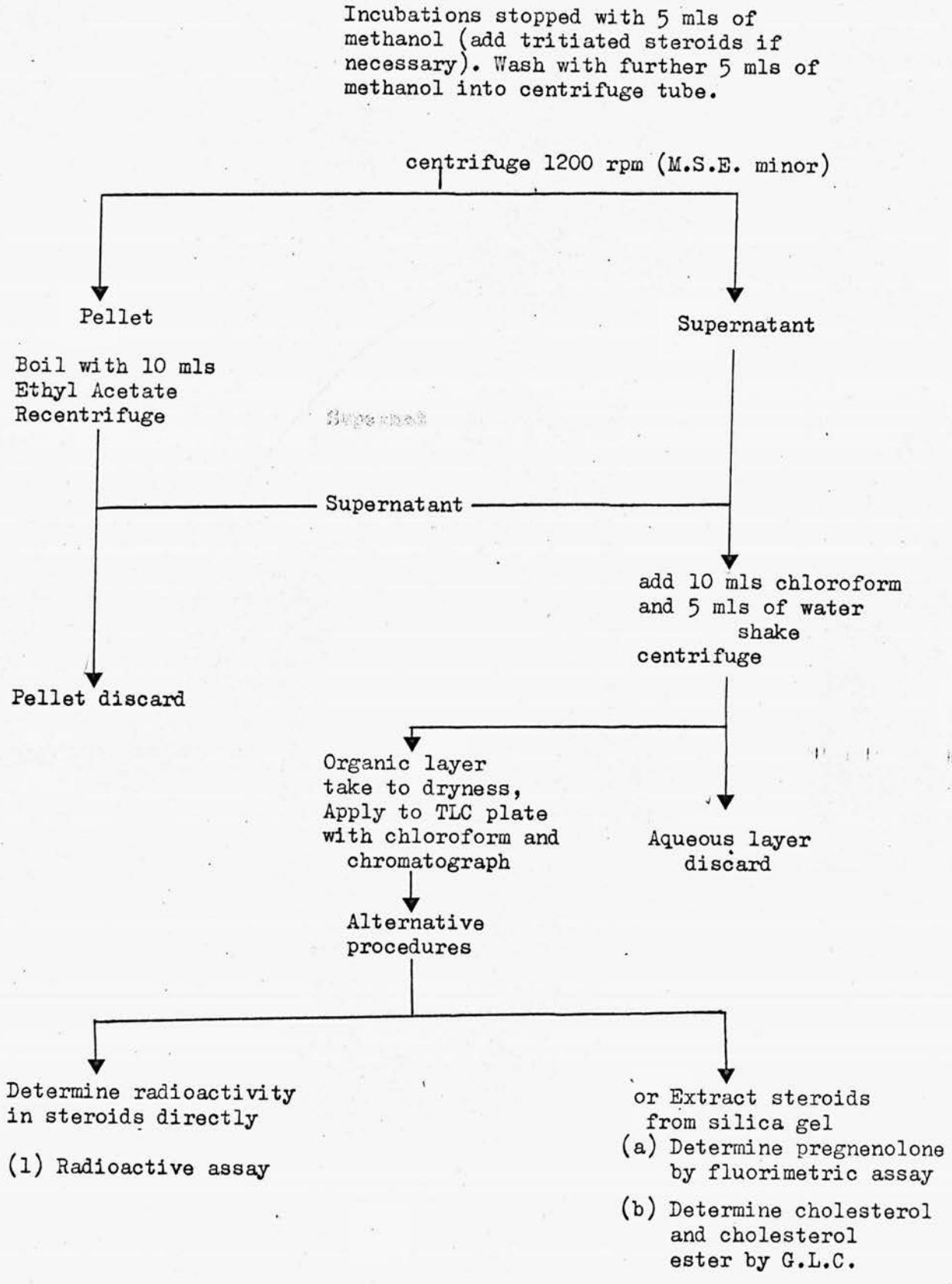
the side chain cleavage activity of the mitochondria. This incubation medium is called medium A. Luteal mitochondrial protein concentrations in these incubations were 1 ——— 2 mgs/ml and incubations were 1, 2 or 3 mls in volume depending on the method of assay. Incubations of 1 ml were suitable for radioactive assays; those of 2 and 3 mls were used for assays involving the measurement of total pregnenolone formed. Cyanoketone was present in incubations, to prevent further metabolism of pregnenolone to progesterone by a 3 β -hydroxysteriod dehydrogenase in the luteal mitochondria. Cholesterol side chain cleavage was initiated in the mitochondria by addition of a suitable electron donor (Chapter 3). Incubations of above 2 mls in volume were performed in 25 ml Erlenmeyer flasks in air in a rapidly shaking waterbath ($<$ 60 times min) at 29°C. Incubations below 2 mls in volume were performed in glass centrifuge tubes (5" x $\frac{1}{2}$") or 10 ml Erlenmeyer flasks under conditions similar to those described above. Reactions were terminated by addition of 5 mls of Methanol.

(iii) Radioactive assay

This method is based on the cholesterol side chain cleavage assays used by Sulimovici and Boyd (1968a) for luteal mitochondria and the assays used for adrenal mitochondria by Simpson and Boyd (1967b) and Simpson et al (1972). [4 -^{14}C] Cholesterol (60,000 c.p.m. 50 μC/μmol) in 5 μl acetone was preincubated with the mitochondria for 10 minutes before addition of a suitable electron donor, to initiate cholesterol side chain cleavage. This preincubation ensured that the [4 -^{14}C] cholesterol had equlibrated with the endogenous

Table 2.3

Summary of Cholesterol Side Chain Cleavage Assays



cholesterol (See figs. 3.1 and 3.2). The [4 -^{14}C] cholesterol was stored in acetone solution at -15°C and checked regularly by thin layer chromatography for autoxidation products. Over periods of six months no autoxidation of the [4 -^{14}C] cholesterol could be detected, providing care had been taken to keep it stored at -15°C.

When the enzymic reaction had been stopped by the addition of 5 mls of methanol to the incubation flasks, the mixture was poured into a stoppered centrifuge tube. A further 5 mls of methanol was used to rinse the incubation flask and this was transferred to the centrifuge tube. This ensured that at least 97% of the radioactivity was transferred to the centrifuge tube. The denatured protein was resedimented and the methanolic supernatant transferred to another centrifuge tube. The pellet was then re-extracted with 10 mls of boiling ethyl acetate. This was combined with the first extract and mixed with 10 mls of chloroform and 5 mls of water, shaken and recentrifuged to separate the aqueous and organic layers. The aqueous layer was then removed with a pasteur pipette. The organic layer was taken to dryness on a hot water bath under a stream of nitrogen or air. The organic extract was redissolved in 0.3 mls of choloroform (Table 2.3).

Separation of components of organic extract

To separate steroids the organic extract was subjected to thin layer chromatography on a glass plate (20 cm x 5 cm) coated with silica gel H. After the organic extract had been applied to the thin layer plate, a further two washes of 0.1 mls of chloroform were used to transfer the steroids to the thin layer plates. The plates

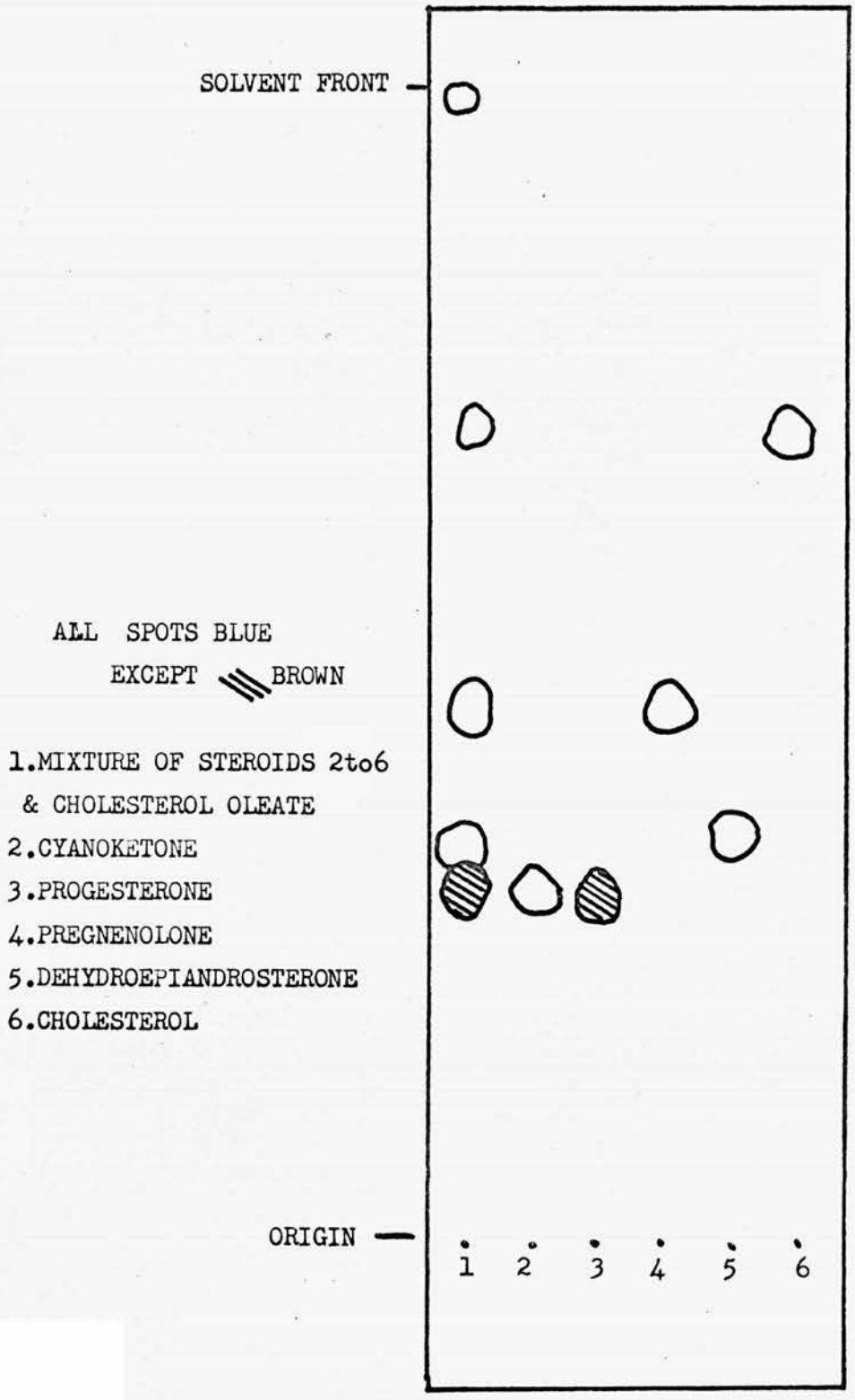


Figure 2.2. The separation of steroids on a thin layer plate run twice in the solvent system di-iso propyl ether-petroleum ether (60/80) - glacial acetic acid (70:30:2, vvv).

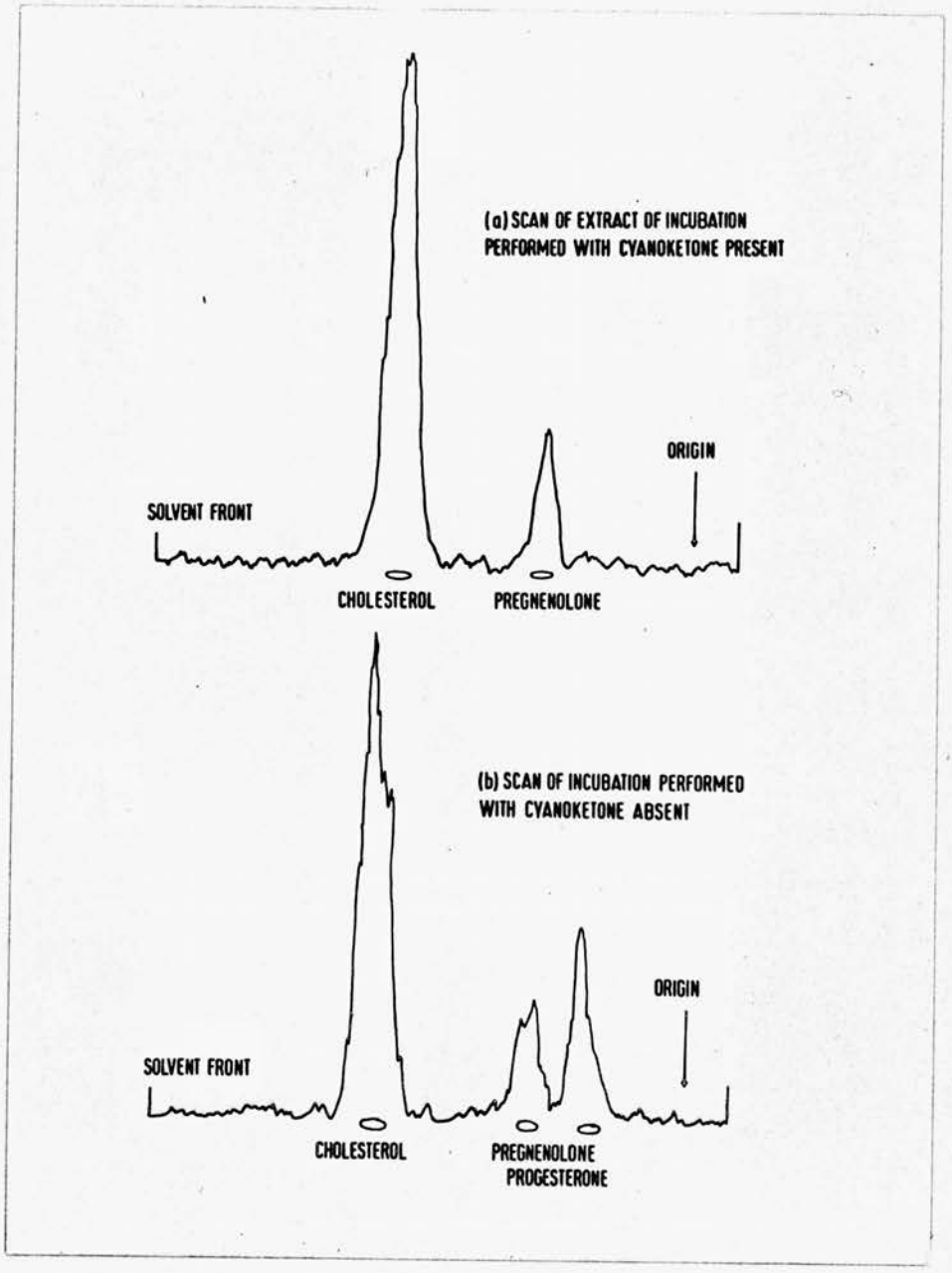


Figure 2.3. Radiochromatograms of the organic extracts of incubations of luteal mitochondria with [4-^{14}C] cholesterol.

were developed by running them twice in a solvent system consisting of di-isopropyl ether, petroleum spirit -60°/80° boiling fraction and glacial acetic acid in the ratio 70 : 30 : 2 by volume. Figure 2.2 shows a typical separation of standard steroids obtained in the solvent system described above. The plates were developed by spraying them with phosphomolybdic acid in ethanol and then heating at 120°C for 5 minutes. The steroids appeared as dark blue spots.

The plates with incubation extracts were scanned with a Panax radioactive thin layer scanner to localise the radioactive peaks. Fig. 2.3 shows scans from extracts of two incubations. Also shown are the positions of standard steroids run at the side of the plate. In incubations performed in the presence of cyanoketone (Fig. 2.3(a)) only one radioactive product, [4 -^{14}C] pregnenolone, is formed from [4 -^{14}C] cholesterol. In the absence of cyanoketone 2 radioactive products [4 -^{14}C] pregnenolone and [4 -^{14}C] progesterone were formed (Fig. 2.3(b)).

(iv) Determination of radioactivity on TLC plate

When the radioactive peaks had been located on the thin layer plates the areas corresponding to the peaks were scraped into scintillation vials. This is a very convenient procedure which obviates the need to extract the steroids from the silica gel. The silica gel does not produce any loss of counting efficiency (Sulimovici (1968)) e.g. by preventing the steroids from dissolving into the scintillation fluid. The scintillation fluid consisted of 4 grms 2,5 diphenyloxazole (P.P.O.) and 0.03 grms 1,4 di [2 - (5 phenyloxazolyl)] benzene (P.O.P.O.P.) in 1 litre of dry toluene containing 5% methanol.

The methanol was added to ensure the radioactive steroids dissolved from the silica gel (Simpson (1967)); 5 mls of scintillation fluid were used when counting the radioactive steroids in a Packard tri-carb 3320 liquid scintillation spectrometer. Under these conditions carbon 14 was counted at an efficiency of 82%. Cholesterol side chain cleavage could now be expressed as percentage conversion of $[4\text{-}^{14}C]$ cholesterol to radioactive products. The radioactive assay and the absolute assay (described below) are summarised in Table 2.3.

(v) Assay of pregnenolone and cholesterol

Pregnenolone and cholesterol were measured in luteal mitochondrial side chain cleavage assays, using the methods described by Simpson et al (1972) for the assays with adrenal mitochondria. The incubations, extractions and chromatography were carried out as described previously for the radioactive assay. The difference between this and the radioactive assay was that $[7\alpha\text{-}^{3}H]$ pregnenolone and $[7\alpha\text{-}^{3}H]$ cholesterol were added to the incubations prior to extraction of steroids. These internal standards were added to correct for procedural losses during the assays. When the steroids had been separated by thin layer chromatography, the areas containing cholesterol and pregnenolone were scraped into centrifuge tubes. The silica gel containing pregnenolone was extracted twice with 5 mls of chloroform/methanol (1 : 1 by vol.). The silica gel containing cholesterol was similarly extracted twice with redistilled acetone.

The pregnenolone containing extracts were taken to dryness and then redissolved in 0.2 mls of acetone; this was then mixed with 2.0 mls of 0.1 M triethanolamine hydrochloride buffer pH 7.4. 0.2 Mls of this pregnenolone extract were taken for counting in a dioxan based

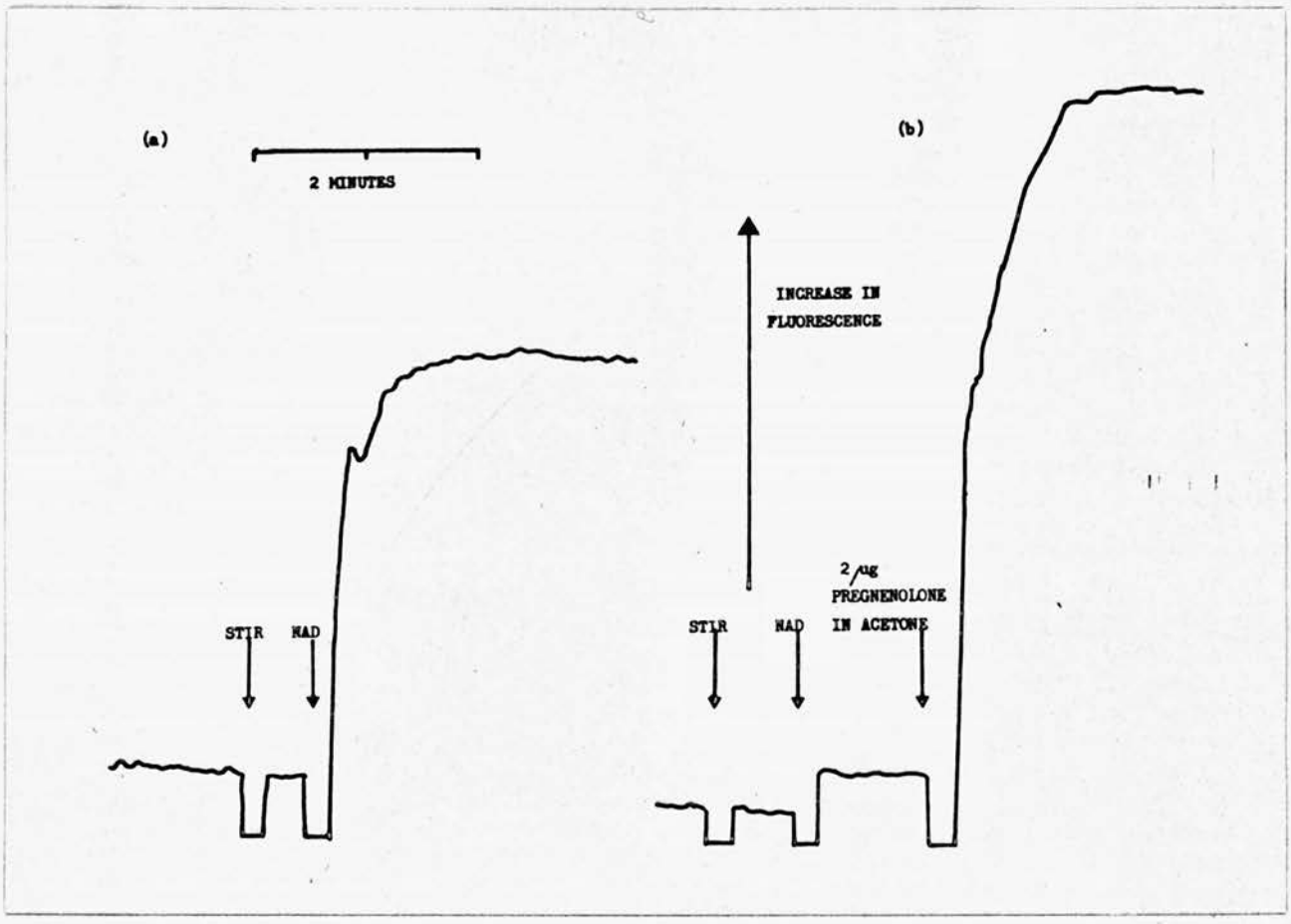


Figure 2.4. The fluorescence changes observed in the assay of pregnenolone; (a) an unknown sample (b) standard pregnenolone solution (2 µg in 10 µl acetone). Note the small increment in fluorescence caused in (b) by the addition of NAD.

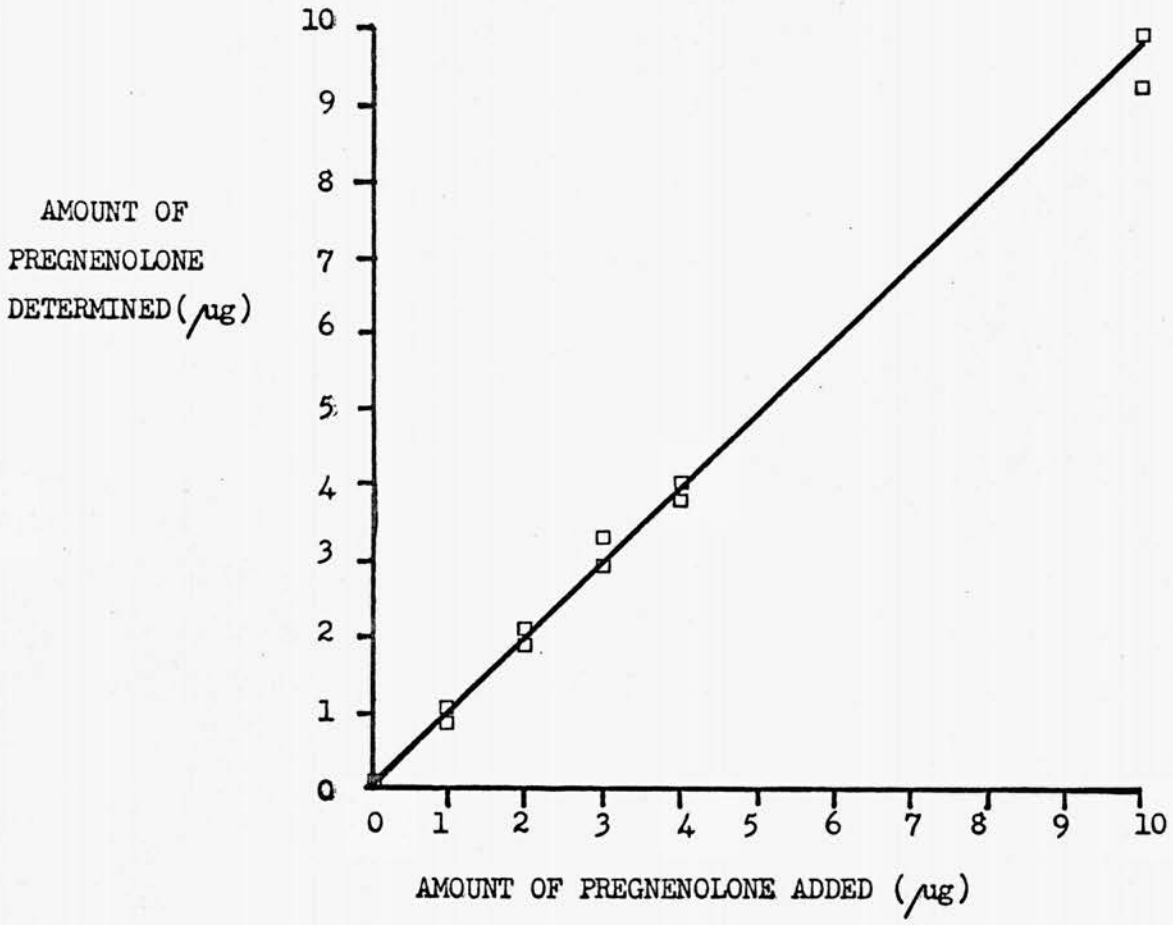


Figure 2.5. Different amounts of pregnenolone were added to samples of luteal mitochondria. The pregnenolone was then measured in these samples using the method of assay described for determination of cholesterol side chain cleavage activity.

scintillation fluid, containing 0.3 grms POPOP, 6 grms PPO and 100 grms napthalene in 1 litre of dioxan (modification of Bray (1960)). This allowed the percentage recovery of the pregnenolone through the extraction procedure to be assessed; this varied from 70% to 80% most of the loss was due to the material left in the centrifuge tube when the plates were spotted. A further 1.0 ml of the pregnenolone extract was mixed with 1.3 mls of 0.1 M triethanolamine hydrochloride buffer pH 7.4 and 0.2 mls of a steroid 3- β-ol-dehydrogenase and Δ^4 - Δ^5 isomerase preparation. This enzyme preparation allowed the conversion of pregnenolone to progesterone with conversion of NAD^+ to NADH. The reaction was initiated by addition of saturating amounts of NAD^+ (10 μl of 100 mg/ml solution). The appearance of NADH was monitored using a Perkin Elmer MPF2A spectrofluorimeter using an exitation wavelength of 340 nm and an emission wavelength of 460 nm. The fluorescence change caused by the unknown pregnenolone solution was quantitated by comparison with the changes produced by standard pregnenolone solutions. The fluorescence produced in the unknown solutions was always corrected for a small increment produced by the addition of the NAD^+. Figure 2.4(a) shows the fluorescence changes produced in the assay of an unknown pregnenolone sample and figure 2.4(b) shows that produced by 2 μg of a standard pregnenolone solution. Known amounts of pregnenolone were added to aliquots of luteal mitochondria (3 mg protein) and taken through the assay procedure (table 2.3); starting at the addition of the methanol - the results of this experiment are shown in figure 2.5. It can be seen that there is a good correlation between the amount of pregnenolone added to the mitochondria and that determined by the assay.

The enzyme used in the assay, the 3 β -ol-dehydrogenase and the Δ^4 - Δ^5 isomerase preparation, was prepared from an extract of freeze dried Pseudomonas Testeroni (Sigma London) (10 mg/ml in 0.1 M Triethanolamine hydrochloride pH 7.4). The buffer and freeze dried bacteria were homogenised and stored at 4°C for 30 minutes before centrifugation at 20,000 x g for 20 minutes. The supernatant was now used without being purified further (Marcus and Talalay (1956)). No alcohols could be in solutions used for the pregnenolone assay, as the enzyme preparation contained alcohol dehydrogenase activity which caused conversion NAD^+ to NADH above that caused by the pregnenolone. Care had also to be taken to make sure the cyanoketone present in mitochondrial incubations was separated from the pregnenolone before assay of the steroid. When the separation was incompletely carried out NAD was not converted to NADH. All samples in which no pregnenolone could be detected had standard pregnenolone solutionsthen added to make sure incomplete separation of cyanoketone had not occurred.

For the assay of cholesterol the acetone extracts, from thin layer plates (table 2.3), were taken to dryness and redissolved in 2.2 mls of acetone; 0.2 mls of this were taken for determination of radioactivity and thus recovery of steroid up to this point. The cholesterol in the rest of the sample was determined by gas liquid chromatography. Pregnenolone acetate (generally 2 μg) was added to the cholesterol sample to act as an internal standard to correct for losses on gas liquid chromatography. After addition of the pregnenolone acetate the acetone extracts were taken to dryness under a stream of nitrogen and redissolved in a small volume of acetone. This solution was then subjected to gas liquid chromatography on a

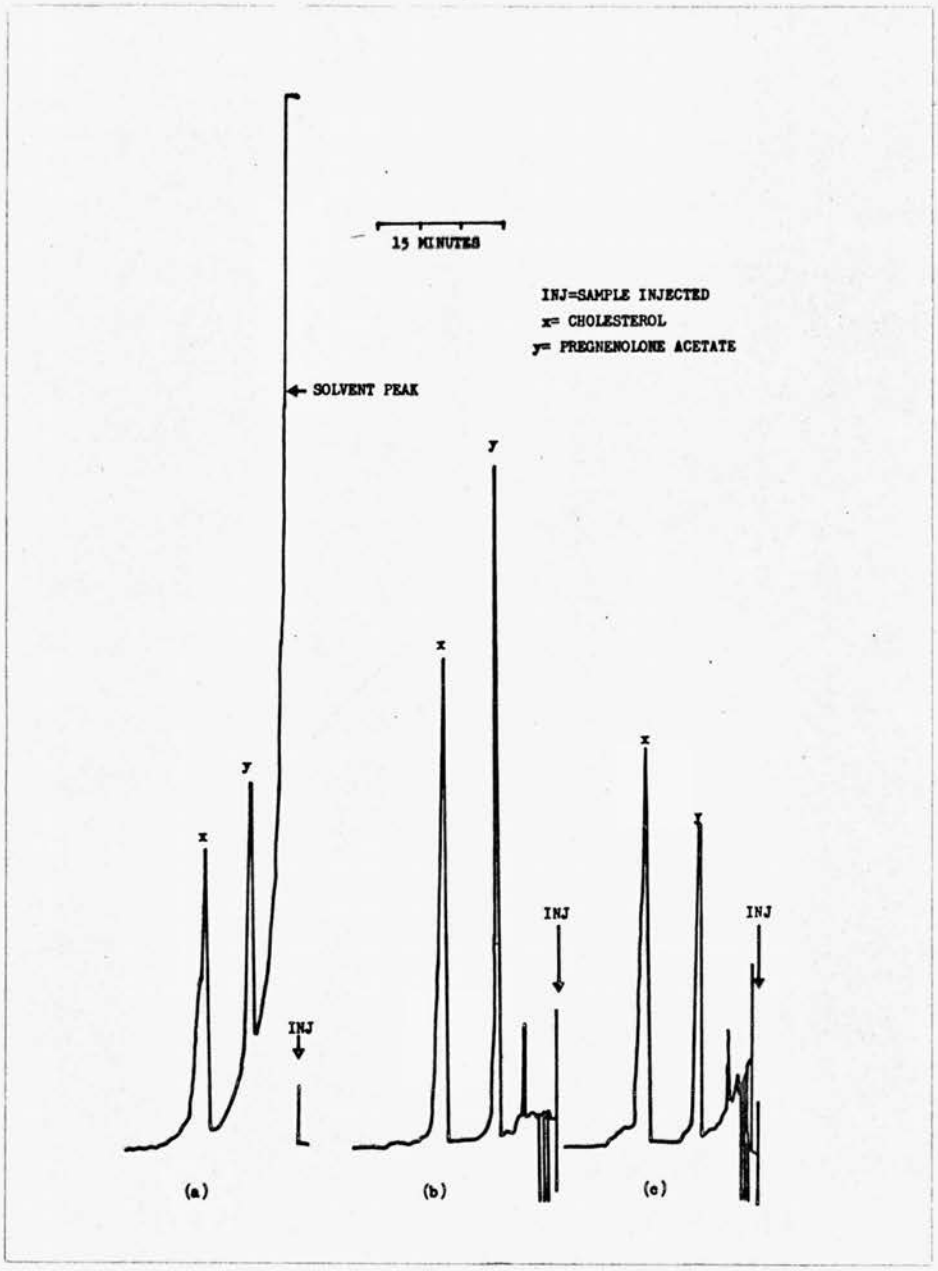


Figure 2.6. Gas Liquid Chromatographs obtained in the assay of cholesterol; (a) Standard mixture of cholesterol and pregnenolone acetate (2:1) injected onto the column in 4 µl of acetone. (b) Standard mixture of cholesterol and pregnenolone acetate (1.5:1) applied to column using Pye 106 auto solids injector. (c) Unknown amount of cholesterol mixed with known amount of pregnenolone acetate and applied to column as in (b).

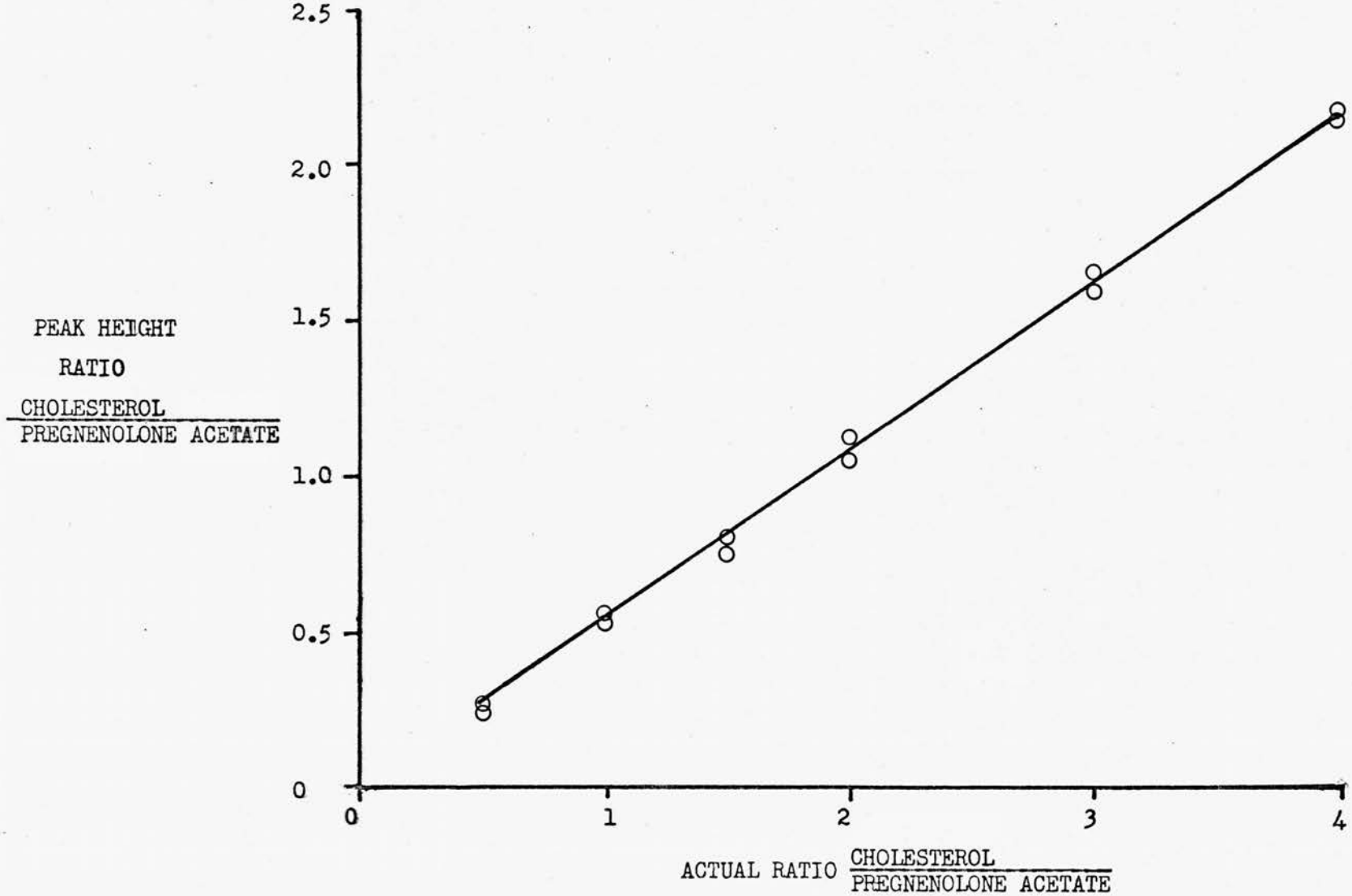


Figure 2.7. A calibration curve constructed for the assay of cholesterol. The actual ratio of cholesterol/pregnenolone acetate in standard solutions is plotted against the ratio of the heights of the peaks the steroids produce on gas liquid chromatography.

Pye 104 gas chromatograph. The column used was 5 feet in length containing 1% SE-30 on Gas Chrom Q 100/120 as support. Nitrogen (30 ml/min) was used as a carrier gas after being dried by passage through a molecular sieve. The column was at 245°C and the chromatograph used a flame ionisation detector. Under these conditions retention times were approximately 14 minutes and 7 minutes for cholesterol and pregnenolone acetate respectively. Solutions were made up of known ratios of cholesterol and pregnenolone acetate. These were subjected to gas liquid chromatography and the ratios of the peak heights of cholesterol and pregnenolone acetate were calculated. The peak heights of cholesterol and pregnenolone acetate were difficult to determine as the peaks appeared on the solvent peak (Fig. 2.6(a)) when the steroids dissolved in acetone were injected onto the column. However when the Pye 106 auto solids injector was used to apply the samples to the column no such problem was encountered as the solvents are evaporated after the steroids are applied to the sample carrier (Figure 2.6(b)). Using this auto solids injector 36 samples could be applied to the column without needing attention during the run. Fig. 2.7 shows a typical calibration curve produced from known ratios by weight of cholesterol and pregnenolone acetate run through the gas liquid chromatograph, using the auto solids injector. As known amounts of pregnenolone acetate were added to the unknown cholesterol samples, the actual amount of cholesterol could be determined from the peak height ratios from gas liquid chromatography (Fig. 2.6(c)). A full scale deflection on the gas liquid chromatographic trace was produced by 0.8 µg of cholesterol.

Cholesterol and cholesterol esters were also determined using a

modified Lieberman-Burchard technique (Kabara (1954)). A minimum of 50 µg of cholesterol were needed for this technique, therefore gas liquid chromatography was more generally used for quantitation of the steroid.

(vi) Assay of cholesterol esters

Mitochondrial cholesterol ester was measured after enzymatic activity had been stopped by the addition of 10 mls of methanol. Tracer amounts of [4 -^{14}C] cholesterol oleate were added to the incubations to allow correction for procedural losses during the isolation of the cholesterol esters. The solvent system described above for the cholesterol side chain cleavage assay was used to isolate cholesterol esters which ran with the solvent front (Fig. 2.2). The cholesterol esters were eluted from the silica gel with acetone, dried under a stream of nitrogen and then hydrolysed at 37°C for 3 hours in 1 ml of 30% methanolic KOH. The reaction mixture was neutralised with 5N HCl and extracted with chloroform. The extract was subjected to thin layer chromatography using the solvent system described above (Fig. 2.2). The free cholesterol was then eluted from the silica gel with 2 x 5 mls acetone and the combined extract taken to dryness. The cholesterol was then redissolved in 2.2 mls of acetone and 0.2 mls of this were kept for radioactivity determinations to assess recovery of cholesterol through the procedure, this was generally 55 —— 65%. The cholesterol content was determined by gas liquid chromatography as described above.

(5) Measurement of cholesterol esterase activity

The method used was that described by Trzeciak and Boyd (1973) for the enzyme activity in rat adrenal 100,000 x g supernatant. Ovarian luteal 100,000 x g supernatant (1 to 2 mgs of protein) was incubated in a reaction mixture containing 50 mM Tris-HCl buffer, 25 mM KCL and 5 mM $MgCl_2$ (pH 7.4) in a final volume of 1 ml. The assay was initiated by addition of 40 μM [4 -^{14}C] cholesterol oleate (30,000 counts/min) in 10 μl of acetone. Incubations were 30 minutes in duration and were carried out in air with vigorous shaking in a water bath. Control incubations contained boiled supernatant. At the end of the incubation period the reaction was stopped with 1 : 1 (v/v) acetone: ethanol (5 mls). This caused the precipitation of protein which was removed by centrifugation, 2.5 mls of the remaining supernatant was taken for the determination of radioactive cholesterol. Carrier cholesterol (0.1 mg) was added to the 2.5 mls of supernatant and the cholesterol precipitated with digitonin (0.5 ml 0.5% in 9 : 11 (v/v) ethanol:water). The digitonin precipitate was centrifuged down and washed with 5 ml 1 : 1 (v/v) acetone:ether and 5 mls of ether. The precipitate was then dissolved in 1 ml of methanol and 0.5 mls of this taken for counting in the toluene based scintillation fluid used for the cholesterol side chain cleavage assay. From the counts in the [4 -^{14}C] cholesterol liberated from the 40 μM [4 -^{14}C] cholesterol oleate it was possible to calculate the cholesterol esterase activity as cholesterol liberated (p mols/min/mg).

(6) Analysis of cholesterol and cholesterol esters in lipid droplets

Cholesterol and cholesterol esters were extracted from the lipid droplets obtained floating on the 10,000 x g and 100,000 x g supernatant (Table 2.1). The same extraction procedure was used as employed for the cholesterol side chain cleavage assays. There was enough cholesterol and cholesterol ester $< 100/\mu g$ each in the lipid droplets to allow their measurement by the Lieberman-Burchard technique.

(7) Optical spectral analysis of luteal mitochondria

Optical difference spectra of rat luteal mitochondria were recorded using an Aminco Chance split beam, dual wavelength, spectrophotometer. The measurements were made using glass cuvettes; the mitochondria being suspended in the medium used for cholesterol side chain cleavage assays at protein concentrations of 1 to 2 mgs/ml.

Cytochrome P450 was measured in mitochondria and microsomes using the method described by Omura and Sato (1964a,b). Mitochondria or microsomes suspended in 6 mls of incubation medium were reduced with a few crystals of dithionite. The reduced suspension was transferred into two,three ml cuvettes and a baseline recorded. The sample cuvette was then gassed with carbon monoxide for 20 seconds resulting, on rescanning, in the appearance of a characteristic peak at 450 nm due to the cytochrome P450.CO complex. The quantity of P450 was measured using an extinction coefficient of 91 mM^{-1} cm^{-1} for the wavelength pair 450 nm - 490 nm (Omura and Sato (1964a)).

Difference spectra induced by steroids were recorded by the successive additions of $1/\mu l$ of a solution of the compound in ethanol

Table 2.4

Extinction coefficients used in measurement of mitochondrial cytochromes

Cytochrome	Wavelength pair	$\triangle A(mM^{-1} cm^{-1})$
a (+ a_3)	605 - 630	16
b	562 - 575	20
c (+ c_1)	551 - 540	19
*P450	450 - 490	91

Cytochromes measured, as described by Cammer and Estabrook (1967), from the reduced - oxidised difference spectrum of luteal mitochondria.

* For cytochrome P450 the reduced Co - reduced difference spectrum was used; Omura and Sato (1964a).

to the sample cuvette, using a Hamilton syringe. An equal volume of solvent was always added to the reference cuvette. After each addition of steroid the spectrum was scanned between 360 nm and 500 nm. This process was usually continued until the spectral change produced had reached a maximum. The binding of the steroids was quantitated by measuring the Δ Absorbance (Δ A) between the peak and trough of the difference spectrum. The apparent binding constant for the interaction and the maximum absorbance change were calculated from a plot of $\Delta A / [\text{steroid}]$ against $[\text{steroid}]$ (Leibman and Estabrook (1971)). A similar procedure was used for compounds which were soluble in water e.g. puromycin. In some experiments titrations were not performed and saturating amounts of steroid were used to produce the ΔA_{max} for the difference spectrum.

Mitochondrial respiratory chain cytochromes were measured using the methods of Cammer and Estabrook (1967b). The extinction coefficients quoted by these authors were used in the calculation of the amounts of cytochromes from their reduced - oxidised difference spectra. Table 2.4 shows the extinction coefficients used in these studies.

(8) Electron paramagnetic resonance spectra

Electron paramagnetic spectra of the rat luteal mitochondria were recorded with a Varian E-4 spectrometer. The samples to be investigated were introduced with a Pasteur pipette into quartz tubes and rapidly frozen to -176°C in liquid nitrogen. They were stored in liquid nitrogen until their spectrum was recorded.

(9) Oxygen electrode

Oxygen uptake by mitochondria was studied using a Clark oxygen electrode (Clark, et al (1954)). The design of the electrode was essentially similar to that described by Estabrook (1967), except the reaction vessel was surrounded by a water jacket. A constant flow of water at 29°C was maintained through this water jacket throughout experiments. The reaction vessel was three mls in volume and the contents were constantly stirred. Changes in oxygen tension measured by the electrode were recorded on a Servoscribe RE 511 recorder. Buffer A was used for the oxygen electrode studies as it was for the spectroscopic and cholesterol metabolism studies. Protein concentrations were 0.5 - 2 mgs/ml in all experiments. Additions of chemicals to the contents of the electrode vessel were made with a Hamilton syringe; additions were 5 or 10 µl in volume. Krebs cycle intermediates ADP, $NAD(P)^+$ and NAD(P)H were dissolved in medium A. Steroids were dissolved in ethanol or acetone. Respiratory control ratios and ADP/O ratios were worked out as described by Chance and Williams (1955).

The calibration of the amount of oxygen dissolved in the buffer was similar to that used by Estabrook and Mackler (1957). Mitochondria were prepared in 0 . 25 M sucrose from 35 grms of rat liver using the method described previously in this section for rat luteal mitochondria. The mitochondria were washed three times with 0.25 M sucrose instead of twice. Sub-particles were prepared from these mitochondria using the method described by Gregg (1967). These sub-particles were used in the electrode to oxidise standard solutions of NADH. The oxygen

concentration in the buffer was then calculated assuming one atom of NADH caused the uptake of one atom of oxygen. The NADH solutions were calibrated by monitoring their absorbance at 340 nm (ϵ mol, $6.22 \times 10^3 \times M^{-1} \times CM^{-1}$).

(10) Protein determinations

Protein determinations were carried out using the biuret method of Layne (1957), using bovine serum albumin as a standard. Correction for turbidity of lipids was made by testing the absorbance of the protein solution at 560 nm in 0.3% NaOH.

(11) Materials

(i) Solvents

All solvents were purchased from BDH Chemicals Ltd. Poole, England and were 'Analar' grade. Diisopropyl ether, ethanol, methanol and acetone were redistilled before use. Ethanol and methanol were refluxed 2 hrs with 0.1% KOH before redistillation. Acetone was refluxed with 0.1% potassium permanganate before redistillation.

(ii) Chemicals

The following chemicals were purchased from the Sigma Chemical Co. London.

NADPH, NADH, NAD^+, ADP, ATP, cyclic AMP, cyclic GMP, cyclic UMP, bovine serum albumin (Type F), isocitrate, malate, succinate, α-ketoglutarate, 5α-androstan - 17 β-ol - 3 - one; pregnenolone, progesterone, cholesterol, triethanolamine hydrochloride, EDTA, EGTA and hydroxysteroid dehydrogenase. The following chemicals were

purchased from BDH Chemicals Ltd., Poole, England.

Sucrose, phosphate buffer (potassium salts) magnesium chloride, potassium chloride, naphthalene and digitonin. The following chemicals were purchased from Boehringer Mannheim: $NADP^+$, glucose-6-phosphate, glucose-6-phosphate dehydrogenase. PPO, POPOP and cycloheximide were obtained from Koch-Light Laboratories, Colnbrook, Bucks; silica gel H from E.Merck; puromycin hydrochloride from Calbiochem, San Diego; chloramphenicol from Parke-Davis and dexamethasone from Merck-Sharp and Dohme. Pregnenolone acetate, pregnant-mare serum gonadotrophin (Gestyl) and human chronic gonadotrophin (Pregnyl) were obtained from Organon. Gas chromatography column packing (1% SE-30 by weight on 100/120 mesh gas chrom Q) was obtained from Applied Science Laboratories Inc. Cyanoketone was a gift from Dr. J.L. McCarthy (Southern Methodist University, Dallas, Texas). LH-S18 ovine was a gift from N.I.H. Bethesda U.S.A. [4 -^{14}C] cholesterol (50 μC/μmol) [7 α - ^{3}H] cholesterol (9.4 Ci/m mol) [7 α - ^{3}H] pregnenolone (9.4 Ci/m mol) were purchased from the Radiochemical Centre (Amersham). [4 -^{14}C] Cholesterol oleate was synthesised by Mr. G. Beckett using the method of Goodman (1969). All other solvents and chemicals used were of the purest grade obtainable.

Summary

(a) A preparation of sub-cellular fractions from ovaries of rats, pretreated by the method of Parlow (1958), is given.

(b) A radioactive tracer assay and an absolute assay can be used to assay cholesterol side chain cleavage in rat luteal mitochondria.

(c) The measurement of cholesterol esterase activity in the luteal cell supernatant is described.

(d) Optical difference spectroscopy as a method of investigating the cytochromes in rat luteal mitochondria and microsomes is outlined.

(e) Use of the Clark oxygen electrode to measure oxygen uptake by luteal mitochondria is described.

(f) The sources of all chemicals, solvents and hormones used in this study are given.

CHAPTER 3

GENERAL PROPERTIES OF THE LUTEAL MITOCHONDRIAL CHOLESTEROL SIDE CHAIN CLEAVAGE SYSTEM

Introduction

Methods for the determination of cholesterol side chain cleavage activity in rat luteal mitochondria have been described in chapter 2. This chapter describes the application of these methods to luteal mitochondria in differing incubation conditions. Thus the most favourable incubation conditions for measuring pregnenolone production are investigated and discussed. The role of calcium ions, cyclic nucleotides and prostaglandin $F_{2\alpha}$ in the control of luteal steroidogenesis are discussed.

Products of cholesterol side chain cleavage in rat luteal mitochondria

When mitochondria from rat corpora lutea are incubated with a source of reducing equivalents (NADPH) it has been reported [4-^{14}C] cholesterol is converted to [4-^{14}C] pregnenolone and [4-^{14}C] progesterone with variable amounts of [4-^{14}C] 20α-hydroxy-progesterone (Sulimovici and Boyd (1968a)).

In porcine and bovine luteal mitochondria [4-^{14}C] cholesterol is converted to [4-^{14}C] pregnenolone and [4-^{14}C] progesterone in the presence of reducing equivalents (Robinson and Stevenson (1971a); Uzgiris *et al* (1971)) supplied by Krebs Cycle intermediates or long chain fatty acids (Robinson and Stevenson (1971b)).

Mitochondria were prepared from rat corpora lutea and incubated in medium A with [4-^{14}C] cholesterol and malate. Two radioactive products could be identified when the extracts of these incubations

Table 3.1

Recrystallisation of [4-^{14}C] pregnenolone and [4-^{14}C] progesterone to constant specific activity

Incubations without cyanoketone

Stage of purification	Crystals
	[4-^{14}C]Pregnenolone disintegrations $min^{-1} \times mg^{-1}$
after addition of standard pregnenolone	143 141
1st recrystallisation	139 176
2nd recrystallisation	141 140
	[4-^{14}C]Progesterone disintegrations $min^{-1} \times mg^{-1}$
after addition of standard progesterone	2237 2061
1st recrystallisation	2015 2070
2nd recrystallisation	2198 2120
3rd recrystallisation	2201 2190

Incubations with cyanoketone

	[4-^{14}C]Pregnenolone disintegrations $min^{-1} \times mg^{-1}$
after addition of standard pregnenolone	3681 3637
1st recrystallisation	3687 3769
2nd recrystallisation	3761 3766

Areas of radioactivity corresponding to standard pregnenolone and progesterone were scraped from thin layer plates of incubation extracts. The radioactive steroids were extracted and mixed with authentic steroid (Organon). Recrystallisations were then carried out from redistilled methanol. Duplicate determinations of specific activity for each recrystallisation are shown in the table.

were subjected to thin layer chromatography (Figure 2.3). These products corresponded to standard pregnenolone and progesterone. When cyanoketone was present in the incubations one product was formed; this corresponded to standard pregnenolone (Figure 2.3(a)). Cyanoketone is known to prevent the conversion of pregnenolone to progesterone in rat luteal mitochondria (Sulimovici and Boyd (1969a)). The identity of the steroids produced, when the luteal mitochondria were incubated with malate, was confirmed by recrystallising them to constant specific activity with authentic steroids. The results of this are shown in table 3.1. Recrystallisations were performed from methanol. After the radioactivity had been determined in each steroid sample a known weight of standard $[1\text{-}^{14}C]$ hexadecane was added to the scintillation vial to determine the counting efficiency. This allowed specific activity to be determined as disintegrations min/mg. Thus these results are in agreement with those of Sulimovici and Boyd (1968a) who found $[4\text{-}^{14}C]$ pregnenolone and $[4\text{-}^{14}C]$ progesterone were produced from $[4\text{-}^{14}C]$ cholesterol in rat luteal mitochondria. However no $[4\text{-}^{14}C]$ 20 α-hydroxy-progesterone could be detected in incubations. Sulimovici and Boyd (1968a) reported that small amounts of $[4\text{-}^{14}C]$ 20 α-hydroxy-progesterone could be detected in incubations of rat luteal mitochondria using NADPH as the source of reducing equivalents. Also Huang and Pearlman (1962) and Channing and Villee have reported that homogenates of rat corpora lutea convert $[7\alpha\text{-}^{3}H]$ cholesterol to radioactive pregnenolone, progesterone and 20 α-hydroxy-progesterone.

Cyanoketone addition to incubations results in the formation of pregnenolone as the only steroid product of cholesterol side chain cleavage in rat luteal mitochondria. No measurements of the

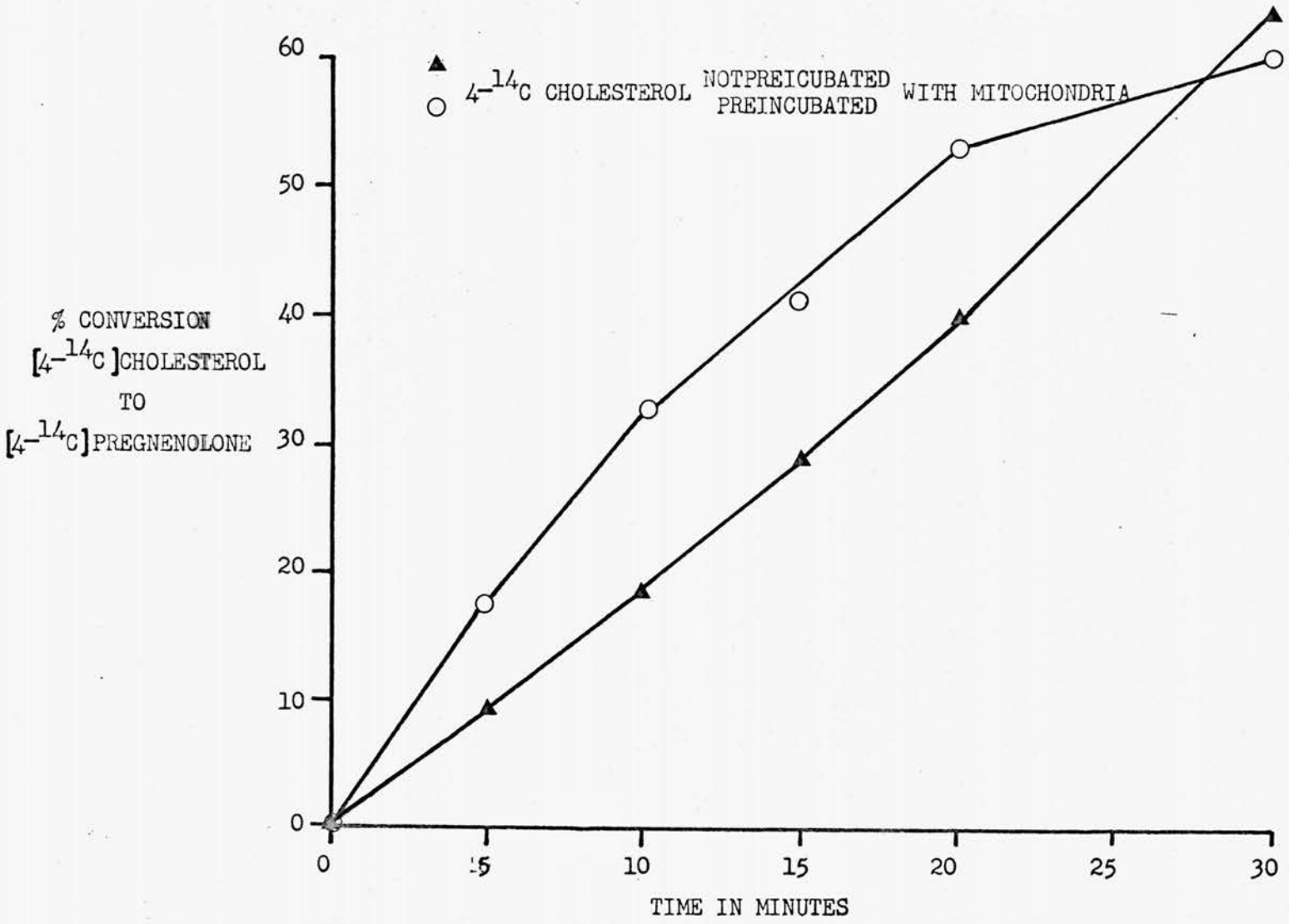


Figure 3.1. The effect of preincubation on the metabolism of [4-14C] cholesterol by luteal mitochondria. [4-14C] pregnenolone production was measured in luteal mitochondria to which [4-14C] cholesterol had been added 10 minutes before or at the same time as malate.

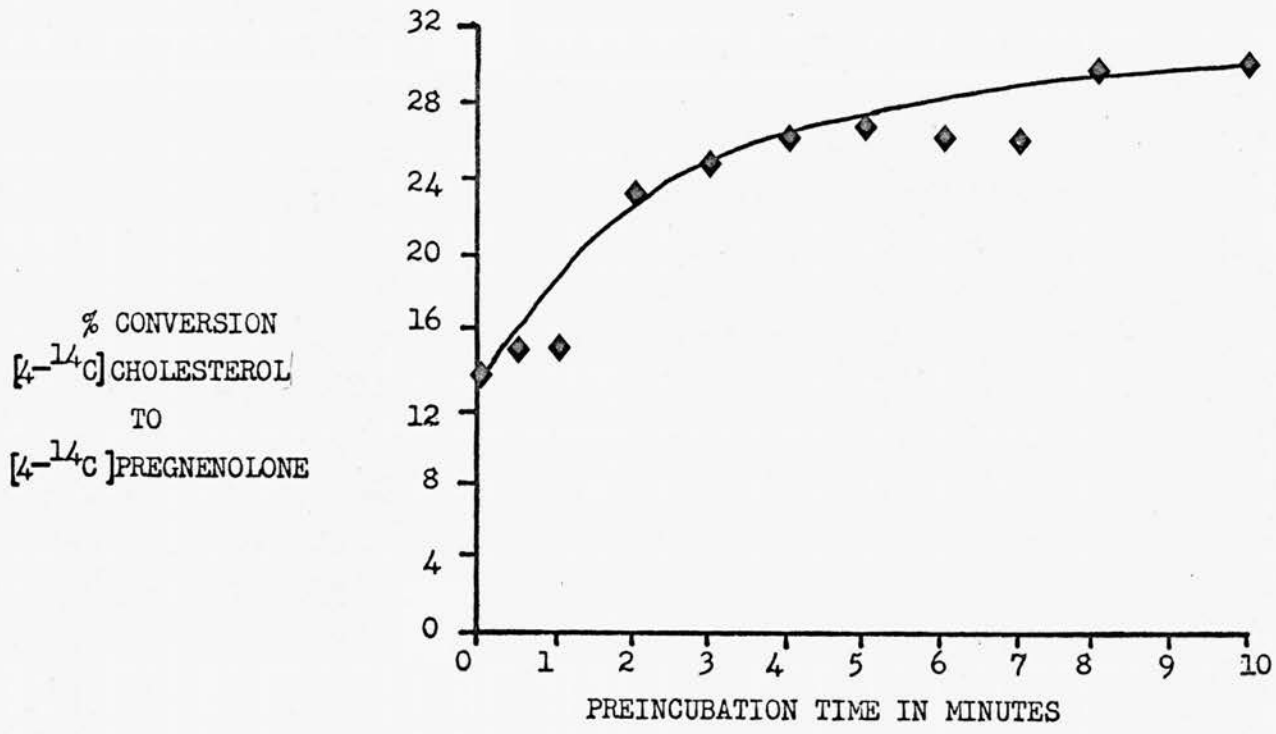


Figure 3.2. The effect of different preincubation times on the metabolism of [4-^{14}C] cholesterol by luteal mitochondria. Luteal mitochondrial [4-^{14}C] pregnenolone production was measured in ten minute incubations. [4-^{14}C] cholesterol was preincubated with the mitochondria for differing times before initiation of cholesterol side chain cleavage with malate.

isocaproic acid side chain, liberated from the cholesterol during side chain cleavage, were made. The fact the steroid products of mitochondrial incubations could be restricted to pregnenolone had important implications. Pregnenolone can be conveniently assayed (Simpson et al (1972)), thus the absolute rate of cholesterol side chain cleavage can be measured by determination of one rather than two or three products.

Effect of preincubation with [4 -^{14}C] cholesterol on the rate of cholesterol side chain cleavage measured by the radioactive assay

Luteal mitochondria were prepared and incubated in medium A, with malate as the electron donor. [4 -^{14}C] Cholesterol was added to the incubations ten minutes before or at the same time as the malate. Figure 3.1 shows the time course of [4 -^{14}C] pregnenolone production in these incubations, performed in the presence of cyanoketone. It will be noticed from the graph that preincubation had the effect of increasing the formation of [4 -^{14}C] pregnenolone during the earlier stages of the incubation. When the [4 -^{14}C] cholesterol was preincubated with the mitochondria for ten minutes [4 -^{14}C] pregnenolone production began to fall five to ten minutes after the addition of malate. When the preincubation period was omitted the formation of [4 -^{14}C] pregnenolone was linear for at least thirty minutes. Figure 3.2 shows the effect of differing preincubation times on the appearance of [4 -^{14}C] pregnenolone in subsequent 10 minute incubations of luteal mitochondria. The effects of 7 to 10 minute preincubations with [4 -^{14}C] cholesterol appeared to be maximal.

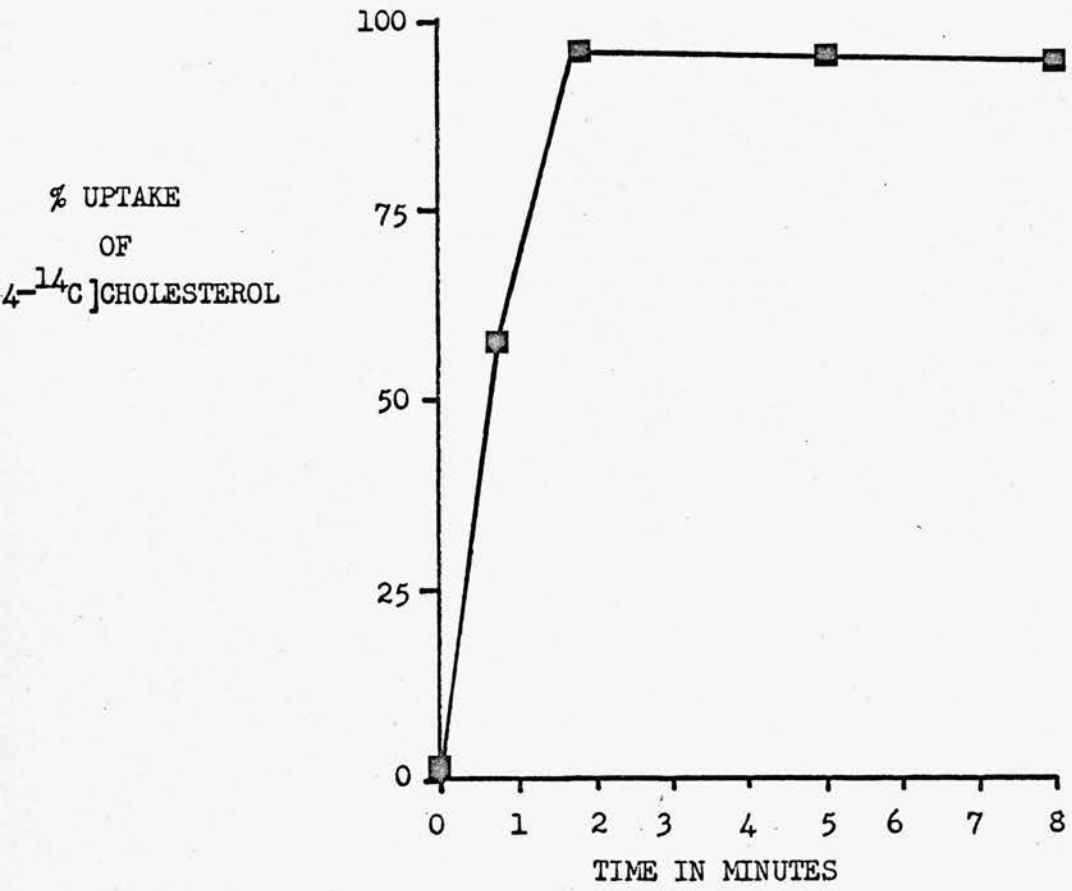


Figure 3.3. The uptake of [4-^{14}C] cholesterol by rat luteal mitochondria. [4-^{14}C] cholesterol in 5 µl acetone was added to luteal mitochondria suspended in medium A. Samples were taken from the incubation and subjected to millipore filtration. The radioactivity bound by the mitochondria was then determined.

An explanation of this preincubation effect is that the metabolism of the added tracer cholesterol was limited by its binding or transport to the intra-mitochondrial cholesterol side chain cleavage enzymes. The preincubation would allow the added [4 -^{14}C] cholesterol to equilibrate with the endogenous pool of cholesterol; this appears to take < 7 minutes at 29°C. As can be seen from Figure 3.1 no conversion of [4 -^{14}C] cholesterol to [4 -^{14}C] pregnenolone was observed during the preincubation period, probably due to the washing of endogenous substrates from the mitochondria during their preparation.

As there appeared to be some constraint on the access of the [4 -^{14}C] cholesterol to the mitochondrial enzymes which metabolise it the uptake of radioactive cholesterol by luteal mitochondria was investigated. [4 -^{14}C] Cholesterol (60,000 cpm in 5 µl acetone) was added to luteal mitochondria incubated in medium A. No malate was added but samples of the incubation were taken at 0.75, 1.75, 5 and 8 minutes. These samples were immediately subjected to millipore filtration. The amount of radioactivity bound to the mitochondria was calculated by determining how much radioactivity had passed through the filter. Aliquots of the filtrate were counted in dioxan based scintillation fluid. Blank incubations were run without mitochondria to find how much [4 -^{14}C] cholesterol was bound by the millipore filter itself. This amounted to less than ten percent of the total [4 -^{14}C] cholesterol added and was corrected for when calculating the amount bound to the mitochondria. Figure 3.3 shows the results of the experiment. Over ninety percent of the [4 -^{14}C] cholesterol was bound to the mitochondria within 1.75 minutes of it

being added to the incubation mixture. In this [4 -^{14}C] cholesterol uptake experiment it was assumed the added cholesterol would not be metabolised, as no source of reducing equivalents had been included in the incubations. Although the mitochondria rapidly took up [4 -^{14}C] cholesterol there was a time lag before it became available to the enzymes which cause its metabolism. Seven minutes preincubation at 29°C are needed before the [4 -^{14}C] cholesterol equilibrates with the cholesterol on the cholesterol side chain cleavage enzymes (Figure 3.2), despite the fact the mitochondria took up the [4 -^{14}C] cholesterol within two minutes of it being added to incubations. This lag may have been due to the transfer of cholesterol from the outer to the inner mitochondrial membrane. In the mitochondria from steriodogenic tissue such as leydig tumour cells, hog and bovine adrenal cortex and pig and rat corpus luteum, cytochrome P450 a component of the cholesterol side chain cleavage enzyme system has been found in the inner mitochondrial membrane (Moyle et al (1973b); Satre et al (1969); Yago and Ichii (1969); Sottocasa and Sandri (1970); Stevenson and Taylor (1971)). Little of the cholesterol in mitochondria from steroid hormone producing leydig tumour cells has been found in the inner mitochondrial membrane (Moyle et al (1973b)). Most of the cholesterol found in isolated liver mitochondria is found in the outer membrane (Graham and Green (1970)). Thus the apparent delay in the metabolism of [4 -^{14}C] cholesterol after it has been bound by the luteal mitochondria (Figs. 3.1 and 3.2) may bedue to it mixing with the endogenous cholesterol in the outer mitochondrial membrane and a subsequent transfer to the cytochrome P450 of the inner membrane. Kahnt et al (1974) have suggested penetration of cholesterol into the

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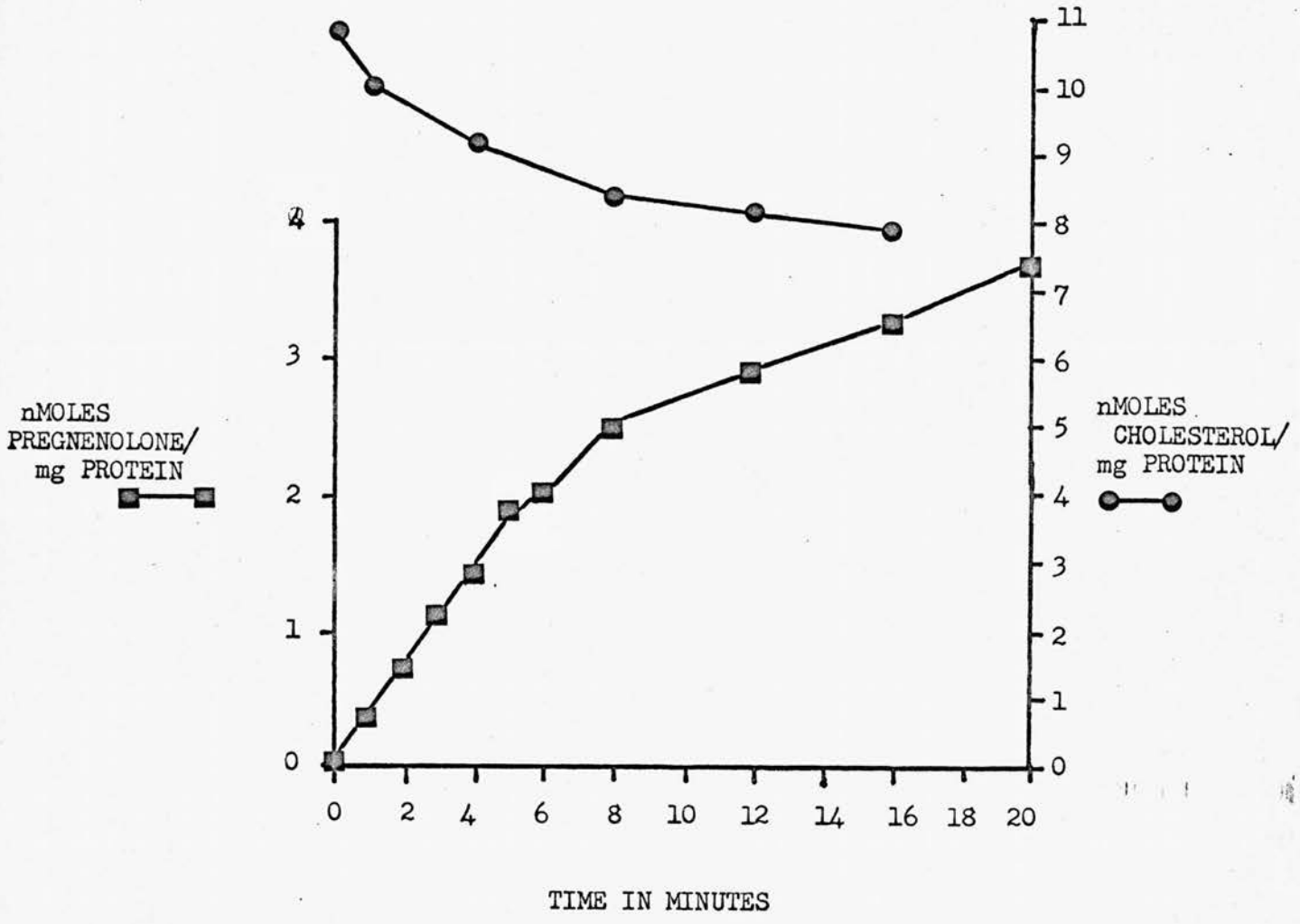


Figure 3.4. The time course of pregnenolone appearance from endogenous cholesterol and the depletion of endogenous cholesterol in luteal mitochondria. Cholesterol and pregnenolone were determined in luteal mitochondria at differing times after cholesterol side chain cleavage had been initiated by addition of malate.

Table 3.2

Measurement of luteal mitochondrial cholesterol side chain cleavage

	Cholesterol Esters	Free Cholesterol	Pregnenolone	% conversion of cholesterol into pregnenolone	
	nMoles steroid/mg protein			Total	Radioactive
Time zero	7.0 ± 0.6	12.9 ± 0.3	not detectable	-	-
After 5 minutes incubation	6.8 ± 1.3	9.6 ± 0.4	3.6± 0.1	28.9±1.4	27.3±0.8

Experimental procedure is described in the text.

Results: Mean ± Standard error of mean for six incubations.

mitochondrion is the rate limiting step in steroidogenesis in bovine adrenal cortex mitochondria. Previously Simpson et al (1971) postulated transport of cholesterol from other mitochondrial sites to the cholesterol side chain cleavage cytochrome P450 was the rate limiting step in cholesterol metabolism in bovine adrenal cortex mitochondria. A similar situation may occur in rat luteal mitochondria. Further evidence for this is discussed in chapters six and seven.

Measurement of cholesterol side chain cleavage in rat luteal mitochondria by the appearance of pregnenolone

The time course of pregnenolone formation in luteal mitochondria, incubated in medium A, is shown in figure 3.4. The reaction was initiated by the addition of 5 mM L - malate in the presence of 6.4 µ M cyanoketone. As can be seen from the graph the time course of the pregnenolone appearance was similar to that of [4 - ^{14}C] pregnenolone observed in figure 3.1 when the [4 - ^{14}C] cholesterol had been preincubated with the mitochondria for 10 minutes before the addition of malate. In both cases the rate of pregnenolone production begins to fall off after five minutes. Thus by preincubating the [4 - ^{14}C] cholesterol with the mitochondria for 10 minutes before addition of malate the actual time course of cholesterol side chain cleavage could be measured as the appearance of [4 - ^{14}C] pregnenolone. This assumption was investigated in more detail as shown in table 3.2. Mitochondria from the same luteal preparation were preincubated for 10 minutes with [4 - ^{14}C] cholesterol in medium A and then malate was added to initiate cholesterol side chain cleavage. Six incubations

were stopped immediately and six were stopped 5 minutes after malate addition. Cholesterol ester, cholesterol and pregnenolone were determined in these incubations. The ^{14}C counts in the cholesterol and pregnenolone were also determined. Tritiated cholesterol and pregnenolone were used as recovery markers during the extractions and assays. The percentage conversion of [4 -^{14}C] cholesterol to [4 -^{14}C] pregnenolone in the same samples as the total steroids were being measured could be calculated. It will be noted (table 3.2) that the actual percentage conversion of cholesterol to pregnenolone is very similar to that measured as percentage conversion of [4 -^{14}C] cholesterol to [4 -^{14}C] pregnenolone. The depletion of cholesterol during the five minute incubations, 3.3 n moles/mg protein, was very similar to the formation of pregnenolone, 3.6 n moles/mg protein. The depletion of cholesterol during a typical incubation is shown in figure 3.4. The formation of pregnenolone in this incubation reflects very closely the cholesterol disappearance. Probably cholesterol rather than cholesterol ester is the substrate for cholesterol side chain cleavage in these mitochondria. Further support for this hypothesis can be seen in table 3.2. The cholesterol ester content of the mitochondria did not change significantly during the five minute incubation. Moyle et al (1973b) have concluded that cholesterol esters are not the immediate substrate for cholesterol side chain cleavage in rat leydig tumour cell mitochondria. Porcine luteal mitochondria can however metabolise added [4 -^{14}C] cholesterol oleate to [4 -^{14}C] cholesterol and then [4 -^{14}C] pregnenolone and [4 -^{14}C] progesterone over two hour incubation periods (Robinson and Stevenson (1971b)). The endogenous cholesterol esters of the rat luteal mitochondrion however do not appear to be hydrolysed in five minute

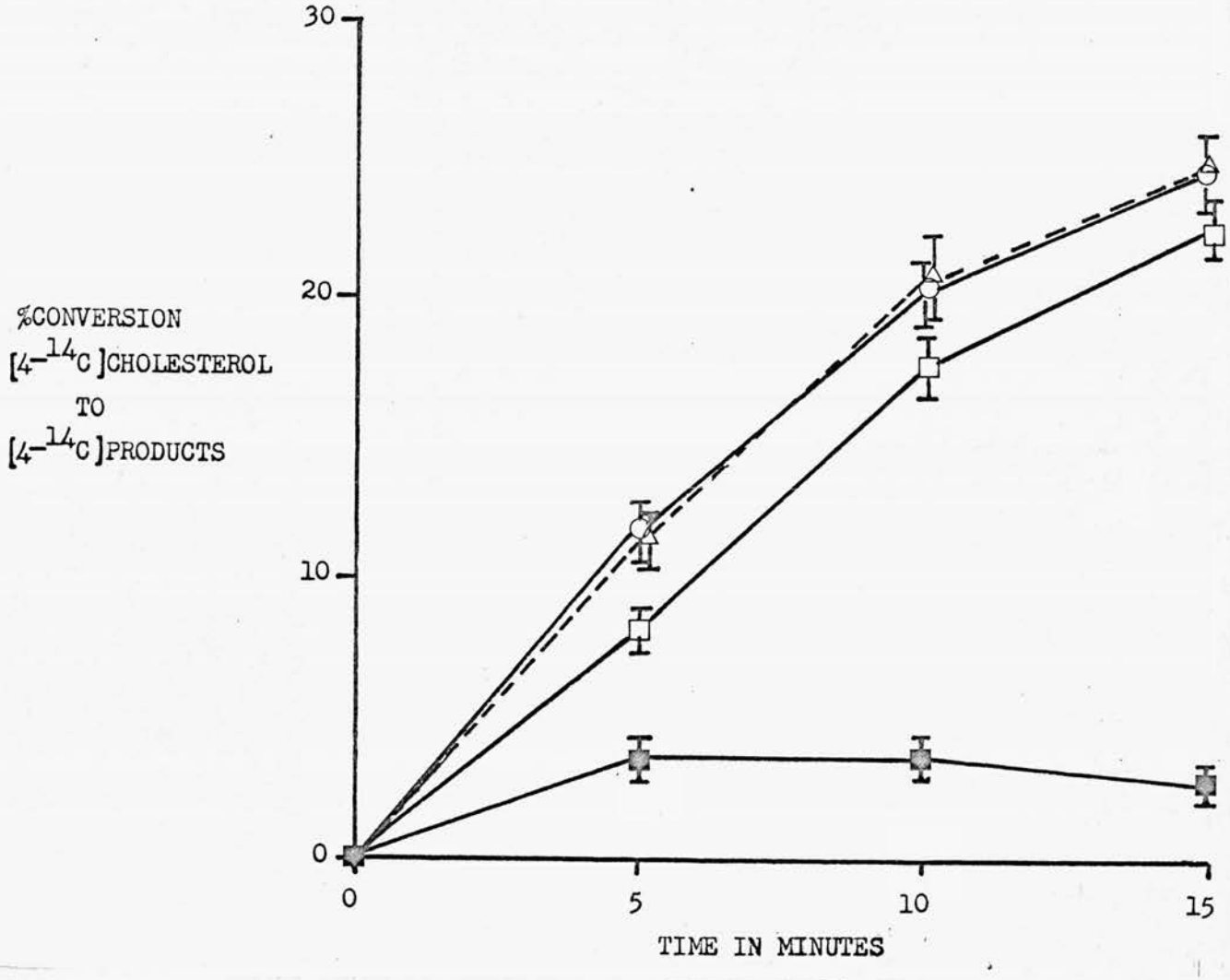


Figure 3.5. The effect of cyanoketone on the time course of cholesterol side chain cleavage of [4-^{14}C] cholesterol. Side chain cleavage was initiated in mitochondria by malate at time zero after they had been preincubated for 10 minutes with [4-^{14}C] cholesterol. △ Total [4-^{14}C] products, ■ [4-^{14}C] pregnenolone and □ [4-^{14}C] progesterone formed in the absence of cyanoketone. ○ [4-^{14}C] pregnenolone formed in the presence of cyanoketone. Results Mean ± SEM for four determinations.

incubations to provide substrate cholesterol for cholesterol side chain cleavage.

Cholesterol side chain cleavage can therefore be conveniently measured as the percentage conversion of [4 -^{14}C] cholesterol to [4 -^{14}C] pregnenolone when the mitochondria have been preincubated with the [4 -^{14}C] cholesterol before the addition of malate.

The effect of cyanoketone on the rate of cholesterol side chain cleavage in rat luteal mitochondria

Cyanoketone causes an inhibition of 3 β-hydroxysteroid dehydrogenases in extracts of the bacterium Pseudomonas Testosteroni and steroidogenic tissue in the rat (Ferrari and Arnold (1963); Goldmann et al (1965); Neville and Engel (1968); McCarthy et al (1966); Sulimovici and Boyd (1969a)). In incubations of luteal mitochondria, cyanoketone was included to prevent the immediate steroid product of side chain cleavage, pregnenolone, from being converted to progesterone. This results in a build up of pregnenolone in incubations. It was therefore of interest to observe if this pregnenolone had any effect on the rate of cholesterol side chain cleavage.

Luteal mitochondria were preincubated in medium A with [4 -^{14}C] cholesterol in the presence and absence of cyanoketone. Malate was then added to the incubations and the time course of the appearance of radioactive products was observed. Figure 3.5 shows the conversion of [4 -^{14}C] cholesterol to products in the presence and absence of cyanoketone. The experiment shows that the total conversion of [4 -^{14}C] cholesterol to products in the presence and absence of cyanoketone was the same. The total percentage conversion to [4 -^{14}C] pregnenolone

and [4 -^{14}C] progesterone in the absence of cyanoketone was the same as the percentage conversion to [4 -^{14}C] pregnenolone in its presence. In the absence of cyanoketone [4 -^{14}C] progesterone was the major product little [4 -^{14}C] pregnenolone remaining. Thus the addition of cyanoketone had no effect on the measured rate of cholesterol side chain cleavage, despite the fact that it blocked further metabolism of the product of the reaction resulting in a large rise in the mitochondrial pregnenolone concentration. In rat adrenal cortex mitochondria and homogenates prevention of pregnenolone metabolism by cyanoketone has no effect on the rate of cholesterol side chain cleavage (Simpson et al (1972); Farese (1970)). Farese (1970) found at high cyanoketone concentrations (above 1 mM) there was a direct inhibition of cholesterol side chain cleavage, however this concentration is over 100 times greater than that used in these studies. Cholesterol side chain cleavage in rat luteal mitochondria can therefore be measured by following the appearance of pregnenolone in the presence of cyanoketone. This method has previously been applied to rat adrenal mitochondria (Simpson et al (1972)). In all measurements of cholesterol side chain cleavage activity which will be reported in this thesis, cyanoketone was included in incubations to restrict the steroid product of the reaction to pregnenolone.

Pregnenolone has been shown to inhibit cholesterol side chain cleavage activity in acetone powders of bovine adrenal cortex and corpus luteum mitochondria (Koritz and Hall (1964a,b); Hall and Koritz (1964a); Ichii et al (1963)). In light of this evidence and further work on the inhibitory effects of pregnenolone, Koritz and Kumar (1970) have advanced the hypothesis that in the rat adrenal cortex the rate

Table 3.3

Inhibition of ovarian mitochondrial cholesterol side chain cleavage in vitro by addition of pregnenolone.

Pregnenolone/uM	% conversion 5 minutes	% inhibition
0	11.0	0
2.5	11.0	0
5	9.5	14
10	7.8	23
20	6.8	37
40	5.1	53

Cholesterol side chain cleavage was measured as % conversion of [4-^{14}C] cholesterol to [4-^{14}C] pregnenolone. Pregnenolone and malate were added after mitochondria were preincubated for 10 minutes with [4-^{14}C] cholesterol. Results are the average of duplicate assays.

limiting step in steroidogenesis is the efflux of pregnenolone from the mitochondrion. ACTH was postulated to stimulate steroidogenesis by allowing the efflux of pregnenolone from the mitochondrion. Such a control of luteal steroidogenesis under the experimental conditions outlined in this thesis would appear not to exist. The amount of pregnenolone produced in incubations can be altered by omitting the 3 β-hydroxysteroid dehydrogenase inhibitor cyanoketone from the incubation medium (figure 3.5). This allows pregnenolone to be converted to progesterone without affecting the rate of cholesterol side chain cleavage. When the rate of pregnenolone production begins to decline in incubations after five to ten minutes (figure 3.4) the concentration of pregnenolone is 2 - 3 μ M. It was therefore of interest to see what effect added pregnenolone had on the rate of cholesterol side chain cleavage. The results of such an experiment are shown in table 3.3. Luteal mitochondria were preincubated in medium A for ten minutes before the addition of malate and pregnenolone. As can be seen from the results 5 μ M pregnenolone was needed to produce appreciable inhibition of cholesterol side chain cleavage, 50% inhibition being produced by addition of 40 μ M pregnenolone. Thus the fall in the rate of pregnenolone production in incubations encountered after five to ten minutes is unlikely to be due to product inhibition. Mitochondrial pregnenolone concentrations <u>in vivo</u> will probably not build up due to the presence of active 3 β-hydroxysteroid and Δ^4 - Δ^5 isomerase enzymes in the mitochondria and microsomes (Sulimovici and Boyd (1969a)).

Table 3.4

Subcellular distribution of the cholesterol side chain cleavage system in ovaries from superovulated rats

Fraction	% conversion(30mins)	pregnenolone(nMoles x mg protein^{-1} x 10 mins)
Mitochondria	47.5%	2.8
12000 x g pellet	0.5%	+
Microsomes	0.2%	ND.
100,000 x g supernatant	N.D.	N.D.

N.D. None detectable

\+ not determined

Cholesterol side chain cleavage activity was determined in each cellular fraction as described in the methods section. Each incubation contained 6.4 μM cyanoketone; where appropriate [4-^{14}C] cholesterol was preincubated with the cellular fraction for 10 minutes before addition of malate or NADPH generator (1mM $NADP^+$ 8mM DG-6-P (final concentrations), D-Glucose-6-Phosphate dehydrogenase 0.5 i.u./3mls.).

Subcellular distribution of the cholesterol side chain cleavage enzymes in the rat corpus luteum

The cholesterol side chain cleavage enzyme complex has been found in the mitochondrial fraction of bovine and superovulated rat corpora lutea (Hall and Koritz (1964b); Yago et al (1967a); Sulimovici and Boyd (1968a); reviewed by Sulimovici and Boyd (1969b)). In contrast to the results mentioned above Flint and Armstrong (1971a,b), (1972) have reported cholesterol side chain cleavage activity in microsomes of bovine and rat corpora lutea. They do not however attribute much quantitative importance to this pathway (Flint and Armstrong (1972)). The subcellular distribution of the cholesterol side chain cleavage enzymes was therefore investigated in the superovulated rat ovary. Mitochondria, 12,000 x g pellet, microsomes and 100,000 x g supernatant were prepared as described in chapter 2. Cholesterol side chain cleavage was assayed in each fraction. Subcellular fractions prepared from 500 mg of luteal tissue were used in each incubation. Each fraction was preincubated for ten minutes with /4 -^{14}C/ cholesterol before initiation of cholesterol side chain cleavage, with malate in the case of mitochondria and NADPH generator with all other fractions. Incubations were carried on for one half hour in medium A before they were terminated with methanol. The percentage conversion of /4 -^{14}C/ cholesterol to /4 -^{14}C/ pregnenolone was determined in each fraction. In a separate experiment the total pregnenolone formation was determined in each fraction after a ten minute incubation. The results are summarised in table 3.4. The measurement of total pregnenolone formed confirmed that the lack of formation of /4 -^{14}C/ pregnenolone in some fractions was not due to dilution of the precursor /4 -^{14}C/ cholesterol

in a large pool of unlabelled cholesterol. Hence it is concluded there is no significant cholesterol side chain cleavage activity in any cellular fraction other than the mitochondria. This confirms the observations of Sulimovici and Boyd (1968a) but disagrees with the findings of Flint and Armstrong (1971a,b), (1972). Flint and Armstrong (1971b) regard finding cytochrome P450 in the microsomal fraction as supportive evidence that there is cholesterol side chain cleavage activity present in this fraction. However this cytochrome is associated with other steroid interconversions e.g. aromatisation (Thompson and Siiteri (1973)), which is a necessary step in the formation of oestrogens. Horikoshi and Wiest (1970) have demonstrated oestrogen formation occurs in the superovulated rat ovary. Siekevitz (1963) has reported that during homogenisation mitochondria can break up and the resultant particles can be found in the microsomal fraction. This may account for the observations of Flint and Armstrong (1971a,b), (1972) although they found little evidence of mitochondrial contamination in their microsomes by using marker enzyme techniques. The mitochondrial markers used were cytochrome oxidase and glutamate dehydrogenase. The ratio, mitochondrial activity over microsomal activity of these markers was greater than the ratio of measured cholesterol side chain cleavage activities in the two fractions (Flint and Armstrong (1971b)). The cholesterol side chain cleavage activity was measured in these studies as radioactivity liberated from added tracer $[26\text{-}^{14}C]$ cholesterol and was not an absolute measure of the rate of the reaction. Therefore comparison of cholesterol side chain cleavage activity in the microsomal and mitochondrial fractions assumes the cholesterol pool sizes were the same, if they were not this comparison could be misleading.

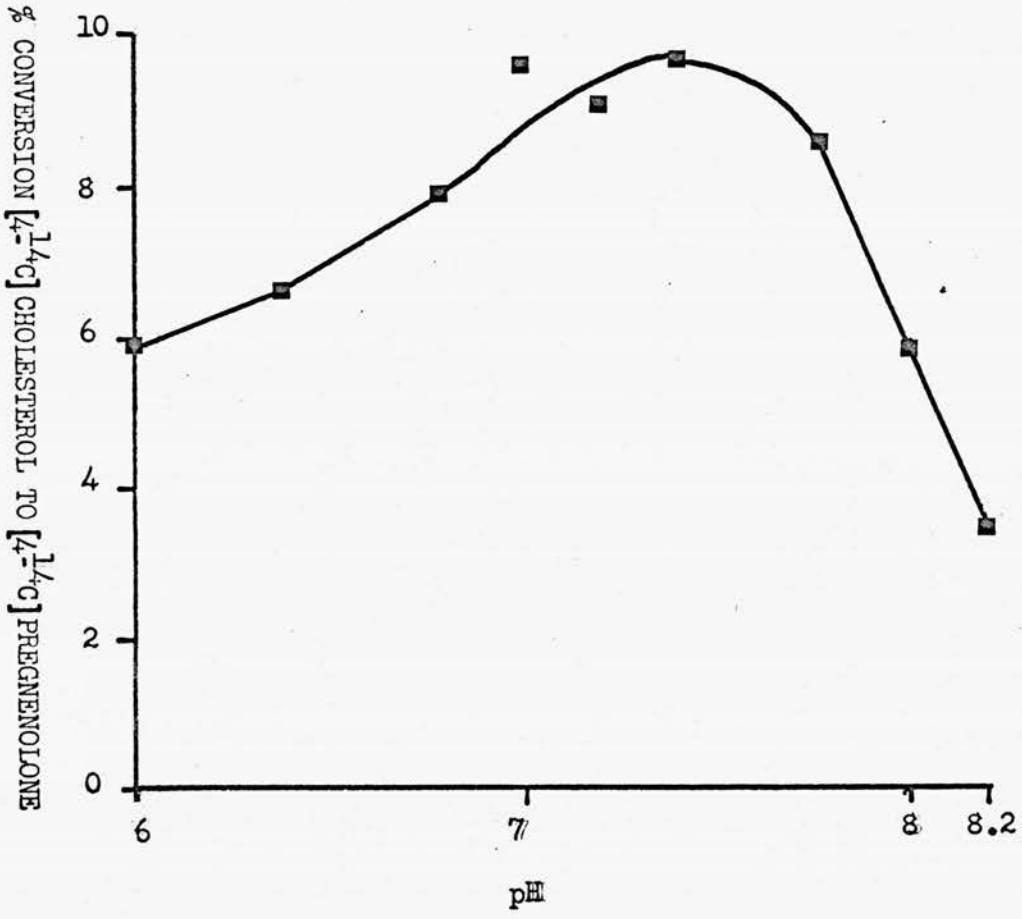


Figure 3.6. The effect of pH on cholesterol side chain cleavage activity in rat luteal mitochondria. [4-^{14}C] Cholesterol was preincubated with the mitochondria for ten minutes before addition of malate to initiate side chain cleavage. Each point represents the average conversion of two five minute incubations.

The effect of pH on cholesterol side chain cleavage in rat luteal mitochondria

Mitochondria were prepared in the usual way from superovulated rat ovaries and cholesterol side chain cleavage activity determined in them as the percentage conversion of [4 -^{14}C] cholesterol to [4 -^{14}C] pregnenolone. Incubations were performed in medium A adjusted to the required pH with 5M HCl or 5M KOH. The pH of the incubations was checked after the addition of the mitochondria. The results of the experiment are shown in figure 3.6. It appeared that the cholesterol side chain cleavage activity had a broad pH optimum between pH7 and 7.8. Incubations in subsequent investigations were therefore carried out at pH 7.4. The pH activity curve did not appear to be symmetrical. Cholesterol side chain cleavage activity decreased more rapidly at high pH than low pH. The reason for this is not however clear. The change in pH could have an effect on the generation of NADPH from reduction of malate or on the flavoprotein, iron sulphur protein and cytochrome P450 enzyme complex which carries out cholesterol side chain cleavage (Sulimovici and Boyd (1968b)). Sulphydryl (SH) groups which are postulated to be associated with the haem group of cytochrome P450 (Mason et al (1965)) are known to be unstable at high pH values. If sulphydryl groups are important in cytochrome P450 dependent reactions this is a possible reason for the luteal mitochondrial cholesterol side chain cleavage activity falling off rapidly at pH values above 7.8.

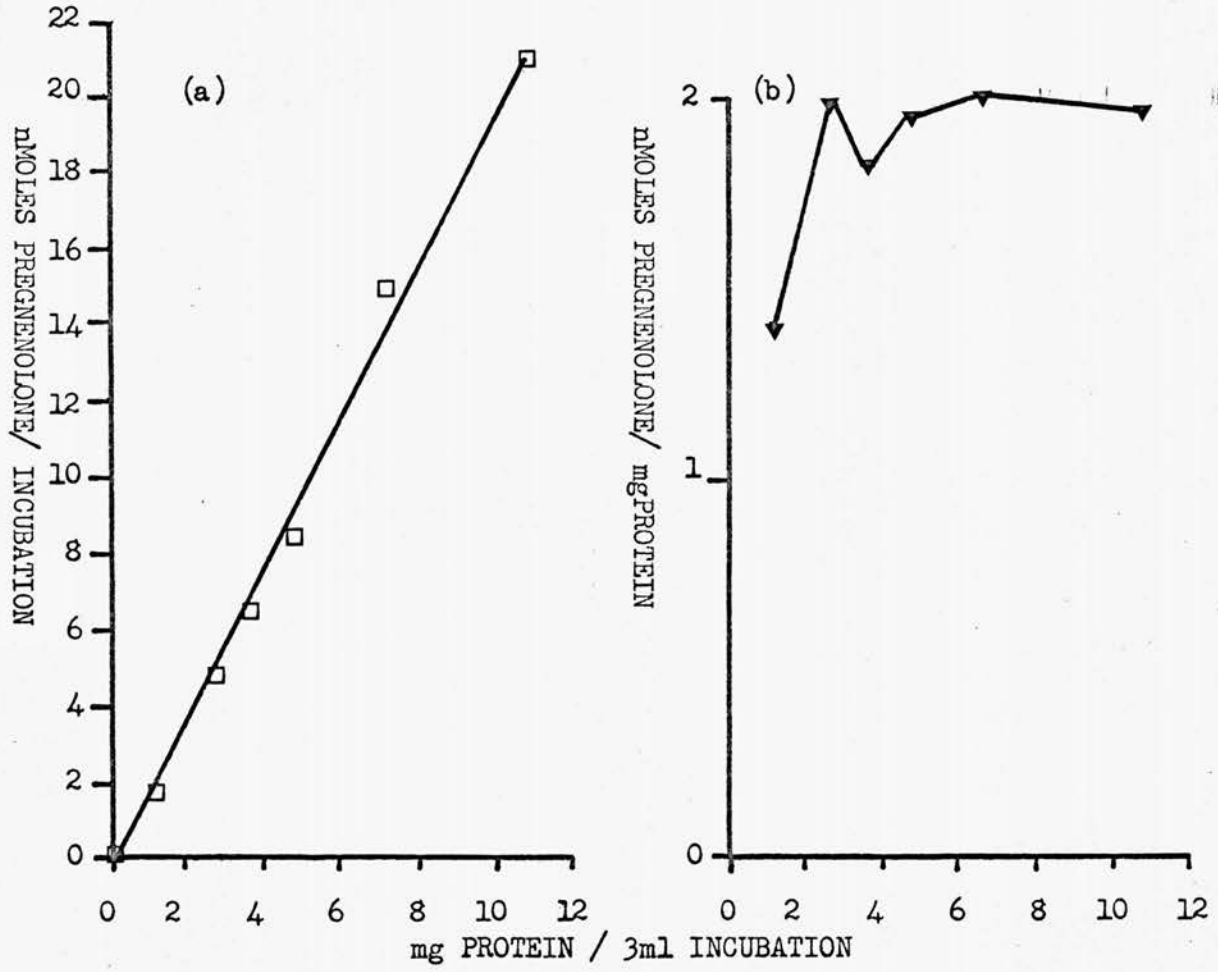


Figure 3.7. The effect of differing concentrations of luteal mitochondrial protein on cholesterol side chain cleavage activity measured in incubations.

The effect of differing protein concentrations in cholesterol side chain cleavage assays

The amount of pregnenolone produced in incubations, with differing protein concentrations, was investigated. The mitochondria were incubated in medium A for 10 minutes in the presence of 6.0 μ M cyanoketone. The resultant pregnenolone formation is shown in figure 3.7(a). There appeared to be a linear relationship between protein concentration and pregnenolone formation as shown in this graph. This was further investigated by calculating pregnenolone formed/mg protein for each protein concentration used in the incubations. The amount of pregnenolone formed per mg of mitochondrial protein appeared to fall off at very low protein concentrations (Figure 3.7 (b)). The fall in pregnenolone production at very low protein concentrations, about 0.3 mg/ml could have at least two explanations.

(a) At lower protein concentrations leakage of NADP(H) from the mitochondria could become significant. The fall in the mitochondrial concentration of NADPH resulting from this would mean that there were no longer enough reducing equivalents available for the cholesterol side chain cleavage reaction.

(b) A significant quantity of mitochondria could stick to the walls of the incubation vessels at low protein concentrations. Resultant denaturation of enzymes would render them inactive thus causing the fall in pregnenolone production.

Of these two explanations the first seems the most likely as in certain circumstances $NADP^+$ can stimulate malate supported cholesterol

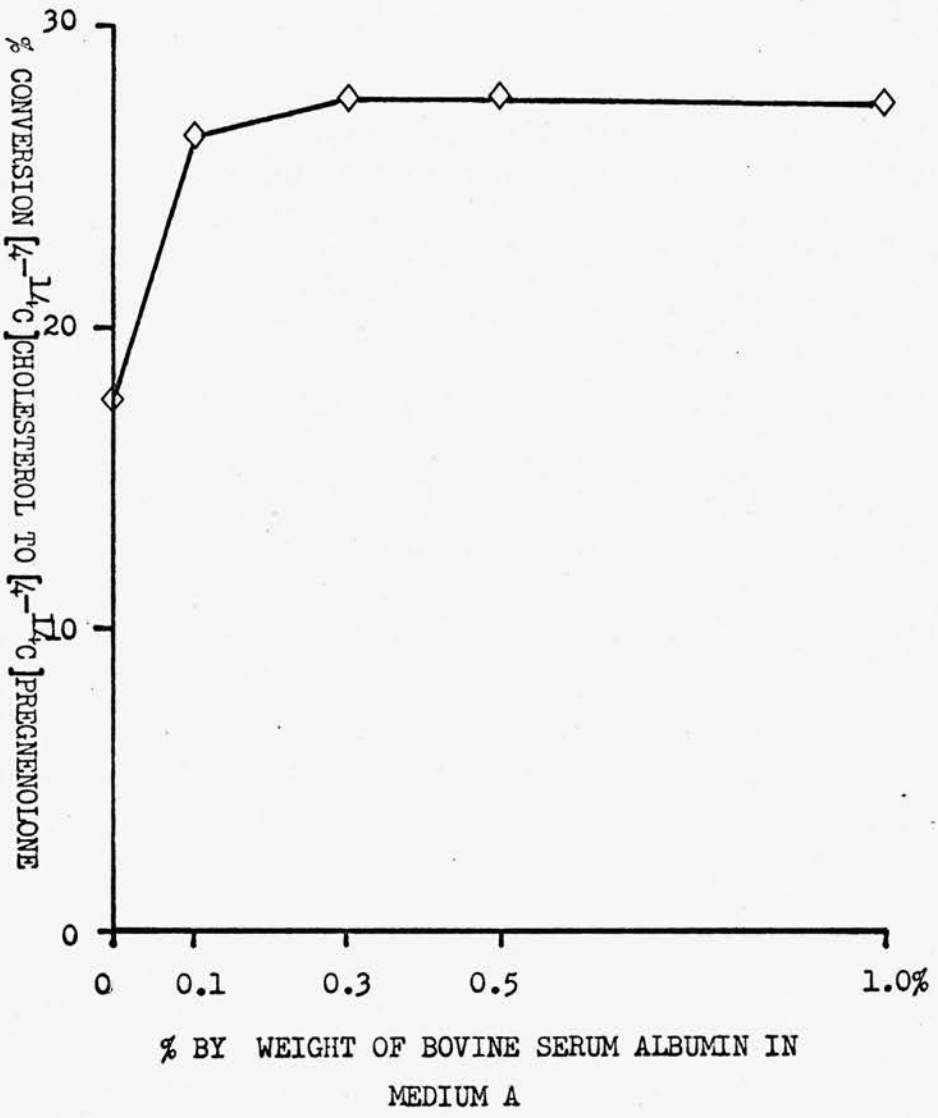


Figure 3.8. The effect of differing concentrations of bovine serum albumin in medium A on cholesterol side chain cleavage activity in luteal mitochondria.

side chain cleavage (figures 3.18 and 3.19). Incubations were generally carried out at protein concentrations of 1 to 2 mg/ml to avoid the loss in pregnenolone production observed at low protein concentrations.

The effect of varying the substituents of medium A on the rate of cholesterol side chain cleavage in isolated rat luteal mitochondria

The concentrations of the substituents of medium A were varied to ensure that the optimal concentration of each one was being used in the cholesterol side chain cleavage assays.

(a) Variation of bovine serum albumin concentration

Bovine serum albumin concentration in medium A was varied from 0.1 to 1%, all other components of the medium were maintained at the concentrations given in table 2.2. Mitochondria were then preincubated in the differing media for 10 minutes with $[4\text{-}^{14}C]$ cholesterol before the addition of L-malate. Formation of $[4\text{-}^{14}C]$ pregnenolone was measured in 10 minute incubations and the results are shown in figure 3.8. Addition of bovine serum albumin to the incubations caused a 60% stimulation in the formation of $[4\text{-}^{14}C]$ pregnenolone. This action is probably due to a "protective effect" of bovine serum albumin on the mitochondria. Free fatty acids have been shown to effect the permeability of mitochondrial membranes, e.g. Van den Bergh (1967); Cannon (1971). It has been suggested that bovine serum albumin protects against this effect by binding the free fatty acids (Van den Bergh (1967)). Free fatty acids are postulated to be released when tissues are homogenised (Davis (1967)). Some of these may bind to the mitochondria and make them permeable, allowing

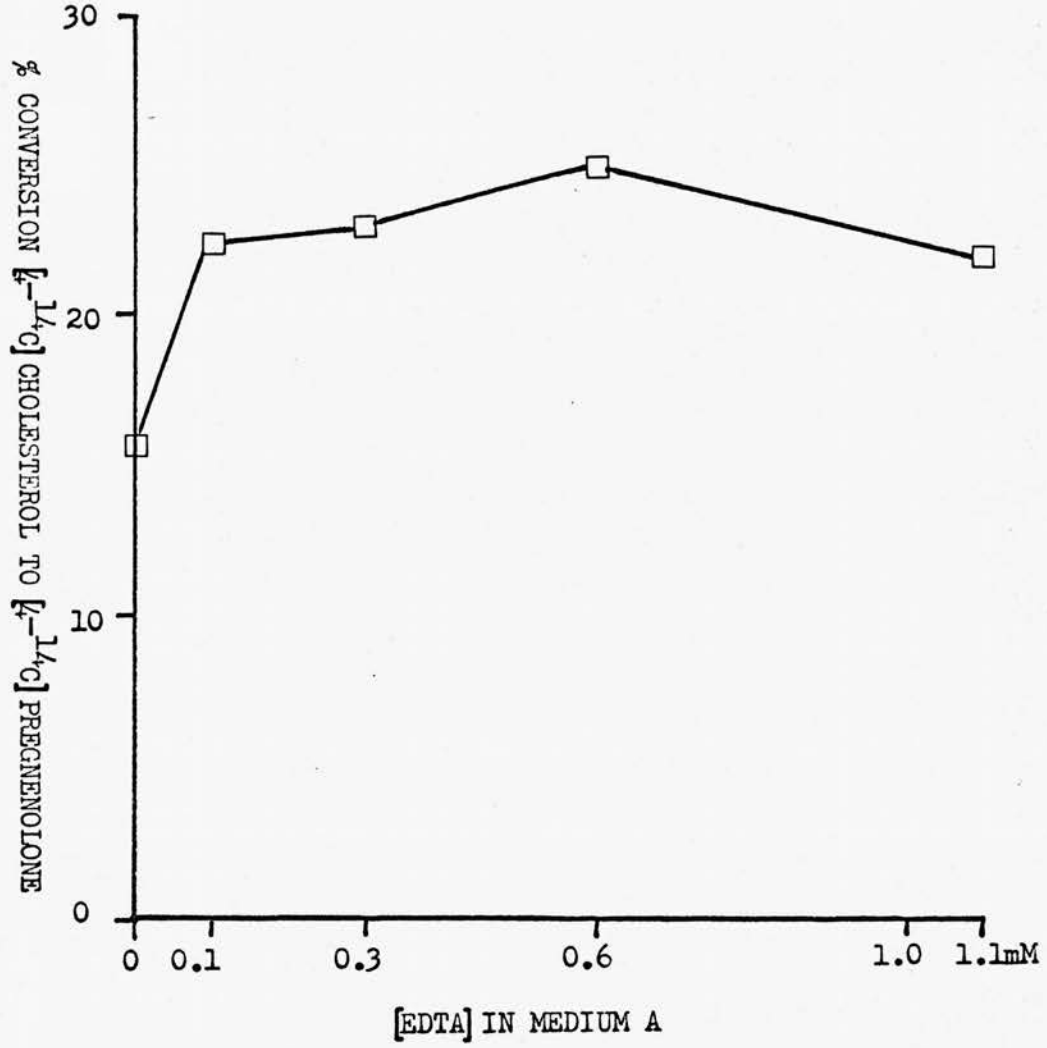


Figure 3.9. The effect of differing concentrations of EDTA in medium A on cholesterol side chain cleavage activity in luteal mitochondria (Experimental procedure as described in text for Figure 3.8).

cofactors such as $NADP^+$ to escape. The cofactors are essential for cholesterol side chain cleavage activity hence it would fall. Peron and McCarthy (1968) have shown that bovine serum albumin has a stimulatory effect on 11 β-hydroxylation of deoxycorticosterone, supported by Krebs cycle intermediates in rat adrenal cortex mitochondria. This was postulated to be due to a change in permeability of the mitochondrial membrane. Fat free bovine serum albumin was used in these investigations as Robinson (1971) has shown that the fatty acids in undefatted albumin may be capable of supporting cholesterol side chain cleavage in porcine corpus luteum mitochondria. 0.1% Bovine serum albumin in medium A was enough to produce an optimal stimulatory effect on production of $[4\text{-}^{14}C]$ pregnenolone. Hence this concentration was used throughout the rest of the studies.

(b) Variation of EDTA concentrations and the substitution of EGTA for EDTA

EDTA was included in medium A as it is well known that it protects mitochondria against the uncoupling effects of free fatty acids (Van den Bergh (1967)). The concentration of EDTA was therefore varied in medium A from 0 to 1.1 mM. Cholesterol side chain cleavage activity was then measured in mitochondria which had been preincubated with $[4\text{-}^{14}C]$ cholesterol (see figures 3.9 and 3.11). It can be seen that the EDTA caused a stimulatory effect on the cholesterol side chain cleavage activity. This stimulatory effect was fairly constant in the range 0.1 to 1.1 mM EDTA and 0.2 mM EDTA was therefore used in medium A in all following experiments. The mechanism by which EDTA exerted its stimulatory effect on cholesterol side chain cleavage was uncertain. It was possible that EDTA chelated small

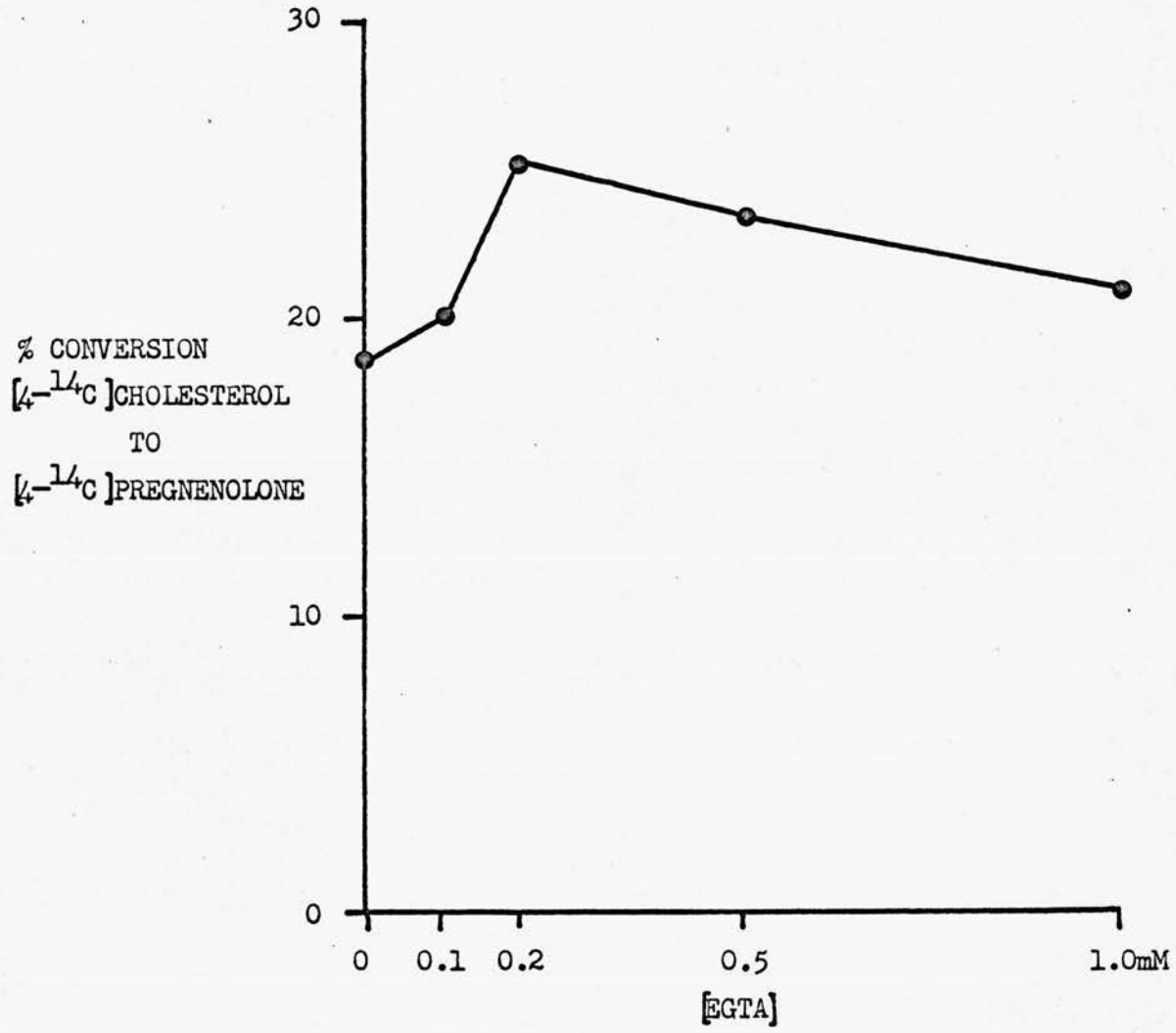


Figure 3.10. The effect of differing concentrations of EGTA in medium A on cholesterol side chain cleavage activity in luteal mitochondria (Experimental procedure as described in text for Figure 3.8).

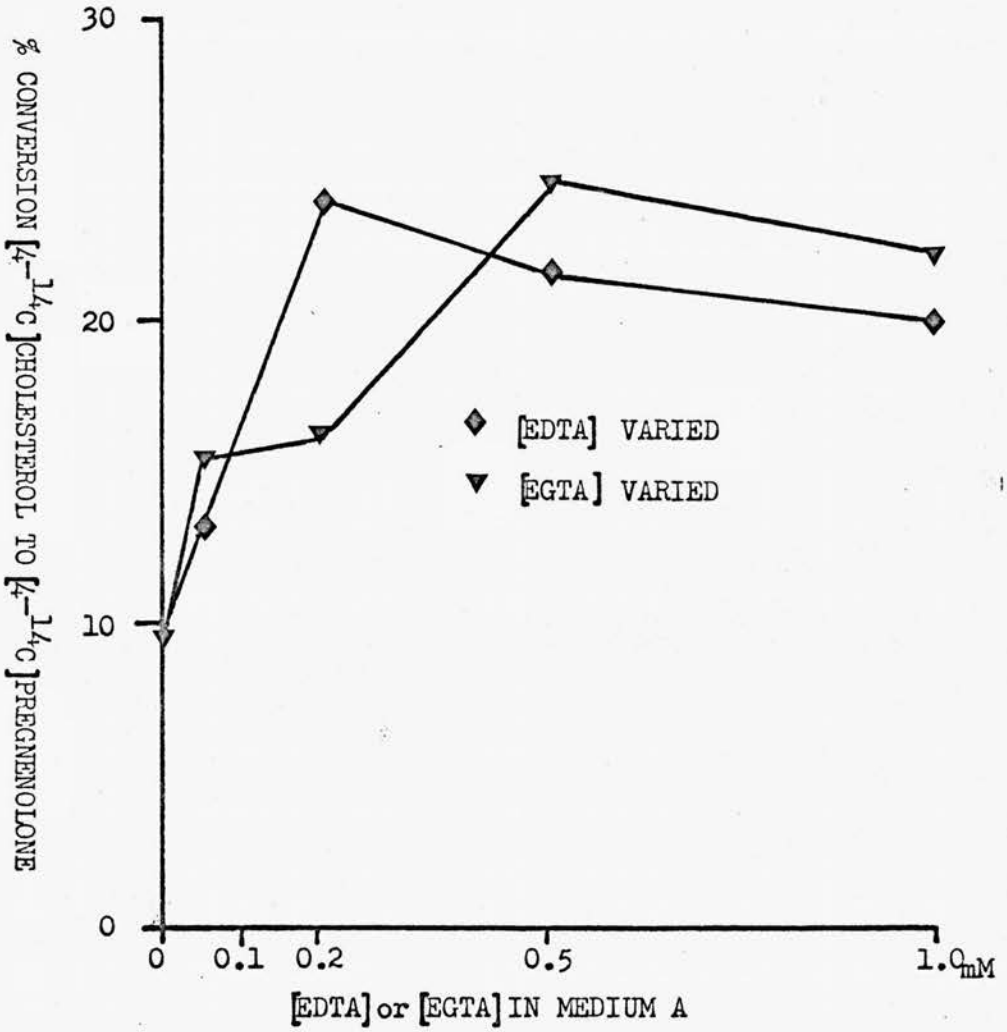


Figure 3.11. The effect of differing concentrations of EGTA or EDTA in medium A on cholesterol side chain cleavage activity in the same preparation of luteal mitochondria (Experimental procedure as described in text for figure 3.8.).

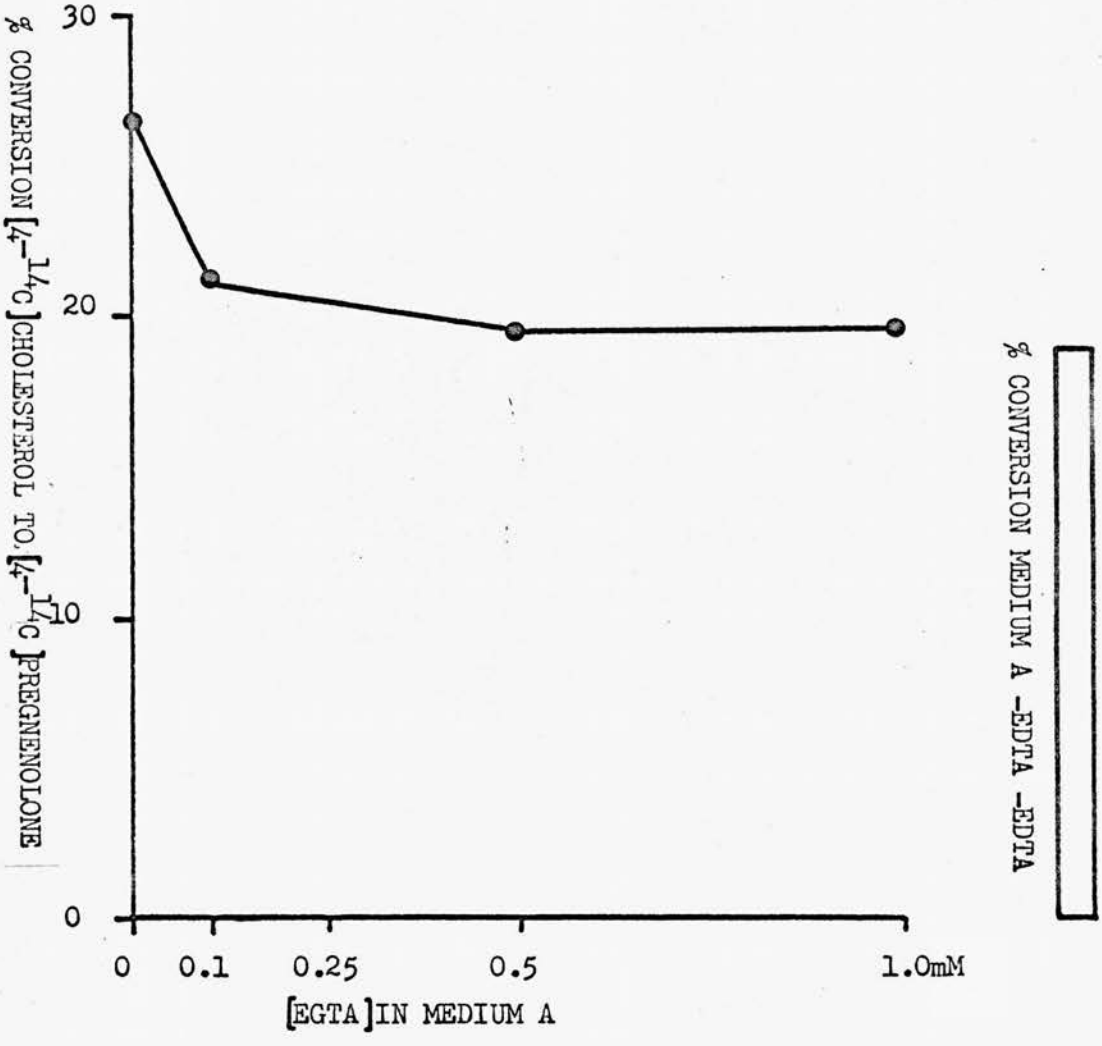


Figure 3.12. The effect of differing concentrations of EGTA, in the presence of 0.2 mM EDTA, in medium A on cholesterol side chain cleavage in luteal mitochondria (Experimental procedure as described in text for figure 3.8.).

amounts of Ca^{++} ions which are known to cause mitochondrial swelling (Allman et al (1970c)). This swelling would allow the escape of cofactors required for the cholesterol side chain cleavage reaction. EGTA is a specific chelator of Ca^{++} ions it was therefore used to replace EDTA in medium A. Cholesterol side chain cleavage assays were then carried out with the mitochondria in medium A containing different concentrations of EGTA. EGTA in the concentration range 0.1 to 1 mM caused a stimulation in Δ^4-^{14}C pregnenolone production (figures 3.10 and 3.11). Figure 3.11 shows the effects of differing EDTA and EGTA concentrations in medium A used for cholesterol side chain cleavage assays on the same luteal mitochondrial preparation. They appear to have caused a similar stimulation in the formation of Δ^4-^{14}C pregnenolone possibly via the same mechanism. The effects of EDTA and EGTA were not additive. When formation of Δ^4-^{14}C pregnenolone was assayed in medium A with 0.2 mM EDTA; concentrations of 0.1 mm 0.5 mM and 1.0 mM EGTA caused a 25% inhibition of activity (figure 3.12). This reason for this effect is not at all clear. In the presence of EDTA and EGTA the formation of Δ^4-^{14}C pregnenolone fell to the levels observed in the absence of both compounds (see figure 3.12 assay carried out on same mitochondrial preparation).

(c) Variations of concentrations of KCl, phosphate and triethanolamine in medium A. Replacement of triethanolamine with Tris - HCl

The concentrations of KCl, phosphate ions and triethanolamine hydrochloride were varied in medium A. The resultant incubation media were used in cholesterol side chain cleavage assays with the same luteal mitochondrial preparation. The mitochondria were

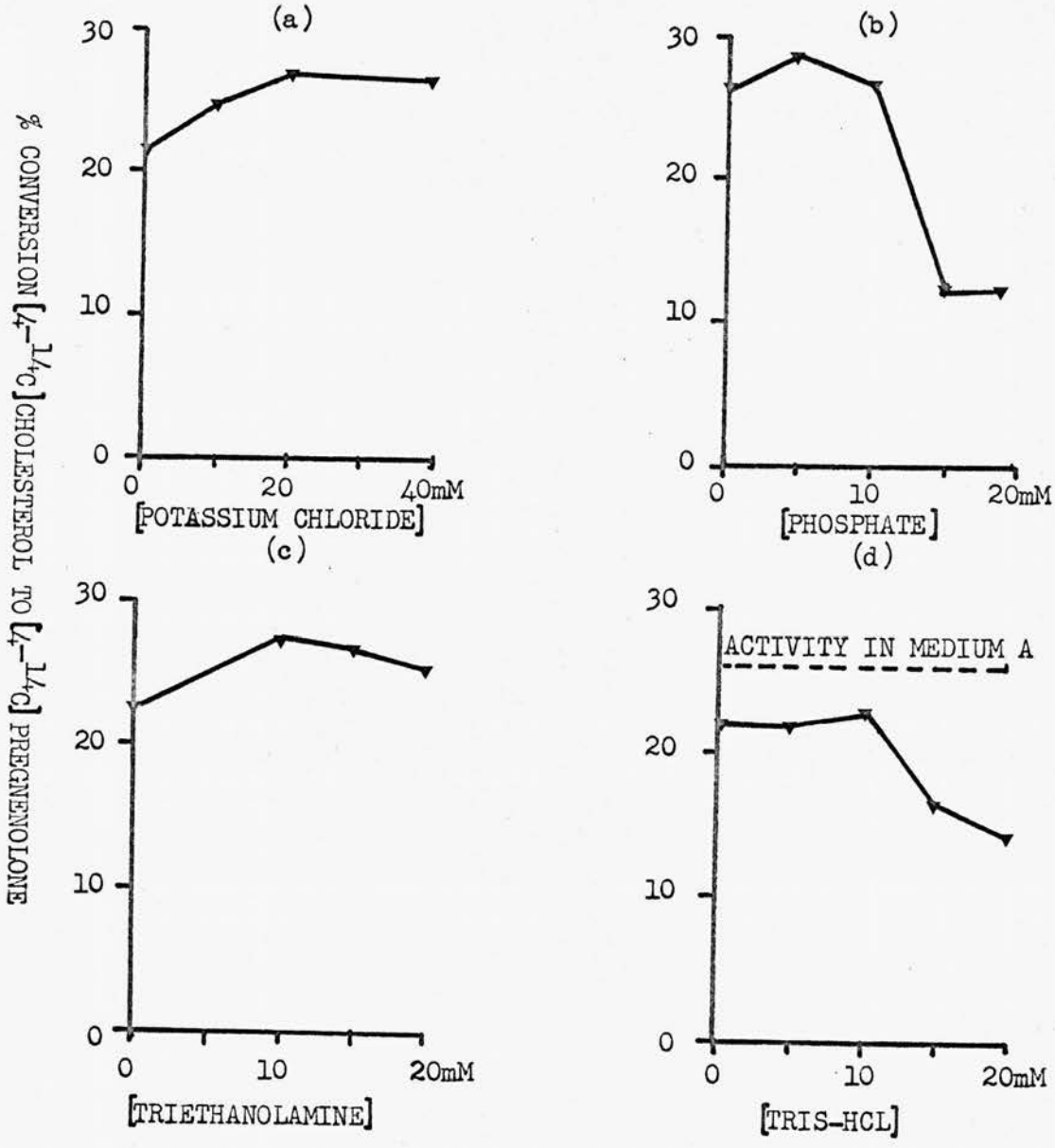


Figure 3.13. The effect of differing concentrations of (a) potassium chloride, (b) phosphate ions and (c) triethanolamine hydrochloride in medium A on cholesterol side chain cleavage activity in luteal mitochondria. Shown also is the effect of replacing triethanolamine hydrochloride in the incubation medium with tris-hydrochloride (d) (Experimental procedure as described in text for figure 3.8).

preincubated with /4 -^{14}C/ cholesterol for 10 minutes before addition of malate. /4 -^{14}C/ Pregnenolone formation was then determined after a ten minute incubation. Care was taken to ensure all incubation media remained at pH 7.4. The results of this experiment are shown in figure 3.13. The results show that none of the variations in the components of medium A tested had a dramatic effect on the /4 -^{14}C/ pregnenolone production. The concentrations of KCl (20 mM) and triethanolamine hydrochloride (15 mM), used in medium A, appear to be optimal for cholesterol side chain cleavage activity. 10 mM Potassium phosphate did not appear to alter the formation of /4 -^{14}C/ pregnenolone but was included in medium A as a buffer and to allow the entrance of Krebs cycle intermediates into the mitochondria. The carriers which allow di- and tricarboxylic acids into the mitochondria are dependent on phosphate exchange for their operation in liver mitochondria (review Klingenberg (1970)) and in bovine and rat adrenal cortex mitochondria (Launay et al (1974); Michejda et al (1973); Sauer and Park (1973); Tsang and Johnston (1973)). It is possible a similar mechanism could exist for rat luteal mitochondria.

Whereas triethanolamine hydrochloride buffer caused a stimulation of /4 -^{14}C/ pregnenolone production use instead of tris-HCl buffer in medium A did not cause any stimulation of activity and at concentrations above 10 mM it caused inhibition. Hence triethanolamine hydrochloride was preferred for use in medium A. No other buffers were tested in the cholesterol side chain cleavage assay system; it is therefore possible that higher activity could be obtained with use of another buffer salt.

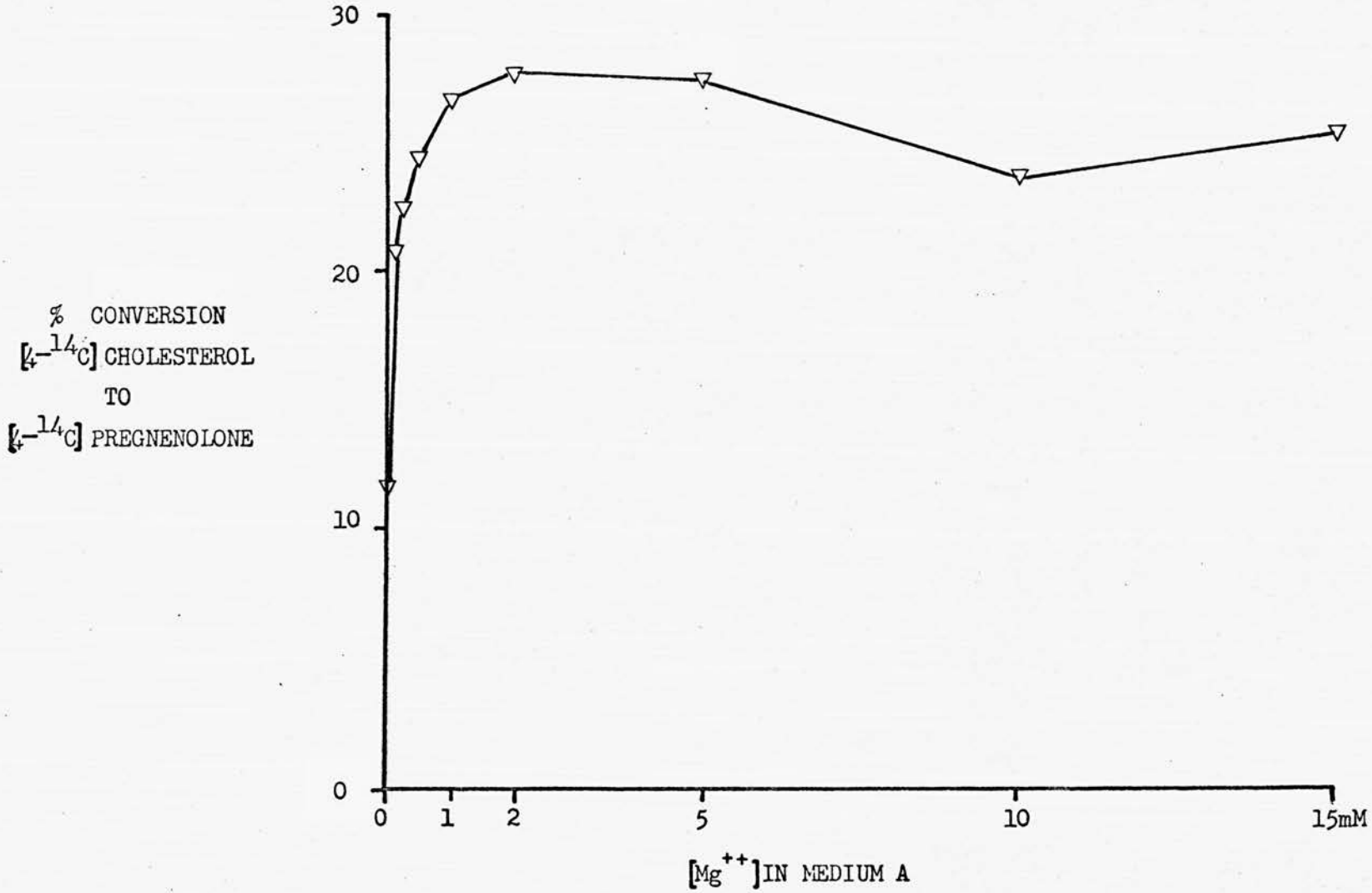


Figure 3.14. The effect of variation in the concentration of magnesium in medium A on cholesterol side chain cleavage activity in luteal mitochondria (Experimental procedure as described in text for figure 3.8.).

(d) Variation of magnesium ion concentration in medium A

Flint and Armstrong (1971b) and Drosdowsky et al (1965) have reported that in the mitochondria of rat corpus luteum and testis, cholesterol side chain cleavage activity supported by NADPH is stimulated by magnesium ions. Koritz (1966) has shown a similar Mg^{++} dependence when cholesterol side chain cleavage was supported by succinate in bovine adrenal cortical mitochondria. Figure 3.14 displays the effect of varying the concentration of magnesium ions in medium A on $[4\text{-}^{14}C]$ pregnenolone production supported by malate, in rat luteal mitochondria. One hundred and fifty per cent stimulation of $[4\text{-}^{14}C]$ pregnenolone formation was obtained with 1 to 2 mM Magnesium ions in comparison to a control with no magnesium ions present. Maximal stimulation of cholesterol side chain cleavage activity was obtained with 10 mM magnesium ions by Flint and Armstrong (1971b) in rat luteal mitochondria. They however were using a different mitochondrial preparation and NADPH to support cholesterol side chain cleavage activity rather than a Krebs Cycle intermediate. Tightly coupled luteal mitochondria have difficulty using externally added NADPH as a source of electrons for cholesterol side chain cleavage (Yago et al (1967a); Robinson and Stevenson (1971a); McIntosh et al (1971)). Externally added NADPH would support very little pregnenolone production in mitochondria used in the studies described in this thesis. Thus they are probably different in permeability to the preparation described by Flint and Armstrong (1971b). This could account for the difference in concentrations at which magnesium ions stimulate cholesterol side chain cleavage in the two preparations.

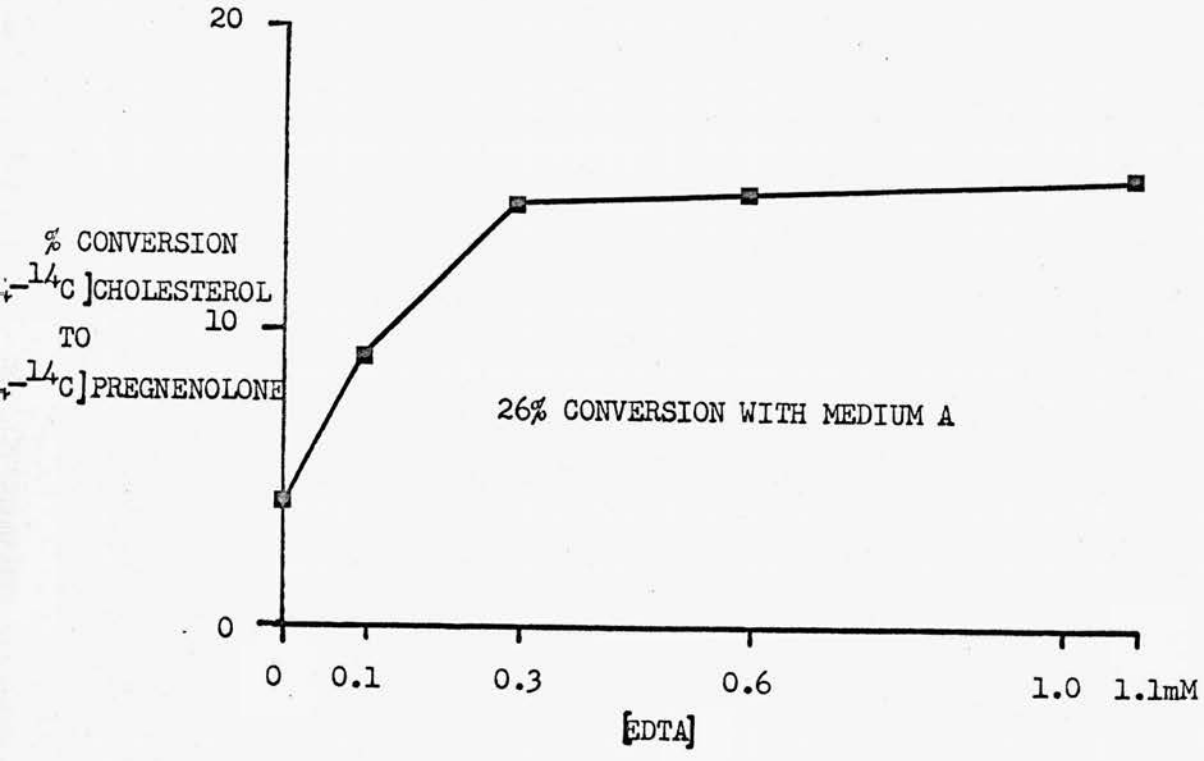


Figure 3.15. The effect of variation in the concentration of EDTA in medium A, without magnesium present, on cholesterol side chain cleavage in luteal mitochondria (Experimental procedure as described in text for figure 3.8.).

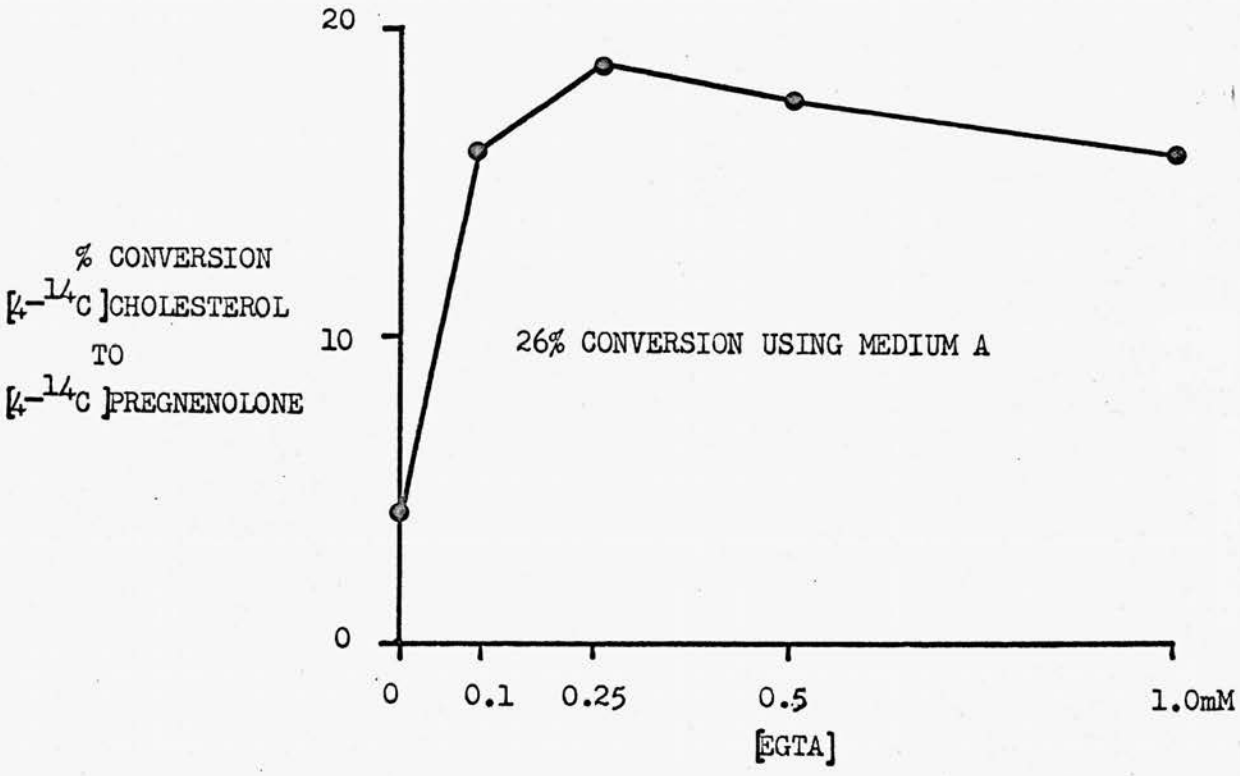


Figure 3.16. The effect of variation in the concentration of EGTA in medium A, without magnesium present, on cholesterol side chain cleavage in luteal mitochondria (Experimental procedure as described in text for figure 3.8.).

There are many ways in which magnesium ions could have affected the system. Magnesium ions could directly activate the cholesterol side chain cleavage enzymes or facilitate the supply of substrates for the reaction. Transhydrogenases have been reported as being important in supplying NADPH, from NADH, for cholesterol side chain cleavage in bovine, rat and porcine luteal mitochondria (Uzgiris et al (1971); Robinson and Stevenson (1971a), (1972)). Malate support for cholesterol side chain cleavage in rat and bovine luteal mitochondria is probably via NADH production as there is reported to be no malic enzyme activity in them (Stevenson and Taylor (1971); Uzgiris et al (1971)). Transhydrogenase activity in rat liver mitochondria is dependent on Mg^{++} ions for activity (Danielson and Ernster (1963)). If the luteal transhydrogenase is similar to the liver enzyme, magnesium ions would be necessary in the production of NADPH, for cholesterol side chain cleavage from NADH produced by malate oxidation.

Another possible effect of magnesium ions would be to protect the mitochondria against other metal ions, such as calcium, which would cause the mitochondria to swell and the pyridine nucleotides to escape from them (McIntosh et al (1971)). Magnesium has been shown to stabilise mitochondria in a coupled condition possibly by reversing the uncoupling effect of Ca^{++} ions (Allman et al (1970c)). The possibility of removing harmful divalent cations in the absence of Mg^{++} was investigated using metal ion chelators. The effects of varying concentrations of EDTA and EGTA on luteal mitochondrial pregnenolone production are shown in figures 3.15 and 3.16. The experiments were performed on preparations of luteal mitochondria incubated in medium A without magnesium. The EDTA and EGTA both caused a stimulation in the production of /4 -^{14}C/ pregnenolone in a

similar range of concentrations to which they had an effect in medium A with magnesium (Figures 3.9, 3.10 and 3.11). The activity obtained in the presence of EDTA and EGTA was only 80% of that obtained with medium A with magnesium. Magnesium ions were needed in the incubation medium to obtain "coupled" mitochondria in the oxygen electrode (Chapter 4) even in the presence of EDTA. Calcium ions are known to cause swelling, loss of coupled reactions and configurational (morphological) transitions in bovine adrenal cortex mitochondria, an effect reversed by magnesium (Allman et al (1970a,b,c)). One of these coupled processes is reversed electron transport, which has been shown to be important for supplying electrons for cholesterol side chain cleavage via NADH generation and a transhydrogenase to form NADPH. This reaction may be important in supporting cholesterol side chain cleavage in adrenal cortex mitochondria (Koritz (1966); Hall (1967), (1972); Cammer and Estabrook (1967a); Oldham et al (1968); Purvis et al (1968); Peron et al (1972)). Also in the adrenal cortex an $NADP^+$ linked malic enzyme may supply NADPH for hydroxylations (Simpson et al (1968); Simpson and Estabrook (1969)). Thus in a steroid hormone producing tissue magnesium ions are important in maintaining mitochondria in a coupled condition necessary to allow Krebs cycle intermediates to support cholesterol side chain cleavage. Magnesium ions probably have a similar effect in rat luteal mitochondria. These studies do not exclude a direct effect of magnesium ions on the cholesterol side chain cleavage enzyme complex.

Variation of the components of the luteal mitochondrial incubation medium A thus had varied effects on the cholesterol side chain cleavage enzymes. Medium A gave the highest cholesterol side chain cleavage activity of the buffers tested, it was therefore used for all

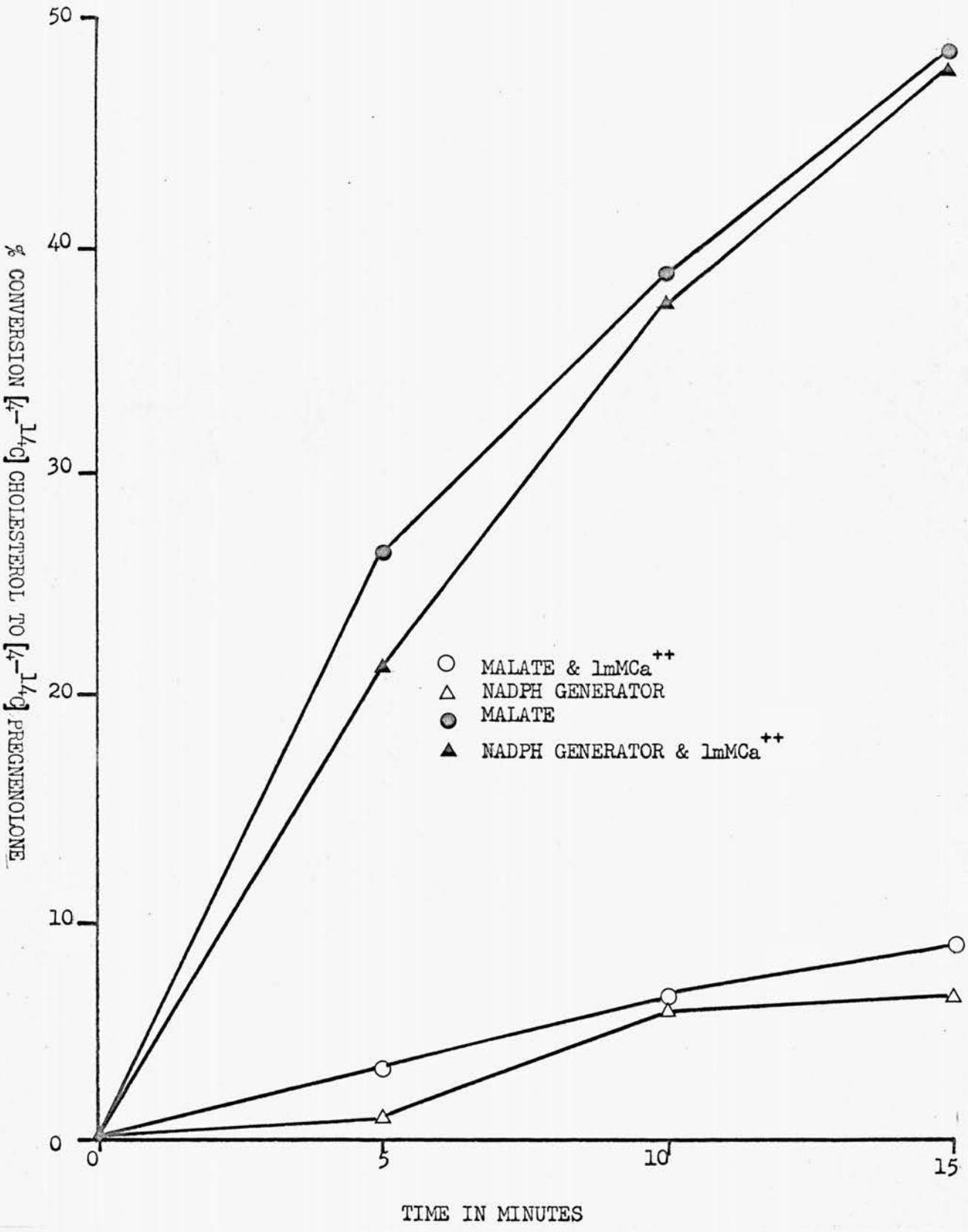


Figure 3.17. The effect of 1mM calcium ions on cholesterol side chain cleavage activity, supported by malate or an NADPH generator, in incubations of luteal mitochondria. The NADPH generating system consisted of 1mM $NADP^+$, 8mM glucose-6-phosphate and 1 unit of glucose-6-phosphate dehydrogenase. The mitochondria were preincubated for ten minutes with [4-^{14}C] cholesterol before initiation of side chain cleavage.

subsequent experiments described in this thesis. Cholesterol side chain cleavage activity measured in luteal mitochondria involved several enzymes; those which convert Krebs cycle intermediates to NADPH or NADH; and those of the cholesterol side chain cleavage complex (see fig. 1.3). Alteration of any one of these enzyme activities could affect the measured rate of the reaction. Mitochondrial permeability to substrates and cofactors will also play a role in altering pregnenolone production. It was therefore important to have an incubation medium which provided stability for all these factors and optimal cholesterol side chain cleavage activity. This allowed consistent results to be obtained in luteal mitochondrial incubations.

The effect of calcium ions on cholesterol side chain cleavage in luteal mitochondria

Calcium ions are known to play a role in the response of steriodogenic tissues to trophic hormone stimulation (Birmingham et al (1953); Carchman et al (1971); Hermier and Jutisz (1969); Sayers et al (1972); Rubin et al (1972)). This requirement is generally thought to be at the level of cyclic AMP production, however evidence is also accumulating for an effect of calcium on steriodogenesis, after cyclic AMP production in adrenal cortex and luteal cells (Rubin et al (1972); Hermier and Jutisz (1969); Haksar and Peron (1972)). It was therefore of interest to see if calcium ions had any effect on cholesterol side chain cleavage in luteal mitochondria. The effect of 1 mM $CaCl_2$ on the time course of $[4\text{-}^{14}C]$ pregnenolone appearance in luteal mitochondria is shown in figure 3.17. As can be seen from

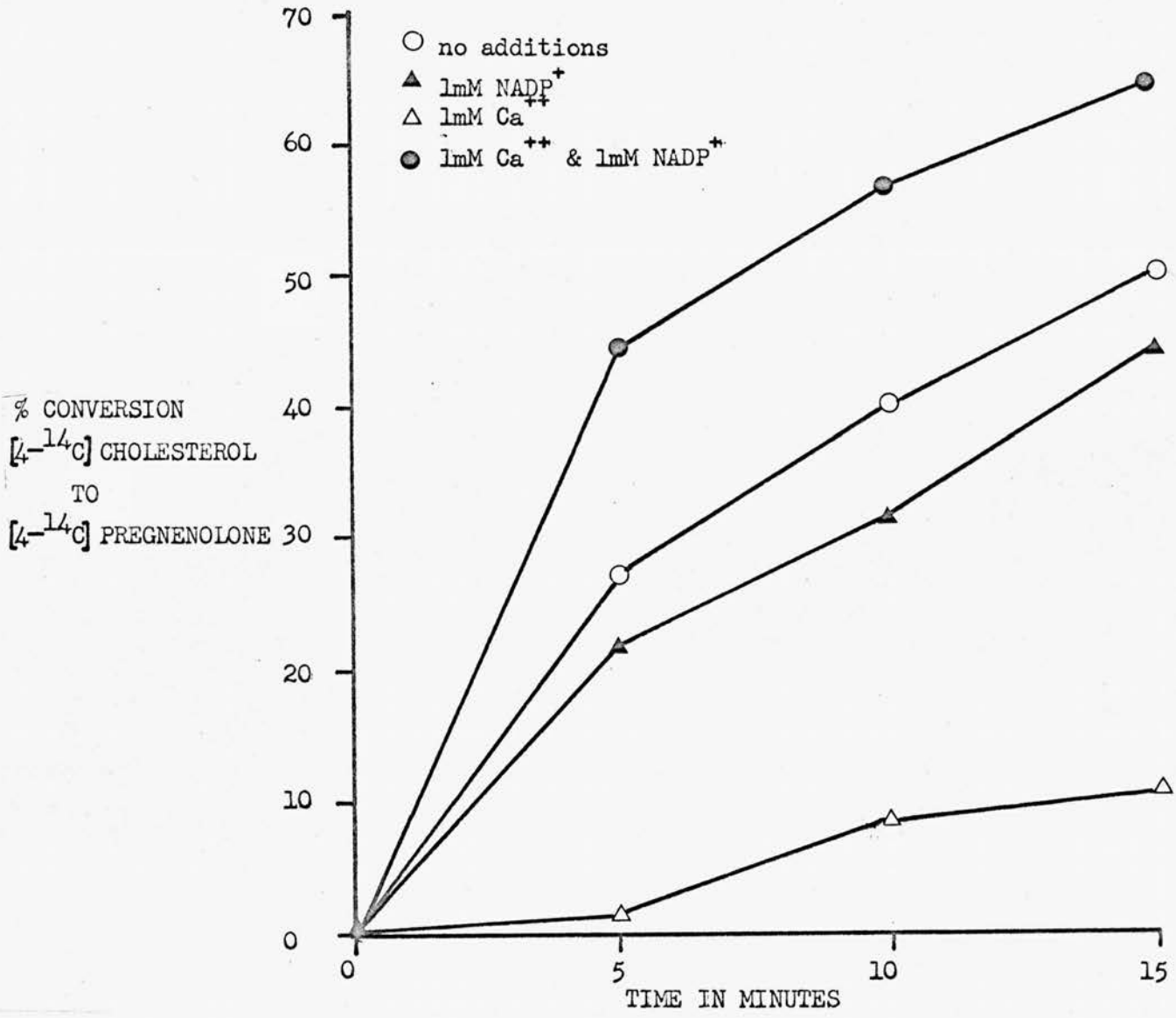


Figure 3.18. The effect of $NADP^+$ on calcium produced inhibition of malate supported side chain cleavage in luteal mitochondria. Mitochondria were preincubated in medium A for 10 minutes with [4-^{14}C] cholesterol. Side chain cleavage was initiated with malate; other additions to the incubations are shown on the graph.

the graph with malate as an electron donor there was a 3% conversion of Δ^4-^{14}C cholesterol to Δ^4-^{14}C pregnenolone in 5 minutes in the presence of calcium ions compared to the 26.5% conversion in its absence. This effect was probably due to calcium altering the mitochondrial permeability to pyridine nucleotides (Vingradov et al (1972)). This would allow NADP(H) to escape and fall below the concentration necessary for maximal steroidogenesis (Simpson et al (1974)). Figure 3.17 also shows the effect of Ca^{++} ions on cholesterol side chain cleavage supported by an NADPH generating system. Luteal mitochondria incubated in medium A show very little cholesterol side chain cleavage activity supported by NADPH generator, 1% formation of Δ^4-^{14}C pregnenolone in five minutes, probably due to the impermeability of intact mitochondria to pyridine nucleotides. This is similar to the findings of Yago et al (1967a) who used bovine luteal mitochondria. In some mitochondrial preparations no cholesterol side chain cleavage activity could be detected with an NADPH generating system, presumably as the reduced pyridine nucleotide was completely excluded from the mitochondria. In contrast to this in the presence of 1 mM $CaCl_2$ the NADPH generating system caused the production of as much Δ^4-^{14}C pregnanolone as malate in the absence of calcium ions. The calcium had probably caused the mitochondria to swell thus allowing the NADPH access to the side chain cleavage enzymes. As calcium probably allowed the escape of NADP(H) from the luteal mitochondria the effect of adding back $NADP^+$ to incubations, with calcium present was investigated. Figure 3.18 shows that addition of 1 mM $NADP^+$ reversed the calcium induced inhibition of malate supported Δ^4-^{14}C pregnenolone production. In fact the addition of 1 mM calcium and 1 mM $NADP^+$ caused a stimulation of Δ^4-^{14}C

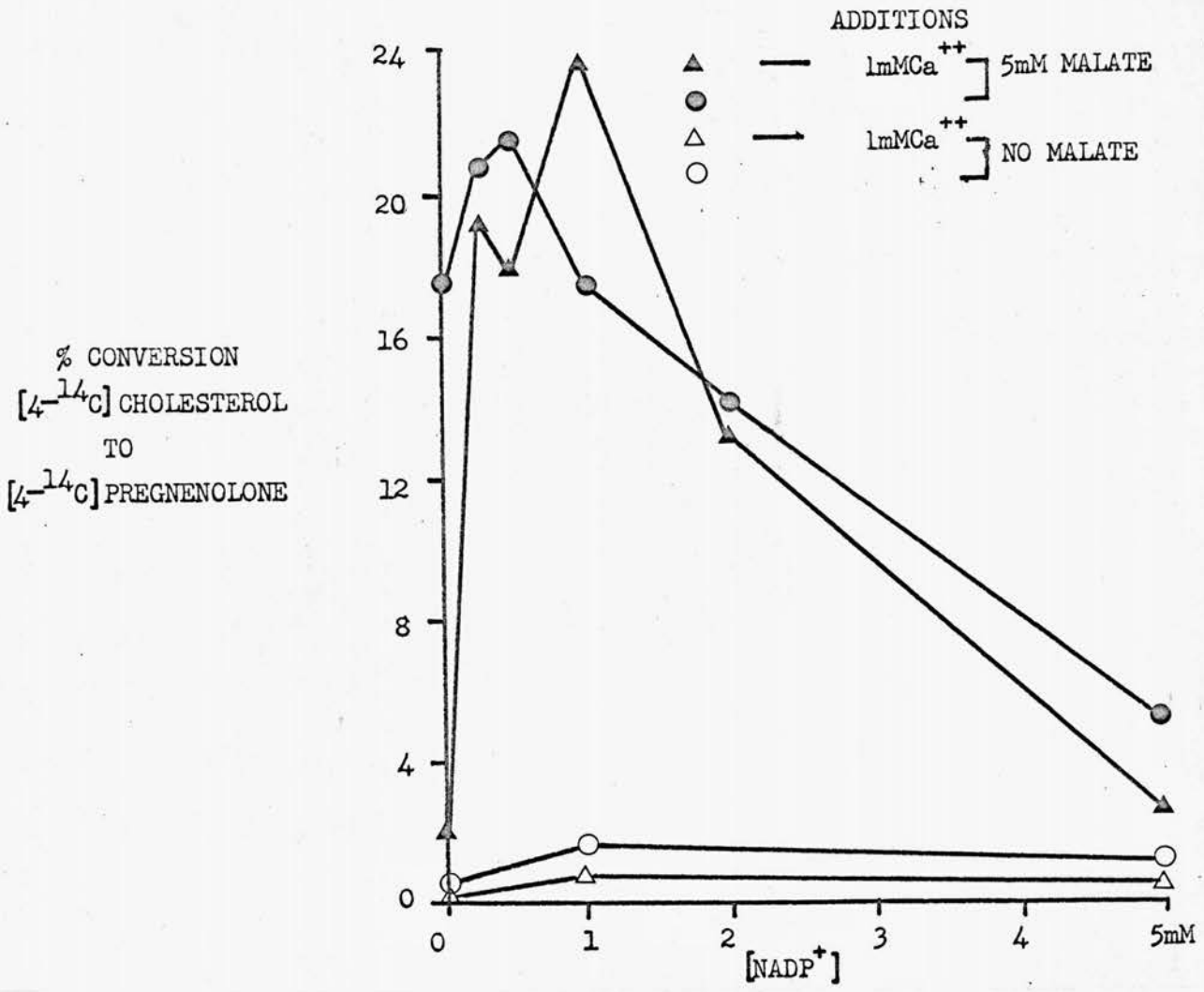


Figure 3.19. The effect of variation in the concentration of $NADP^+$ on luteal mitochondrial cholesterol side chain cleavage activity, supported by malate, in the presence and absence of calcium ions (Experimental procedure as described in text for figure 3.8.).

pregnenolone production above the levels seen with malate alone. Addition of 1 mM $NADP^+$ alone made no difference to the malate supported $[4\text{-}^{14}C]$ pregnenolone production. In some experiments low concentrations of $NADP^+$ (< 1 mM) produced a small stimulation of malate supported $[4\text{-}^{14}C]$ pregnenolone production. Indicating small amounts of NADP(H) may have leaked from the mitochondria. The effects of differing concentrations of $NADP^+$ on luteal mitochondrial cholesterol side chain cleavage activity, in the presence and absence of calcium ions are shown in figure 3.19. In the concentration range 0 to 1 mM, $NADP^+$ had a small stimulatory effect on $[4\text{-}^{14}C]$ pregnenolone production initiated by malate. At concentrations above 1 mM $NADP^+$ was inhibitory on the cholesterol side chain cleavage reaction. Concentrations of NADPH above 1 mM have been reported to be inhibitory on cholesterol side chain cleavage in bovine luteal and adrenal cortex mitochondria and rat luteal mitochondria (Ichii *et al* (1963); Yago *et al* (1967b); Satoh *et al* (1966); Sulimovici and Boyd (1968a)). This observed inhibitory effect on cholesterol side chain cleavage, of NADPH, is probably not physiologically important as the concentrations of $NADP^+$ and NADPH in the luteal cell are of the order of 0.14 mM (Flint and Denton (1970a)). In the presence of 1 mM Ca^{++} up to 1 mM $NADP^+$ also stimulated $[4\text{-}^{14}C]$ pregnenolone production supported by malate. In the absence of $NADP^+$, 1 mM Ca^{++} had caused an eight fold inhibition of $[4\text{-}^{14}C]$ pregnenolone production. There was little difference between the $[4\text{-}^{14}C]$ pregnenolone supported by malate and $NADP^+$ in the presence and absence of Ca^{++}. The large inhibition of $[4\text{-}^{14}C]$ pregnenolone production by calcium in mitochondria was only observed when $NADP^+$ was absent. $NADP^+$ on its own with or without calcium did not support cholesterol side chain

cleavage (figure 3.19). NAD^+ was not effective in reversing the calcium induced inhibition of cholesterol side chain cleavage. The fact that in some incubations that $NADP^+$ could cause a small stimulation of the formation of pregnenolone supported by malate suggested there had been an escape of this cofactor during the preparation of the mitochondria. This may have been due to the presence of small concentrations of calcium in the sucrose used in the preparation of subcellular fractions (20 μM in 0.25 M sucrose) (Allman et al (1970a)). The magnesium, EDTA and bovine serum albumin in medium A counteracted the possible effects of calcium ions from the sucrose. It was important when comparing pregnenolone production in two groups of luteal mitochondria to remember differences in activity could have been due to NADP(H) leakage from one group of mitochondria during their preparation rather than an effect of pre-treatment of rats before hand.

As externally added NADPH was relatively inefficient in supporting cholesterol side chain cleavage in luteal mitochondria the role of extra-mitochondrial NADPH in cellular steroidogenesis is uncertain. The calcium ion concentration in the steroidogenic cell could be altered by trophic hormone stimulation. This could affect the permeability of the mitochondria and possibly allow the entry of NADPH into the cholesterol side chain cleavage enzymes. Supply of electrons and NADPH have been postulated as one of the ways by which trophic hormones stimulate steroidogenesis in endocrine tissues (Haynes and Berthet (1957); Haynes et al (1960); McKerns (1965); Robinson and Stevenson (1973)). However other authors have challenged this hypothesis applied to the adrenal gland and the corpus luteum

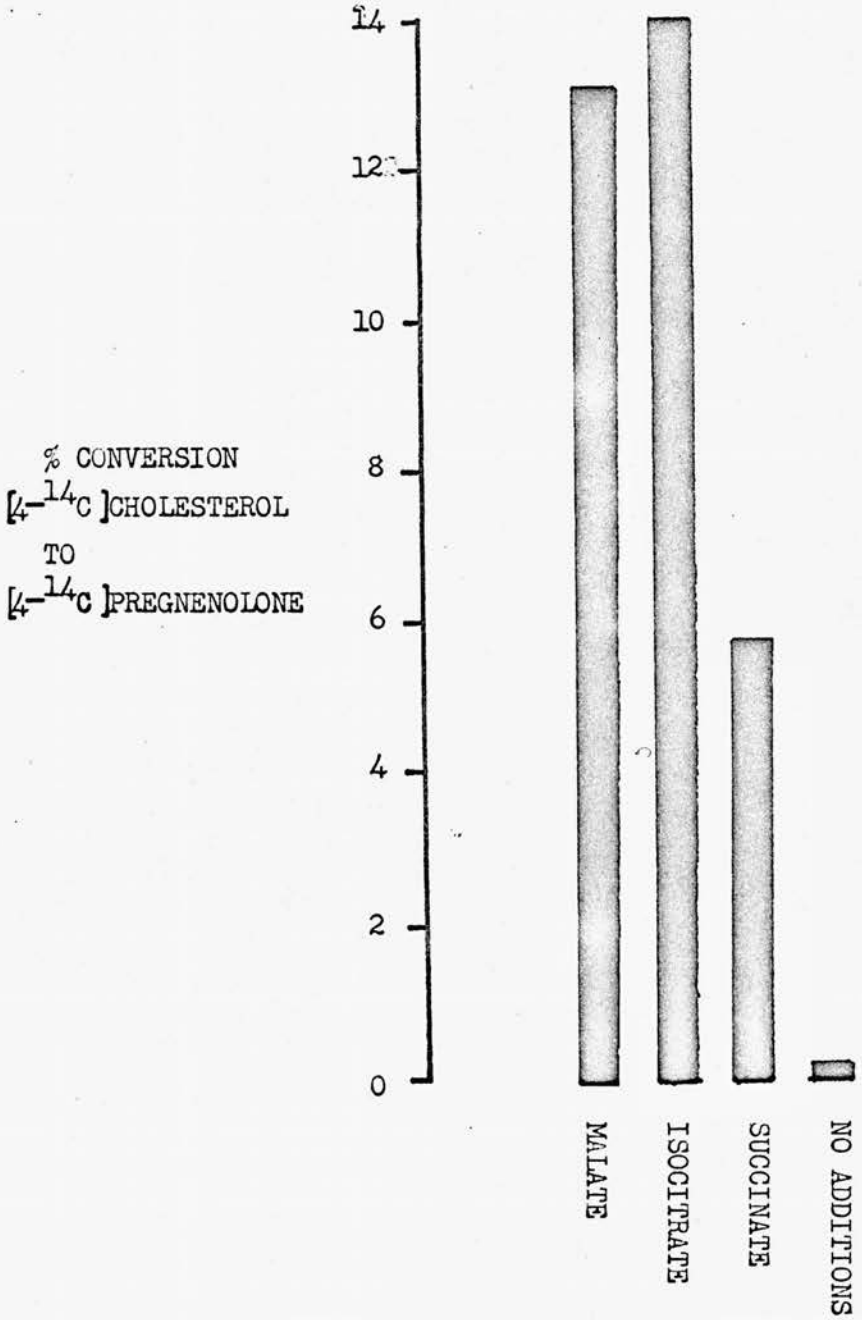


Figure 3.20. The use of different Krebs cycle intermediates to support cholesterol side chain cleavage in luteal mitochondria. [4-14C] Cholesterol was preincubated with the mitochondria for 10 minutes before initiation of side chain cleavage. Results are the average of two determinations of activity.

(Chance et al (1962); Harding and Nelson (1964); Flint and Denton (1970a), (1972); Sulimovici and Lunfeld (1972)). These authors found no changes in cellular NADPH/$NADP^+$ levels when the adrenal cortex or corpora lutea were producing more steroid hormones in response to trophic hormone stimulation. Measuring the cellular NADPH/$NADP^+$ ratios does not exclude the possibility that there may be an increased turnover of NADPH for steroidogenesis or localised changes of concentration in the mitochondrion without the total cellular concentrations changing significantly (Hall (1971)). Haynes (1968) has withdrawn the hypothesis that ACTH increases steroidogenesis in the adrenal cortex by increasing NADPH levels through activation of glycogen phosphorylase and thus glucose 6-phosphate dehydrogenase.

Different electron donors for cholesterol side chain cleavage in rat luteal mitochondria

Intermediates from the Krebs cycle are known to be more effective than NADPH in supplying electrons for hydroxylation reactions in incubations of intact mitochondria (Robinson and Stevenson (1971a); McIntosh et al (1971); Uzgiris et al (1971)). Fatty acids may also be a possible source of respiratory substrate in the rat corpus luteum (Flint and Denton (1970b); Robinson and Stevenson (1971b); Stevenson et al (1973)). NADH is produced by β-oxidation and by reverse electron flow from FADH formed during β-oxidation. This NADH can then form NADPH via a tranhydrogenase.

Three different Krebs cycle intermediates were tested as electron donors for cholesterol side chain cleavage in rat luteal mitochondria. The results are shown in figure 3.20. Each acid was used at a final

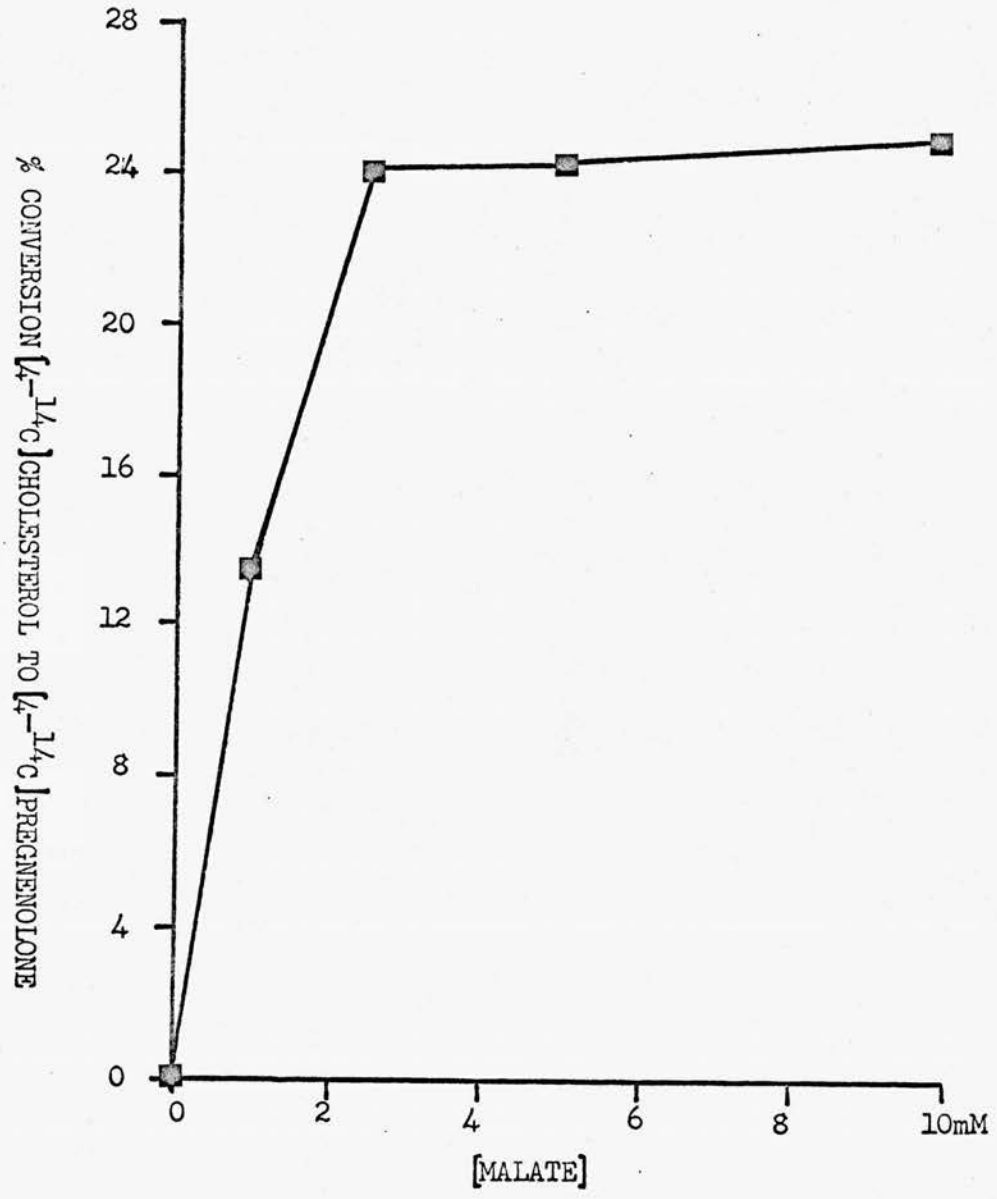


Figure 3.21. The effect of different concentrations of malate on cholesterol side chain cleavage activity in luteal mitochondria. [4-14C] Cholesterol was preincubated with the mitochondria for 10 minutes before initiation of side chain cleavage with malate. The enzymic activity was stopped with methanol ten minutes after the addition of malate.

concentration of 5 mM. /4 -^{14}C/ Pregnenolone formation was negligible in the absence of an added electron donor, and it is possible that endogenous substrate had been washed from the mitochondria during their preparation. Malate and isocitrate appeared to be equally efficient in supporting cholesterol side chain cleavage in five minute incubations. Succinate only supported 40% of the activity produced by malate and isocitrate. Malate and isocitrate were routinely used to measure the cholesterol side chain cleavage activity in luteal mitochondrial preparations. The effect of varying the concentration of malate in incubations is shown in figure 3.21. There was no difference in cholesterol side chain cleavage activity using malate concentrations from 2.5 to 10 mM. In all assays of cholesterol side chain cleavage, malate and isocitrate were used in the concentration range 3 to 6 mM.

The effect of cyclic nucleotides on cholesterol side chain cleavage activity in rat luteal mitochondria

Sutherland and Rall (1957) showed that a cyclic adenine nucleotide was involved in the adrenaline stimulated breakdown of glycogen in the liver. This cyclic nucleotide (cyclic AMP) was shown to accumulate in the adrenal gland in response to stimulation by ACTH. It was proposed by Haynes (1958) that cyclic AMP was an obligatory intermediate in the ACTH induced rise in corticosteroidogenesis in the adrenal gland. Cyclic AMP was thought to stimulate directly cholesterol side chain cleavage and 11 β-hydroxylation in rat adrenal homogenates (Roberts et al (1964), (1967); Creange and Roberts (1965)). These effects required cyclic AMP concentrations of above 500 μM which

Table 3.5

The effects of cyclic 3'5' AMP, cyclic 3'5' GMP and cyclic 3'5' UMP on cholesterol side chain cleavage activity in rat luteal mitochondria

cyclic nucleotide	concentration	% conversion [4-^{14}C] cholesterol to [4-^{14}C]-pregnenolone	
		saline treated	cycloheximide treated
cyclic 3'5' AMP	10^{-5} M	35.1	13.4
	10^{-6} M	35.9	12.8
	10^{-7} M	30.9	14.6
	10^{-8} M	39.3	13.8
	0	37.2	13.0
cyclic 3'5' GMP	10^{-5} M	36.5	13.8
	10^{-6} M	31.2	12.4
	10^{-7} M	29.3	10.9
	10^{-8} M	32.2	10.8
	0	38.8	13.3
cyclic 3'5' UMP	10^{-5} M	33.7	13.9
	10^{-6} M	39.1	12.1
	10^{-7} M	34.5	13.8
	10^{-8} M	38.6	11.9
	0	38.4	12.9

[4-^{14}C] cholesterol was preincubated with the luteal mitochondria for ten minutes before the addition of malate. The mitochondria were prepared from the ovaries of rats which had been treated with 0.9% saline or cycloheximide twenty minutes before they were killed (see chapter 7). Cyclic nucleotides were present in the incubations at the concentrations given above. Incubations were stopped by the addition of methanol 10 minutes after the addition of malate.

are up to one hundred times greater than those of 5 μM found in the adrenal cell (Grahame-Smith et al (1967); Haynes (1958)). Marsh et al (1966) have shown that cyclic AMP accumulates in response to LH stimulation in bovine corpus luteum slices. Increases of progesterone synthesis stimulated by cyclic AMP have been demonstrated in bovine and rat corpora lutea and rabbit interstitial tissue. Marsh and Savard (1964), (1966a); Savard et al (1965); Hall and Koritz (1965); Dorrington and Kilpatrick (1967); Hermier and Jutisz (1969)). Previous investigators could however find no effect of millimolar concentrations cyclic AMP on the cholesterol side chain cleavage activity of luteal mitochondria (Sulimovici and Boyd (1968a); Jackanicz and Armstrong (1968)). Similar findings have now been reported for bovine and rat adrenal cortex mitochondrial cholesterol side chain cleavage (Koritz et al (1968); Cohen and Moriwaki (1969); Kahnt et al (1974); Mahaffee et al (1974)). It was however of interest to see whether cyclic AMP had any direct effect on luteal mitochondria in which cholesterol side chain cleavage activity had been inhibited by prior treatment of the rats with cycloheximide (see chapter 7). Table 3.5 shows the effect of a wide range of cyclic AMP concentrations (10^{-5} to 10^{-8}M) on [4 -^{14}C] pregnenolone production in luteal mitochondria. As can be seen from the results cyclic AMP does not have any effect on production of [4 -^{14}C] pregnenolone in mitochondria from control animals or animals previously treated with cycloheximide. The fact that cyclic AMP does not reverse the effect of cycloheximide treatment, fits in with the observations of other workers who have shown that inhibitors of protein synthesis such as cycloheximide will block the steroidogenic effect on luteal tissue slices of LH without affecting the accumulation

of cyclic AMP (Marsh et al (1966)).

Recently it has been discovered that low concentrations of trophic hormones stimulate steroidogenesis in rat adrenal, rat leydig tumour cells, rat testis interstitial cells and rat luteal cells without a measurable rise in the cellular concentrations of cyclic AMP (Beall and Sayers (1972); Moyle and Ramachandran (1973); Rao and Saxena (1973); Rommerts et al (1973)). Cyclic GMP has been implicated in the steroidogenic response of rat luteal tissue to the subunits of HCG. Rat luteal tissue responds in vivo to subunits of HCG by producing progesterone without a detectable rise in cyclic AMP concentrations of the tissue. At the same time the tissue concentration of cyclic GMP rises. However no change in cyclic GMP concentration could be detected in response to HCG treatment (Rao and Carman (1973)). In cell cultures of adrenal cortex concentrations of cyclic GMP have been reported to rise in response to small doses of ACTH which do not cause a rise in cyclic AMP concentrations but cause increased steroid output (Wilson and Kitabachi (1973)). Glinsmann et al (1969) reported a stimulatory effect of cyclic GMP on steroid output in adrenal cortex slices. Kowal (1973) has failed to show a stimulatory effect of cyclic GMP on steroidogenesis in a line of mouse adrenal cortex cells in culture. Steroid output in these cells was stimulated by cyclic CMP and to a lesser extent cyclic UMP and cyclic IMP. It was therefore possible that cyclic GMP,cyclic CMP, cyclic UMP or cyclic IMP could stimulate cholesterol side chain cleavage directly. The results of adding cyclic UMP and cyclic GMP, to luteal mitochondria, on $/4\text{-}^{14}C/$ pregnenolone production are shown in table 3.5. As can be seen from the table no effect was observed

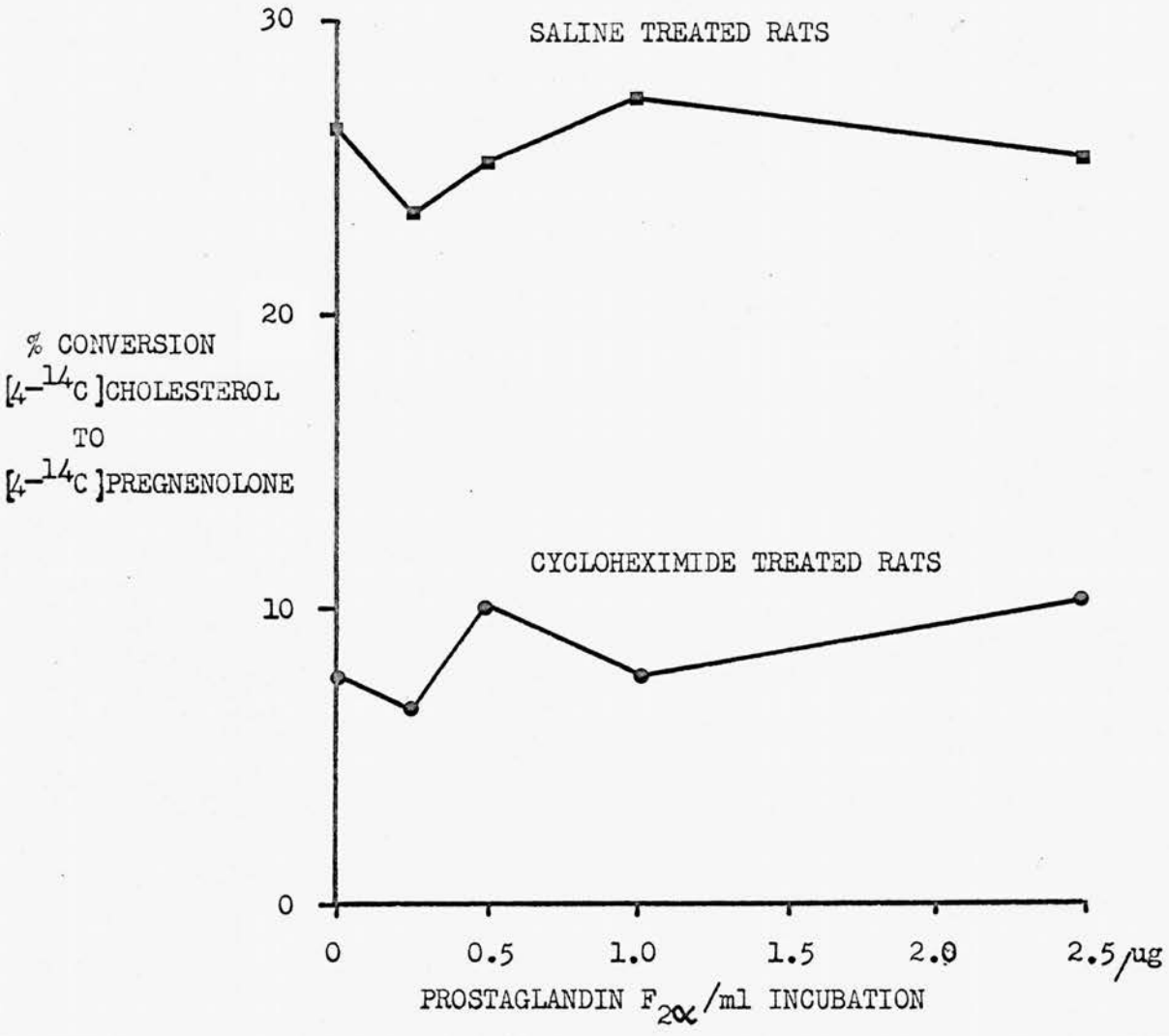


Figure 3.22. The effect of prostaglandin $F_{2\alpha}$ on cholesterol side chain cleavage activity in luteal mitochondria from rats treated with 0.9% saline or cycloheximide twenty minutes before being killed (Experimental procedure as described in text for figure 3.8.).

on luteal mitochondria from saline treated and cycloheximide treated rats. It therefore appears cyclic nucleotides do not directly affect cholesterol side chain cleavage in luteal mitochondria.

The effect of prostaglandin $F_{2\alpha}$ on cholesterol side chain cleavage in luteal mitochondria

Prostaglandin $F_{2\alpha}$ is known to be luteolytic in the rat (Pharris and Wyngarden (1969)). It causes the corpus luteum to regress and the steroid output of the gland to fall (Behrman et al (1971b)). Behrman et al (1971c) have shown one effect of prostaglandin $F_{2\alpha}$ in rat luteal tissue is to stop the storage of cholesterol as cholesterol ester. Another possible way prostaglandin $F_{2\alpha}$ could alter steroidogenesis would be to interact with the cholesterol side chain cleavage enzymes. Prostaglandin $F_{2\alpha}$ was added to incubations of luteal mitochondria from saline treated and cycloheximide treated rats. The formation of $[4\text{-}^{14}C]$ pregnenolone in these incubations is shown in figure 3.22. Prostaglandin $F_{2\alpha}$ would appear to have had no effect on the cholesterol side chain cleavage activity. However in vivo prostaglandin $F_{2\alpha}$ could still affect the output of the cholesterol side chain cleavage enzymes by restricting the availability of the substrate cholesterol.

Summary

This chapter has described the general properties of the mitochondrial cholesterol side chain cleavage system of rat corpus luteum.

(a) The products of the reaction in incubations have been shown to be pregnenolone and progesterone in the absence of cyanoketone and pregnenolone only in its presence. Cyanoketone does not affect the overall rate of cholesterol side chain cleavage.

(b) Assay of cholesterol side chain cleavage in luteal mitochondria has been discussed. The activity could be measured either as the appearance of pregnenolone or $[4\text{-}^{14}C]$ pregnenolone when trace amounts of $[4\text{-}^{14}C]$ cholesterol had been preincubated with the mitochondria for 10 minutes prior to the addition of an electron donor.

(c) The constituents of medium A (table 2.2) have been found to be optimal for pregnenolone production in incubations. The pH optimum of the cholesterol side chain cleavage enzyme was found to be in the range 7 to 7.8. The activity resided mainly in the mitochondrial fraction of the corpus luteum.

(d) Malate, isocitrate and succinate have been shown to support cholesterol side chain cleavage in intact luteal mitochondria. NADPH could only do so when the mitochondria were swollen with Ca^{++} ions. The possible significance of this calcium effect to the control of steroidogenesis is discussed.

(e) Cyclic nucleotides and prostaglandin $F_{2\alpha}$ had no direct effect on luteal mitochondrial cholesterol side chain cleavage activity in the incubation conditions described in this thesis.

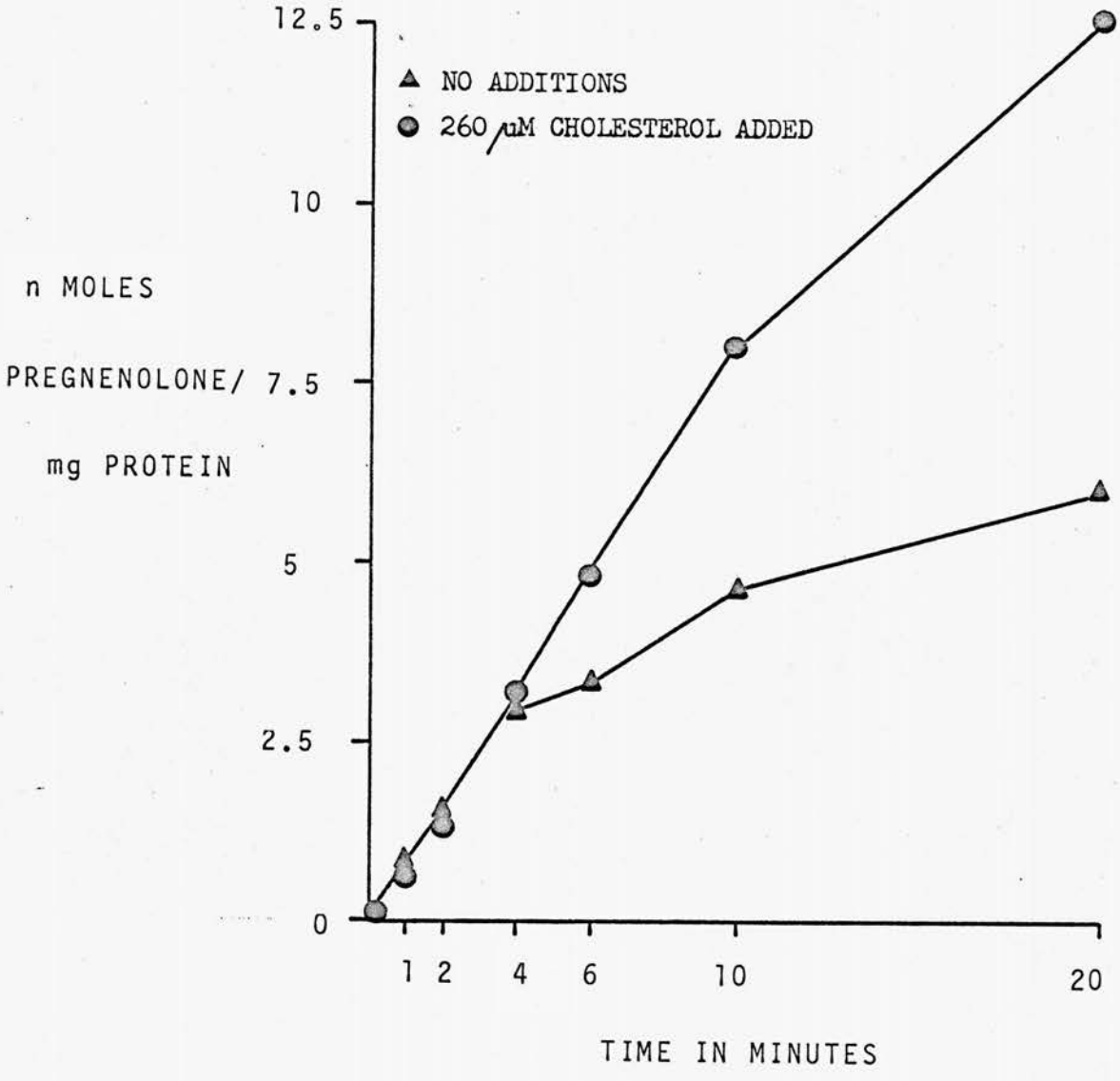


Figure 4.1. Formation of pregnenolone, from endogenous and added cholesterol by luteal mitochondria. Pregnenolone formation, initiated by the addition of malate, was measured in luteal mitochondria in the presence and absence of 260 uM cholesterol.

CHAPTER 4

SOME CHOLESTEROL AND OXYGEN METABOLISING PROPERTIES OF RAT LUTEAL MITOCHONDRIA

A. Cholesterol metabolising properties

Utilisation of endogenous and added cholesterol for steroidogenesis in incubations of rat luteal mitochondria

In vitro luteal mitochondrial pregnenolone production, from endogenous cholesterol, continued at a linear rate for five to six minutes (figure 3.1 and 3.4). After this time the rate of pregnenolone production began to fall. This fall was not caused by product inhibition of the reaction (figure 3.5). It was therefore of interest to see how the addition of more substrate cholesterol to incubations affected the rate of pregnenolone production in rat luteal mitochondria.

The effect of added cholesterol on the rate of cholesterol side chain cleavage in rat luteal mitochondria

Figure 4.1 shows the effect of the addition of cholesterol to mitochondrial incubations. There was no obvious dramatic effect on the initial rate of pregnenolone production. When the production of pregnenolone from endogenous cholesterol shows a decrease after 5-10 minutes, the added (260 μM) cholesterol corrects this and results in the pregnenolone formation continuing at the initial rate (0.8 n moles/

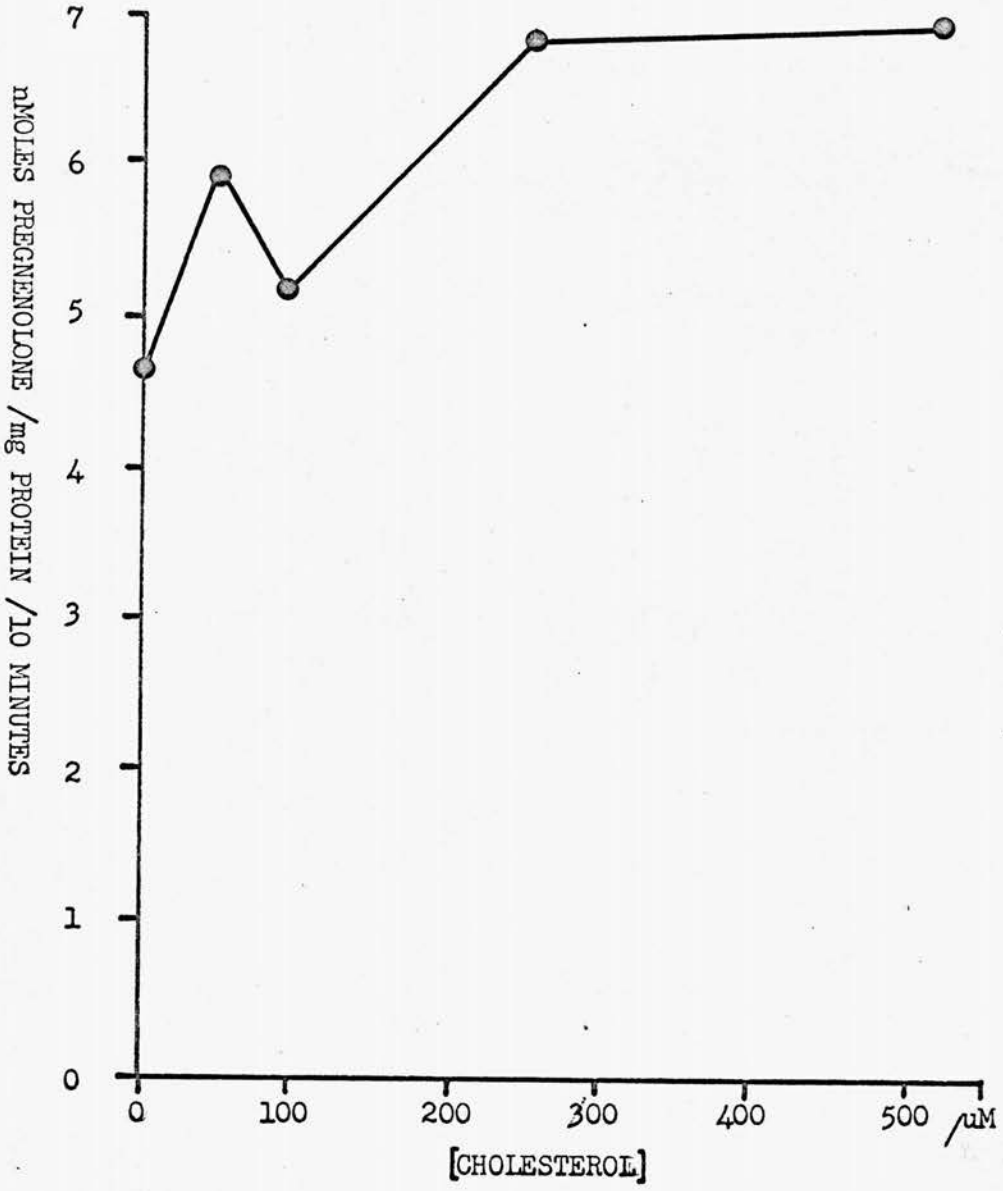


Figure 4.2. Formation of pregnenolone, from endogenous and different amounts of added cholesterol by luteal mitochondria. The mitochondria were preincubated with the cholesterol for ten minutes before initiation of side chain cleavage with malate.

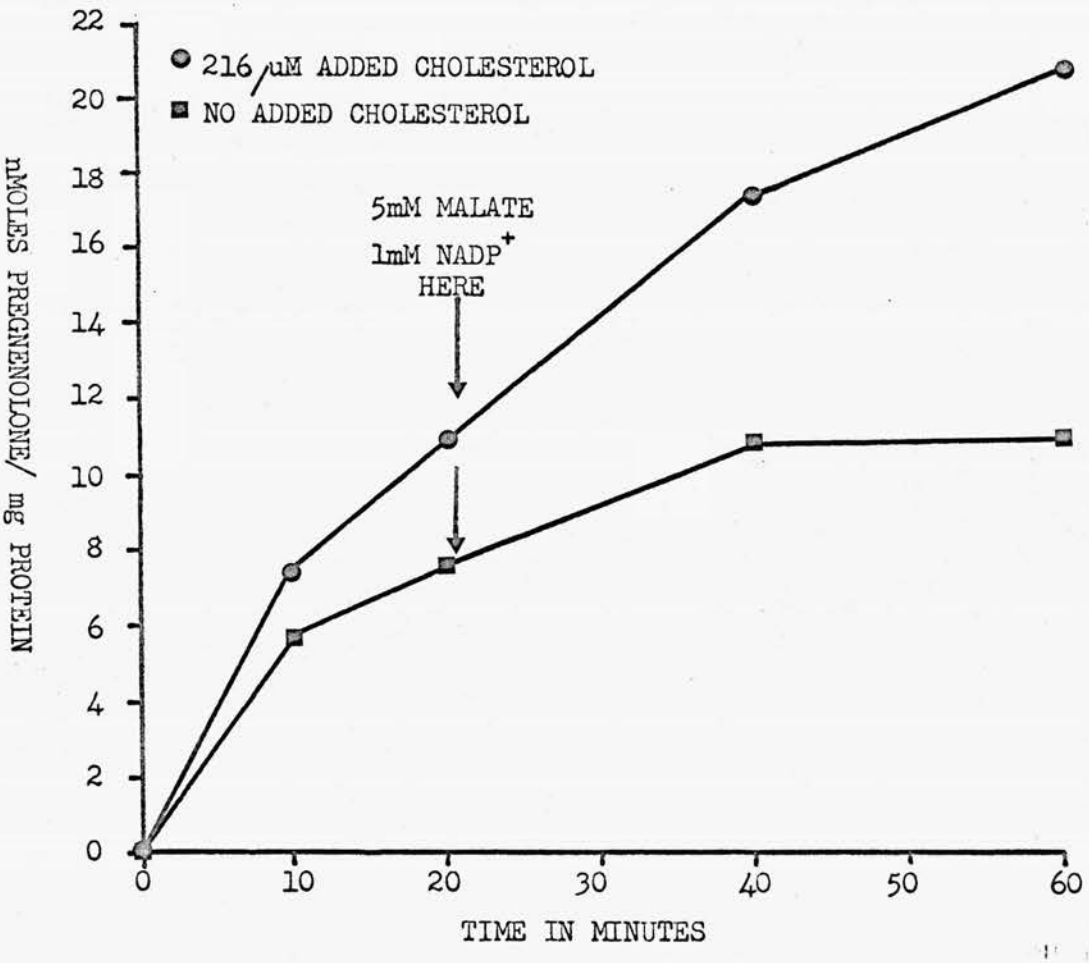


Figure 4.3. The formation of pregnenolone from endogenous and added cholesterol by rat luteal mitochondria. Conditions as for figure 4.1, but more malate (5mM) and $NADP^+$ (1mM) (final concs.) were added to the incubations twenty minutes after they were initiated.

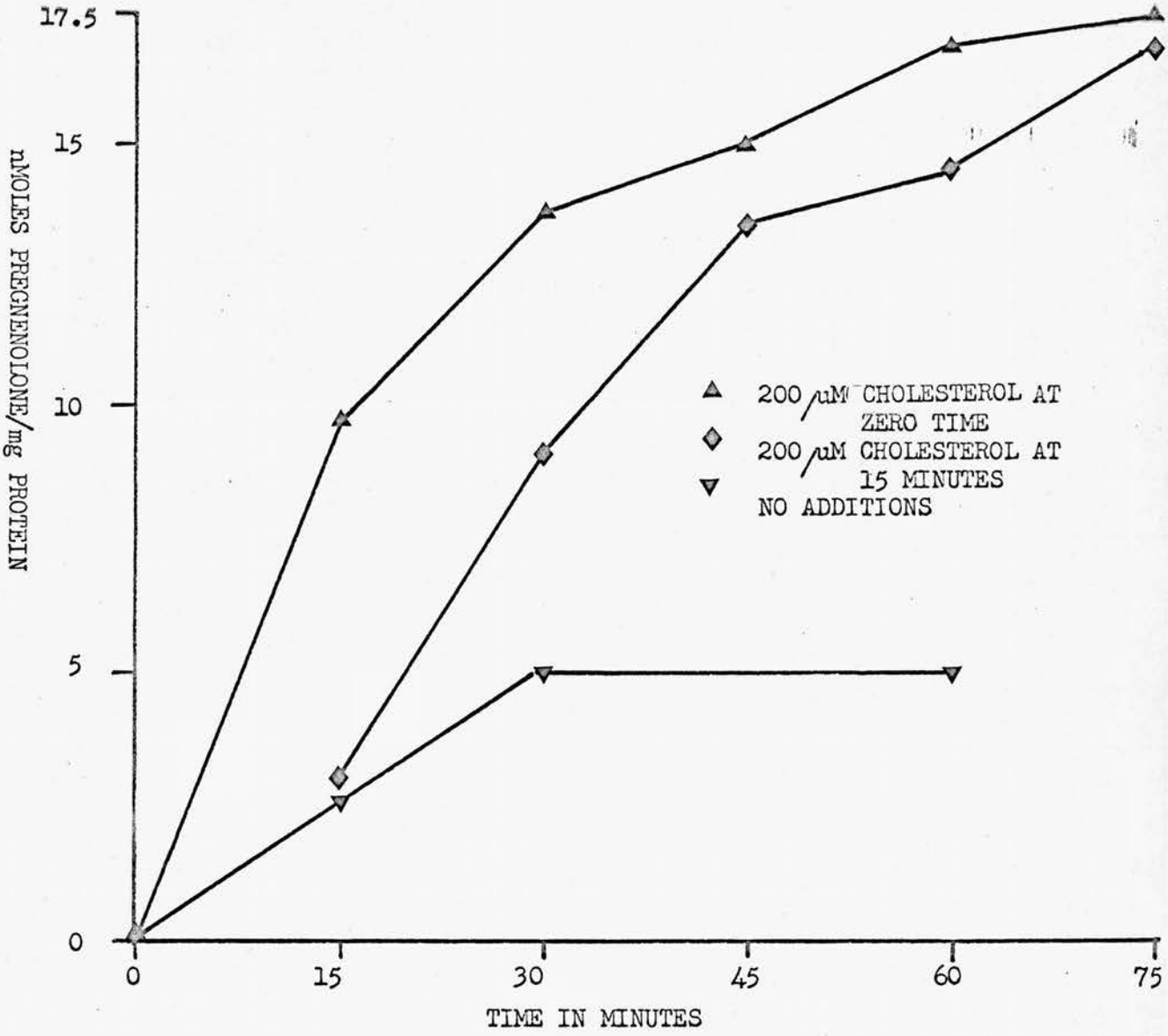


Figure 4.4. The effect of cholesterol, added at different times, on the pregnenolone production of rat luteal mitochondria. Conditions as for figure 4.1. but cholesterol was added at the times indicated on the graph. All incubations contained 1mM $NADP^+$.

mg/protein/min) for at least 20 minutes. The decline in the initial rate of pregnenolone production appears therefore to be due to depletion of substrate cholesterol. The results suggest that, the supply of cholesterol to the luteal mitochondrial cholesterol side chain cleavage system is important and mitochondrial stores of cholesterol can be depleted rapidly. Addition of cholesterol at concentrations above 260 μ M did not cause any further increase in pregnenolone production over that observed with 260 μ M cholesterol (figure 4.2). This suggested that under experimental conditions saturation kinetics for cholesterol had been established.

The fall in the rate of cholesterol side chain cleavage in incubations, after five minutes, could also be in part due to reduced levels of NADPH necessary for the mixed function oxidase reaction. The depletion of cholesterol in the luteal mitochondrial membrane during incubations may alter the permeability of the membrane to pyridine nucleotides. Addition of cholesterol to liver mitochondria in incubations has been shown to render them more stable (Graham and Green (1970)). Therefore extra cholesterol could be preventing the escape of NADPH through the mitochondrial membrane. Figure 4.3 shows the effect of adding 1mMNADP$^+$ and 5mM malate to incubations twenty minutes after they had been started with malate. This did not prevent the fall in pregnenolone production from endogenous cholesterol. Figure 4.4 shows the effect of addition of cholesterol to incubations performed in the presence of malate and NADP$^+$. The cholesterol was added at the same time and ten minutes after the malate. In both cases a great increase in pregnenolone production over that formed from endogenous cholesterol was observed. Thus after endogenous mitochondrial cholesterol has been depleted addition of more can cause an increase in pregnenolone production.

Phospholipid cholesterol micelles as a source of cholesterol for steroidogenesis

Bell and Harding (1974) have used cholesterol phospholipid micelles as substrate for the cholesterol side chain cleavage enzymes of the adrenal cortex. The micelles were used to solubilise the cholesterol, which normally has to be added to incubations dissolved in detergents or organic solvents. The micelles may be very similar to a possible natural substrate for the cholesterol side chain cleavage enzymes. In the corpus luteum and adrenal cortex most of the cholesterol in the cell is stored in the cytoplasmic lipid droplets, esterified at the 3 β-position to long chain fatty acids (see Boyd and Trzeciak (1973); Armstrong and Flint (1973)). These lipid droplets contain about 50% by weight of phospholipid. On trophic hormone stimulation of the adrenal or luteal cell the cholesterol ester in the lipid droplets is hydrolysed by the ACTH or LH sensitive cholesterol esterase enzymes (Trzeciak and Boyd (1973); Armstrong and Flint (1973)). This will leave phospholipid, cholesterol and fatty acids in the lipid droplets, which could be in the form of micelles similar to the ones produced synthetically. Bell et al (1973) and Bell and Harding (1974) quote rates for cholesterol side chain cleavage, in rat adrenal cortex mitochondria, of 1100 n moles/mg N/min (approx 200 n moles product/mg protein/min). These rates were determined at 37°C using lipid micelles as substrate. The quoted rate of cholesterol side chain cleavage (Bell and Harding (1974)) is over twenty times greater than the fastest rates observed with luteal mitochondria with cholesterol and added in acetone (figure 6.3) (Values corrected for incubation temperature and cytochrome P450 content of luteal mitochondria).

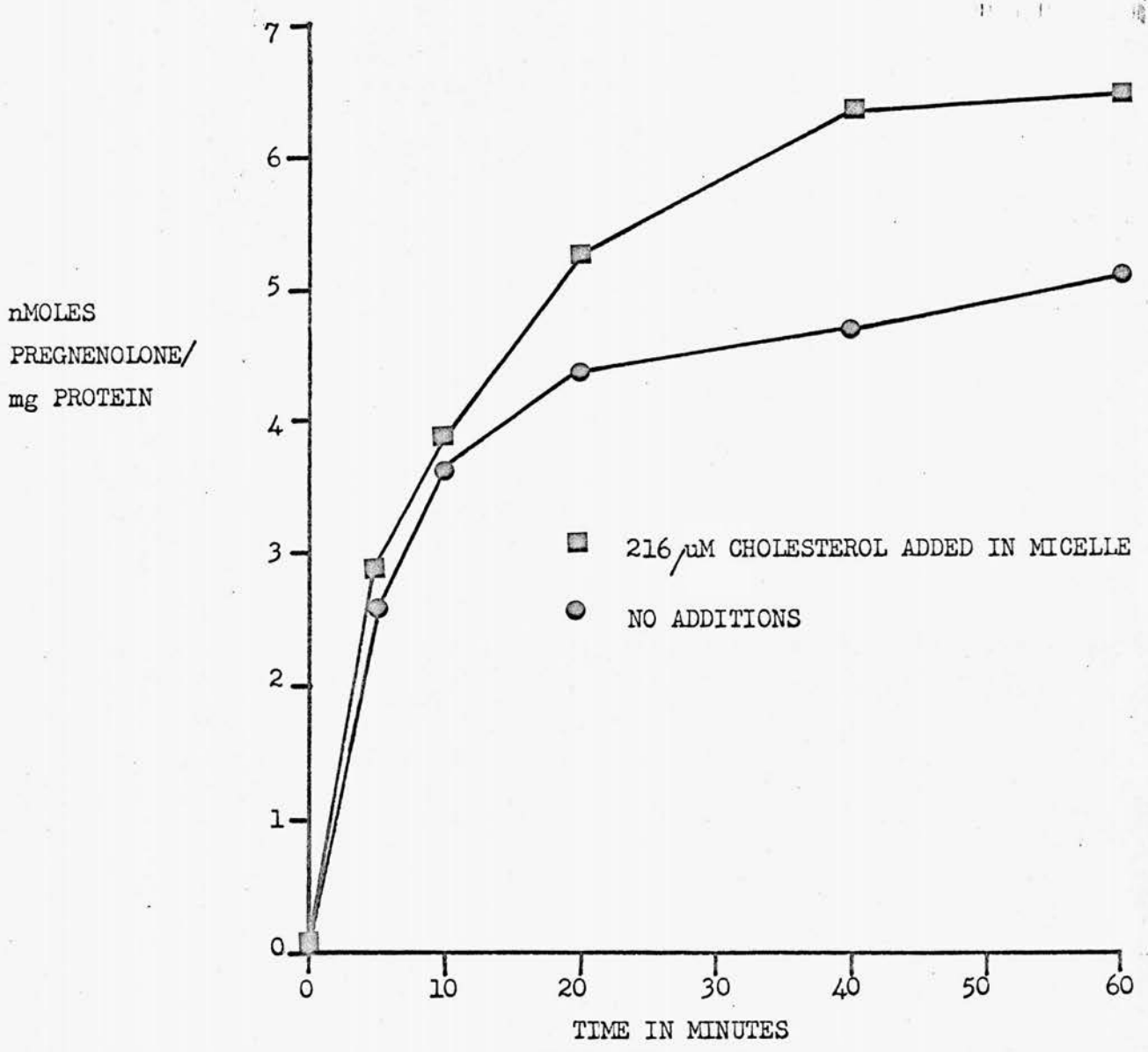


Figure 4.5. The effect of cholesterol, added in phospholipid micelles, on the pregnenolone production of rat luteal mitochondria. The mitochondria were preincubated for 10 minutes, with and without added micelles, before the addition of malate to initiate side chain cleavage.

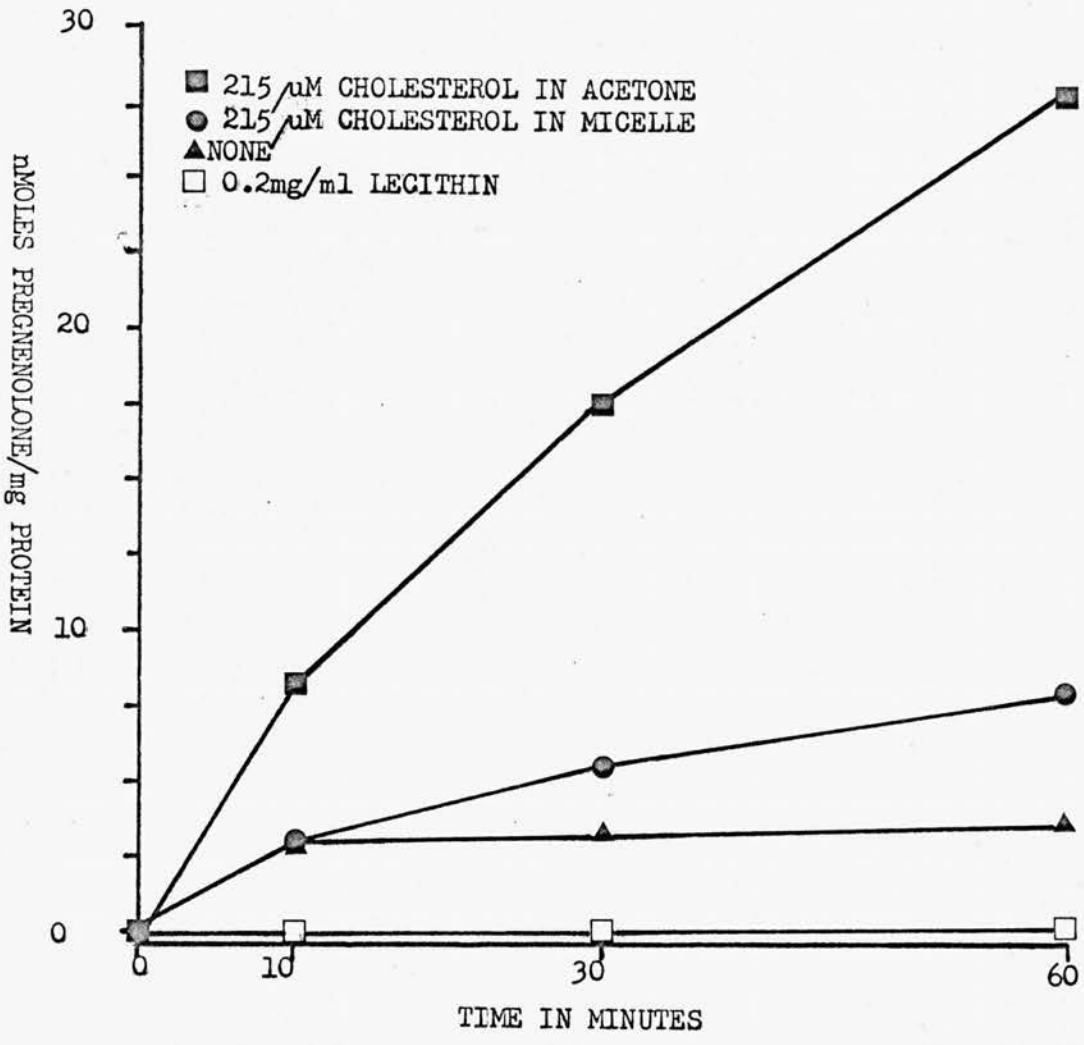


Figure 4.6. The effect of cholesterol added in acetone or in phospholipid micelles on the pregnenolone production of rat luteal mitochondria. The mitochondria were preincubated for ten minutes, alone or with the additions shown on the graph, before the initiation of side chain cleavage with malate.

Lipid micelles were prepared by dissolving 10 mg of cholesterol and 20 mg of egg lecithin in 2 mls of chloroform. After thorough mixing the chloroform was evaporated, under a stream of nitrogen, and 10 mls of medium A were then added to the cholesterol and phospholipid. The mixture was then sonicated at full power, with a Branson Utrasonicator, for twenty minutes. The phospholipid cholesterol suspension was kept under a stream of nitrogen and the temperature was not allowed to rise above 20°C. Figure 4.5 shows the effect of the micelles on the pregnenolone production of rat luteal mitochondria. The addition of micelles, to give a cholesterol concentration of 216 μM, did not appear to cause any extra pregnenolone production in ten minutes of incubation in comparison to the amounts produced in incubations without micelles. After forty minutes the mitochondria incubated with micelles had produced 40% more pregnenolone than those mitochondria incubated alone. After 40 minutes pregnenolone production had almost stopped in both incubations. The addition of cholesterol in phospholipid micelles did not therefore produce the rates of cholesterol side chain cleavage, in luteal mitochondria, recently reported to occur in adrenal cortex mitochondria by Bell and Harding (1974). Addition of cholesterol dissolved in acetone or ethanol appeared to be just as effective a way of presenting it to the luteal mitochondrial cholesterol side chain cleavage enzymes as addition in a phospholipid micelle. Figure 4.6 shows the effect of adding cholesterol dissolved in acetone or as a micelle, with phospholipid, on pregnenolone production in the same preparation of luteal mitochondria. Cholesterol added in acetone was the most effective method of providing substrate for pregnenolone production in these

mitochondria. After incubation for one hour there was four times as much pregnenolone produced by the mitochondria which had cholesterol added in acetone than the mitochondria with cholesterol added in micelles. The addition of lecithin in the quantities found in the micelles caused the complete inhibition of cholesterol side chain cleavage activity (figure 4.6). This inhibitory action of lecithin is probably the reason the cholesterol added in micelles caused less pregnenolone formation in incubations than the cholesterol added in acetone. $NADP^+$ was present in all the incubations shown in figure 4.6 to ensure that lecithin could not cause an inhibition of side chain cleavage through altering mitochondrial membrane permeability. The phospholipid could have allowed escape of $NADP^+(H)$ from the luteal mitochondria, thus decreasing the source of reducing power needed for the conversion of cholesterol to pregnenolone. Lecithin has been shown to inhibit cholesterol side chain cleavage activity in the mitochondria of human term placenta (Mason and Boyd (1971)). This inhibitory effect of lecithin was thought be due to it binding steroid thus making cholesterol unavailable for side chain cleavage. Cholesterol and lecithin are known to interact to form membrane like liposomes (Shimshick and McConnell (1973); De Gier et al (1968)). Incubation of red blood cells with lecithin results in the phospholipid extracting some of the cholesterol from the cell membrane (Masiak and Le Fevre (1974)). The difference in the rates of cholesterol side chain cleavage measured in this study and those of Bell and Harding (1974) are difficult to explain. The rates of steroidogenesis quoted by Bell and Harding (1974) were maintained in incubations for less than fifteen seconds. An error may be introduced

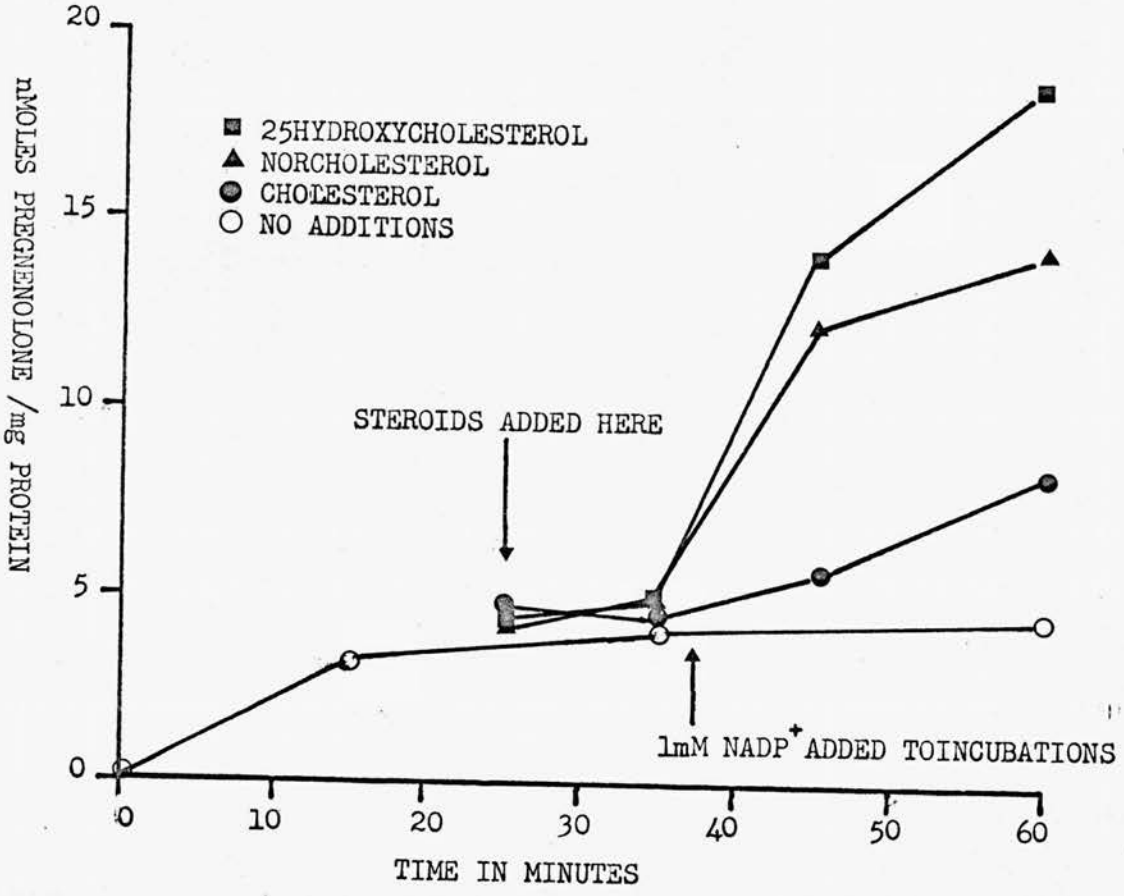


Figure 4.7. Derivatives of cholesterol as substrates for side chain cleavage in luteal mitochondria.

here as only one point on a graph was used to measure the rate. In incubations of luteal mitochondria, linear rates of pregnenolone production could be maintained for up to one half hour (figure 4.6) but were $\frac{1}{20}$ of the initial rate quoted by Bell and Harding (1974).

Different steroids as substrates for side chain cleavage in rat luteal mitochondria

Figure 4.7 shows the addition of 25-hydroxycholesterol or nor-cholesterol or cholesterol to luteal mitochondria which had been incubated for 25 minutes with malate, to deplete their endogenous cholesterol. The amount of pregnenolone in each incubation was determined in samples taken before the extra steroids were added. In a further ten minutes no more pregnenolone appeared to be formed in any of the incubations with added steroid. The addition of 1 mM$NADP^+$, ten minutes after the steroids, had a dramatic effect on the pregnenolone formation in the incubations. Pregnenolone was formed in the incubations with added 25-hydroxycholesterol, nor-cholesterol and cholesterol. The incubation without added steroid produced no more pregnenolone on the addition of $NADP^+$. During the depletion of the endogenous cholesterol, the $NADP^+(H)$ must have escaped from the mitochondria, thus decreasing the supply of reducing power for the cholesterol side chain cleavage enzymes. The absence of further pregnenolone production in the incubation without any added steroid after the addition of $NADP^+$ shows that the endogenous substrate cholesterol in the mitochondria had been severely depleted. Both 25-hydroxycholesterol and nor-cholesterol appear to be better substrates for pregnenolone production than cholesterol. 25-Hydroxycholesterol, with its bulky hydroxyl group on the side chain

and nor-cholesterol, which has one methyl group less on the side chain (C26 or C27), may not interact with the mitochondrial membrane to the same extent as cholesterol. Thus there would be less competition between membrane binding sites and cytochrome P450 for the modified steroids than there would be in the case of cholesterol. This competition for binding sites may result in the smaller rate of side chain cleavage of cholesterol in comparison to nor-cholesterol and 25-hydroxycholesterol. In another cytochrome P450 dependent reaction, alterations of the side chain structure of cholesterol have been shown to reduce the 7 α-hydroxylation of the steroid (Boyd et al (1974)). This is direct contrast to the effect of side chain alterations on the conversion of cholesterol to pregnenolone.

B. Respiratory properties of rat luteal mitochondria

Oxygen content of medium A

The oxygen content of medium A was determined using the method of Estabrook and Mackler (1957). Submitochondrial particles were prepared, from rat liver, using the method described in chapter 2. These particles were then used to oxidise known amounts of NADH. Assuming that during the oxidation of NADH one atom of oxygen is consumed the amount of the gas in medium A could be determined. Using this method the oxygen content of medium A was calculated to be 0.48 μ atoms/ml at 29°C.

Table 4.1

Oxidative, phosphorylative and respiratory control capacities of luteal mitochondria

Substrate	oxygen consumption n atoms/mg protein/min State 4 (-ADP)	State 3 (+ADP)	Respiratory control ratios	ADP/O
Succinate 2.2 mM	88 ± 4(7)	221 ± 20(5)	2.6 ± 0.2(5)	1.6 ± 0.2(4)
Malate 5.0 mM	30 ± 3(9)	84 ± 7(8)	2.9 ± 0.3(7)	2.5 ± 0.2(7)
Isocitrate 5.0 mM	37 ± 2(4)	107 ± 17(3)	2.8 ± 0.5(3)	2.3 ± 0.2(3)
∝-Ketoglutarate 5.0 mM	33 ± 2(4)	85 ± 15(3)	2.5 ± 0.4(3)	2.0 ± 0.3(3)
NADH 1.0 mM	no detectable uptake of oxygen supported by NADH, no stimulation on addition of ADP			
NADPH 1.0 mM	similar results to NADH above			

Reaction conditions were similar to those shown in figure 4.8. Respiratory control ratios and ADP:O ratios were determined as described in the methods section. Results expressed as mean ± SEM; number of determinations in parenthesis.

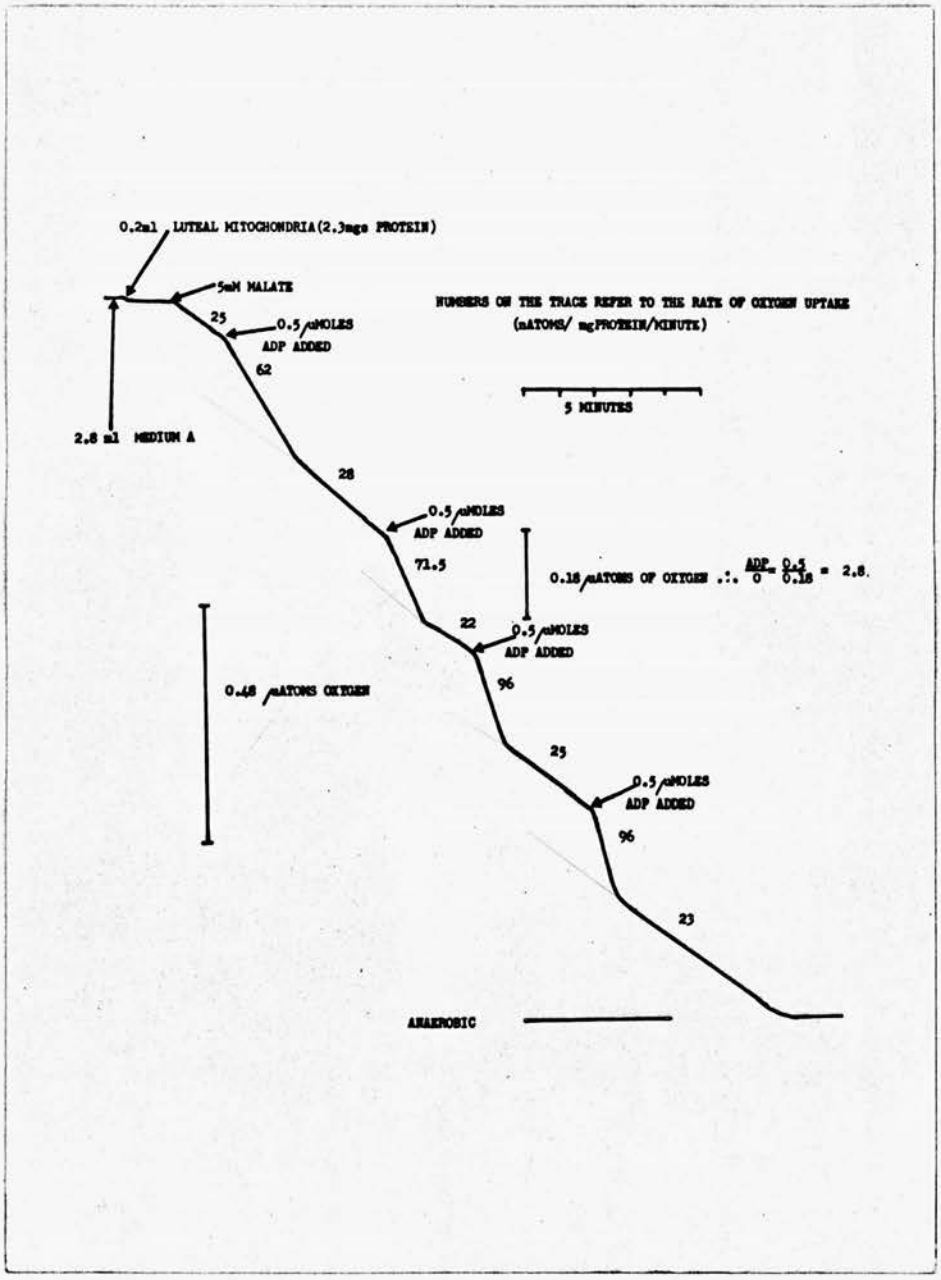


Figure 4.8. The oxygen uptake of luteal mitochondria in the presence of malate.

Oxygen uptake by rat luteal mitochondria

The rat luteal mitochondrial preparation oxidised several Krebs cycle intermediates (Table 4.1). Succinate caused the greatest oxygen uptake in the presence and absence of ADP. The mitochondria did not appear to oxidise added NADPH or NADH to any appreciable extent under the conditions used to measure ADP/O ratios. ADP did not stimulate oxygen uptake in the presence of NADPH or NADH. The conditions of incubation for oxygen uptake experiments were the same as those used for cholesterol side chain cleavage assays. Thus the lack of oxygen uptake on the addition of NADPH reflects its inability to effectively support cholesterol side chain cleavage in the intact mitochondria. The NADPH probably can not penetrate the inact mitochondrial membrane. This is in agreement with the results of Yago et al (1967a), McIntosh et al (1971), and Robinson and Stevenson (1971a), who worked with bovine and porcine luteal mitochondria. Unlike NADH and NADPH supported oxygen uptake that caused by Krebs cycle intermediates was stimulated by ADP (Table 4.1). Figure 4.8 shows a recording of the oxygen uptake in luteal mitochondria metabolising malate. No oxygen uptake was observed until the addition of 3.3 mM malate to the mitochondria. Addition of ADP caused a two to four fold stimulation of oxygen uptake. The conversion of all the added ADP to ATP resulted in a sharp decrease in the respiratory rate. Four additions of ADP are shown in figure 4.8. The sharp changes in the rate of oxygen uptake in the absence of ADP (state 4) and presence of ADP (state 3) show the mitochondria have a high respiratory control capacity. High respiratory control is an indication of intact mitochondria (Chance and Williams (1956)). The

Table 4.2

The effect of changes in the composition of medium A on coupling and respiratory control in rat luteal mitochondria

change in medium A	coupling	respiratory control ratio
none	yes	1.8
no Mg^{++}	none	none
no BSA	ADP addition caused a 50% increase in the rate of oxygen uptake	no return to state 4 respiration after ADP addition
no EDTA	ADP causes 45% increase in oxygen uptake	no return to state 4 respiration after ADP addition
0.2 mM EDTA	yes	1.7
0.3 mM EDTA	yes	1.7

Oxygen uptake was measured in luteal mitochondria in the presence of 2.2 mM succinate. The ability of ADP to stimulate oxygen uptake (coupling) was investigated in varied incubation media. It was also noted whether the mitochondrial respiration could return to state 4 rates seen before the addition of ADP (Respiratory control).

amount of oxygen consumed for conversion of the added ADP to ATP (ADP/O ratios) could be calculated from the traces (Chance and Williams (1955)). Table 4.1 shows the respiratory control values and ADP/O ratios calculated for luteal mitochondria with different Krebs cycle intermediates.

The composition of medium A was important in determining whether the luteal mitochondria could display respiratory control and be used to measure ADP/O ratios. Without BSA or EDTA in the incubation medium ADP addition still increased the oxygen uptake of mitochondria in the presence of succinate. The mitochondria continued to take up oxygen and did not show a return to state 4 respiration as observed with medium A (See table 4.1 and 4.2). An ATPase must have been converting ATP formed back to ADP which continued to stimulate the oxygen uptake. BSA or EDTA alone in the incubation medium could not reverse this effect. Respiratory control only returned when 0.1 mMEDTA and 0.1% BSA were included in medium A. EDTA (0.1, 0.2 or 0.3mM) in the presence of 0.1% BSA for incubations in the oxygen electrode gave similar values for mitochondrial coupling and respiratory control (Table 4.2). Omission of magnesium from medium A resulted in the loss of respiratory control and coupling in luteal mitochondria even in the presence of BSA and EDTA. BSA, EDTA and magnesium as well as being necessary to maintain coupling stimulated cholesterol side chain cleavage in luteal mitochondria (chapter 3).

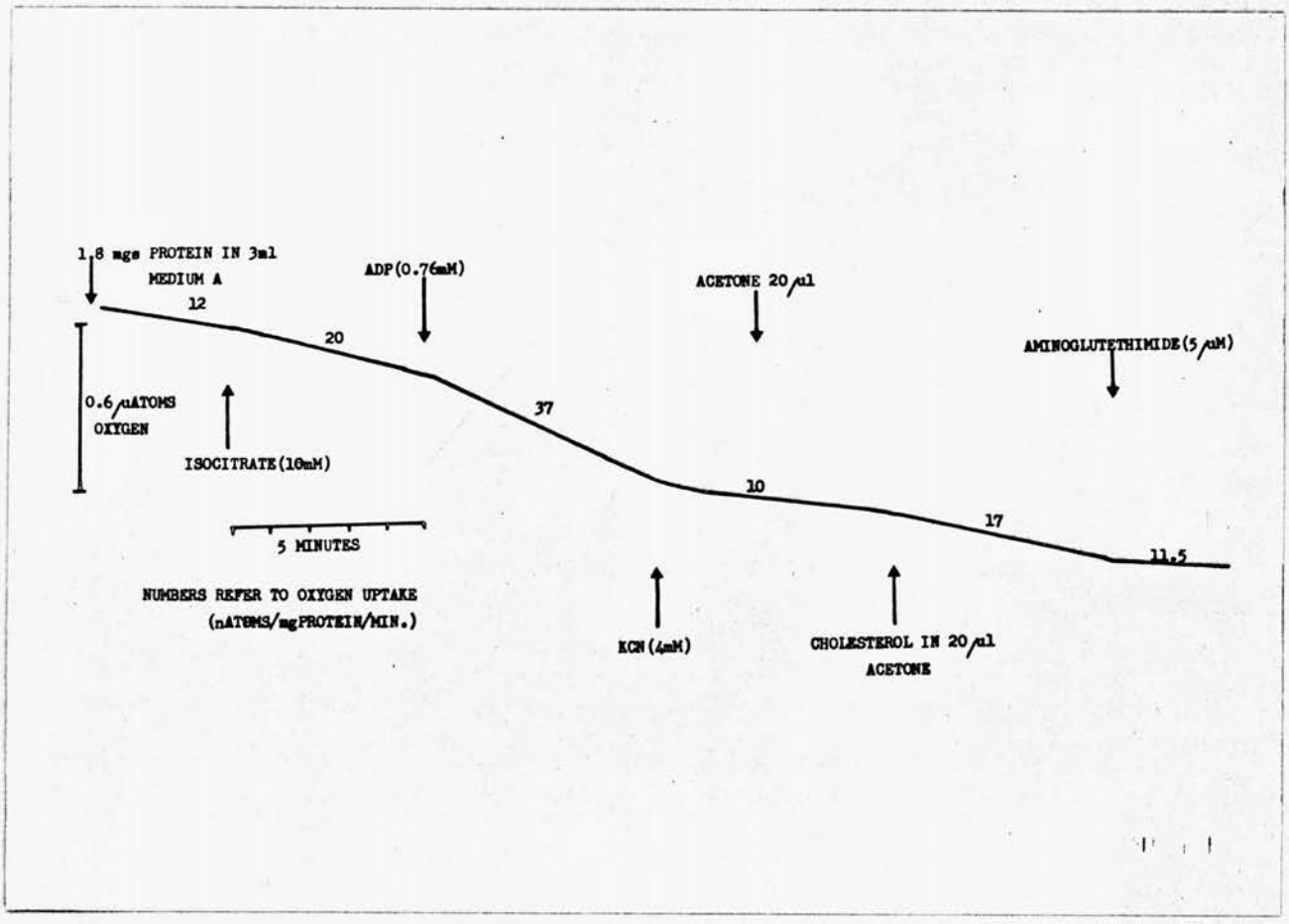


Figure 4.9. The effect of the addition of cholesterol on the oxygen uptake of rat luteal mitochondria. The final concentrations of the compounds added to the incubations are given on the figure.

The effect of the addition of cholesterol on oxygen uptake by rat luteal mitochondria

The addition of cholesterol to mitochondria previously incubated with malate or isocitrate to deplete their endogenous cholesterol resulted in an increased production of pregnenolone (figure 4.6). It was of interest to see how this increase in pregnenolone production affected the oxygen uptake of the mitochondria. Figure 4.9 shows the result of an experiment to investigate this. Isocitrate was added to the mitochondria, in the oxygen electrode, to initiate cholesterol side chain cleavage. ADP was then added to test for coupling in the mitochondria. In this case ADP caused a two fold increase in isocitrate supported oxygen uptake. Potassium cyanide was then added to prevent the flow of electrons down the respiratory chain, but it did not completely inhibit oxygen uptake by the mitochondria. The mitochondria had been previously incubated, in the oxygen electrode, for fifteen minutes so that most of their endogenous cholesterol would have been converted to pregnenolone. Acetone was then added to the electrode vessel but did not cause any increase in oxygen uptake. Then after this cholesterol (216 μM final concentration) was added to the incubation. Addition of cholesterol can be seen so have stimulated oxygen uptake from 5.0 n moles/mg protein/min to 8.5 n moles/mg protein/min. In incubations the rate of steroidogenesis in the presence of added cholesterol is about 1 n mole pregnenolone produced/mg protein/min (figure 4.1). Thus molar oxygen uptake exceeds molar pregnenolone production by a factor of eight to nine. The accepted stoichiometry of the side chain cleavage reaction is three molecules of oxygen consumed for each molecule of cholesterol converted to pregnenolone. This has been

confirmed using a preparation of cholesterol side chain cleavage cytochrome P450 from bovine adrenal cortex (Shikita and Hall (1974)). In intact luteal mitochondria, cyanide may not completely inhibit the respiratory chain or there may be other hydroxylation or peroxidation events occurring. Another reason for the apparent deviation from stoichiometry of the cholesterol side chain cleavage reaction could be so called "non-productive cytochrome P450 cycles", which result in oxygen consumption without the production of hydroxylated intermediates, other than H_2O_2. This type of phenomenon has been observed in liver microsomal preparations (Ullrich and Didhl (1971)). When cholesterol side chain cleavage was inhibited by aminoglutethimide (figure 4.9 see also figure 5.7) there was still an oxygen consumption rate of 5.6 n moles/mg protein/min. Thus it is not really practical to use intact mitochondria to study the stoch-iometry of cholesterol side chain cleavage as there appear to be too many other processes occurring which cause a background consumption of oxygen. It was however possible to show that addition of cholesterol to incubations, which causes increased pregnenolone production, also causes an increase in oxygen uptake.

Summary

(a) In incubations of luteal mitochondria the fall off in the rate of cholesterol side chain cleavage, observed after five to ten minutes of reaction, is due to depletion of substrate cholesterol.

(b) The fall off in rate can be corrected by addition of cholesterol in acetone to the incubations. This does not cause any appreciable increase in the initial rate of cholesterol side chain cleavage.

(c) Cholesterol added to incubations in phospholipid micelles was found to be less efficiently metabolised by the side chain cleavage enzymes than cholesterol added in acetone.

(d) Nor-cholesterol and 25-hydroxycholesterol were found to be more rapidly converted to pregnenolone than cholesterol by luteal mitochondria.

(e) Luteal mitochondria in medium A were shown to exhibit respiratory control and coupling properties in the oxygen electrode.

(f) Cholesterol was shown to stimulate oxygen uptake by luteal mitochondria, which had been incubated with isocitrate or malate, to deplete the endogenous supplies of steroid.

CHAPTER 5

SPECTRAL PROPERTIES OF THE LUTEAL MITOCHONDRIAL CHOLESTEROL SIDE CHAIN CLEAVAGE SYSTEM

Introduction

The discovery and role of cytochrome P450 in the cholesterol side chain cleavage reaction have been discussed in chapter one. In endocrine tissues cytochrome P450 was first discovered in the microsomal and the mitochondrial fractions of the adrenal cortex. (Estabrook et al (1963); Harding et al (1964)). In the mitochondria of the adrenal cortex the cytochrome P450 is involved in cholesterol side chain cleavage, 11 β-hydroxylation and 18-hydroxylation reactions (Simpson and Boyd (1967); Wilson and Harding (1970); Greengard et al (1967)). However in the mitochondrial fraction of the corpus luteum there is only one steroid mixed function oxidase namely the cholesterol side chain cleavage system (Ichii et al (1963)). This cholesterol side chain cleavage system is probably identical to the one found in adrenal cortex mitochondria; consisting of cytochrome P450, a non-haem-iron protein (ovarian ferredoxin) and a NADPH specific flavo-protein dehydrogenase (Sulimovici and Boyd (1968b); Simpson and Boyd (1967a); see figure 1.3).

Cytochrome P450 has been shown to be present in luteal mitochondria (Yohoro and Horie (1967); Sulimovici and Boyd (1968b); McIntosh et al (1971)). Also present in pig luteal mitochondria is a non-haem-iron protein (ovarian ferredoxin) similar to the adrenal non-haem-iron protein (Kimura and Ohono (1968); Ohono et al (1967)).

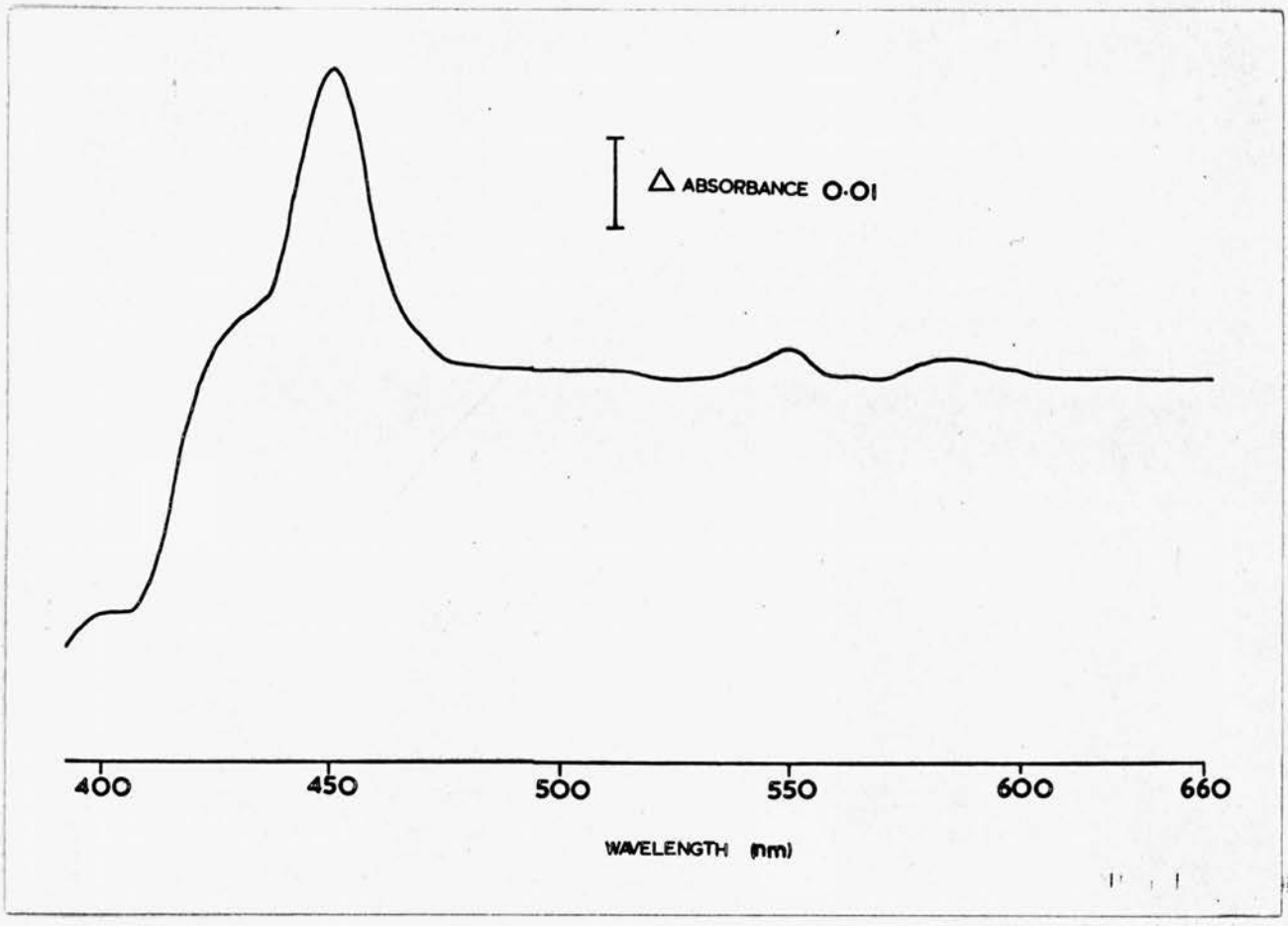


Figure 5.1. Dithionite reduced carbon monoxide minus dithionite reduced difference spectrum of rat luteal mitochondria. Note the peak at 450 nm characteristic of cytochrome P450 (Omura and Sato (1964a)). The mitochondrial protein concentration was 1 mg/ml in medium A. The concentration of cytochrome P450 in the mitochondria was then 0.36 nmoles/mg protein.

Table 5.1

The cytochrome contents of mitochondria from adrenal cortex, corpus luteum and testis

Tissue	a(+ a_3)	b	c(+ c_1)	P450
(a)bovine adrenal cortex	0.23	0.17	0.29	1.5
(b)bovine corpus luteum	0.23	0.15	0.35	0.18
(c)bovine corpus luteum	-	-	-	0.17
(d)rat corpus luteum	0.15	0.12	0.14	0.24
(e)rat corpus luteum	0.349	-	-	0.29
(f)rat corpus luteum	$0.13 \pm 0.01(7)$	$0.22 \pm 0.02(7)$	$0.35 \pm 0.02(7)$	$0.41 \pm 0.01(20)$
(g)pig testis	0.06	0.06	0.12	0.07

The cytochrome content of luteal mitochondria was determined as described in the methods. The results which are shown in (f) are expressed as mean $\pm$ SEM; number of determinations in parenthesis; other values from (a) Cammer and Estabrook (1967b); (b) McIntosh *et al* (1971); (c) Yohoro and Horie (1967); (d) Cooper and Thomas (1970); (e) Flint and Armstrong (1971b); (g) Mason *et al* (1973).

The ovarian ferredoxin can replace adrenal ferredoxin in a reconstituted adrenal cortex mitochondrial 11 β-hydroxylase system (Kimura and Ohono (1968)). The adrenal ferredoxin can replace ovarian ferredoxin in the reconstitution of bovine luteal mitochondrial cholesterol side chain cleavage activity (McIntosh et al (1973)). The adrenal, ovarian and testicular mitochondrial ferredoxins, from rat tissue, have been shown to be immunologically very similar (Baron (1973)). Thus the ovarian ferredoxin seems to have similar functional properties to the more widely studied adrenal ferredoxin. This chapter describes some spectral properties of luteal mitochondrial cytochrome P450 and ovarian ferredoxin.

Cytochrome P450 in rat luteal mitochondrial preparations

Cytochrome P450 was found in rat luteal mitochondria, confirming the results of Sulimovici and Boyd (1968b), Cooper and Thomas (1970) and Flint and Armstrong (1971b). Figure 5.1 shows the carbon monoxide anaerobic minus anaerobic difference spectrum of the luteal mitochondrial preparation. In the example shown the cytochrome P450 content was 0.36 n moles/mg protein, calculated using the extinction coefficient for liver microsomal P450 quoted by Omura and Sato (1964a). In twenty different luteal mitochondrial preparations the average cytochrome P450 content was 0.41 $\pm$ 0.02 moles/mg protein (Mean $\pm$ SEM). This is greater than the values previously reported for rat, bovine and porcine corpora lutea (see table 5.1). The spectrum of cytochrome P450 (figure 5.1) shows no Soret peak at 420 nm, where an enzymically inactive form of cytochrome P450, cytochrome P420 has an absorption band (Ichikawa and Yamano (1967)). The shoulder at 430 nm in the

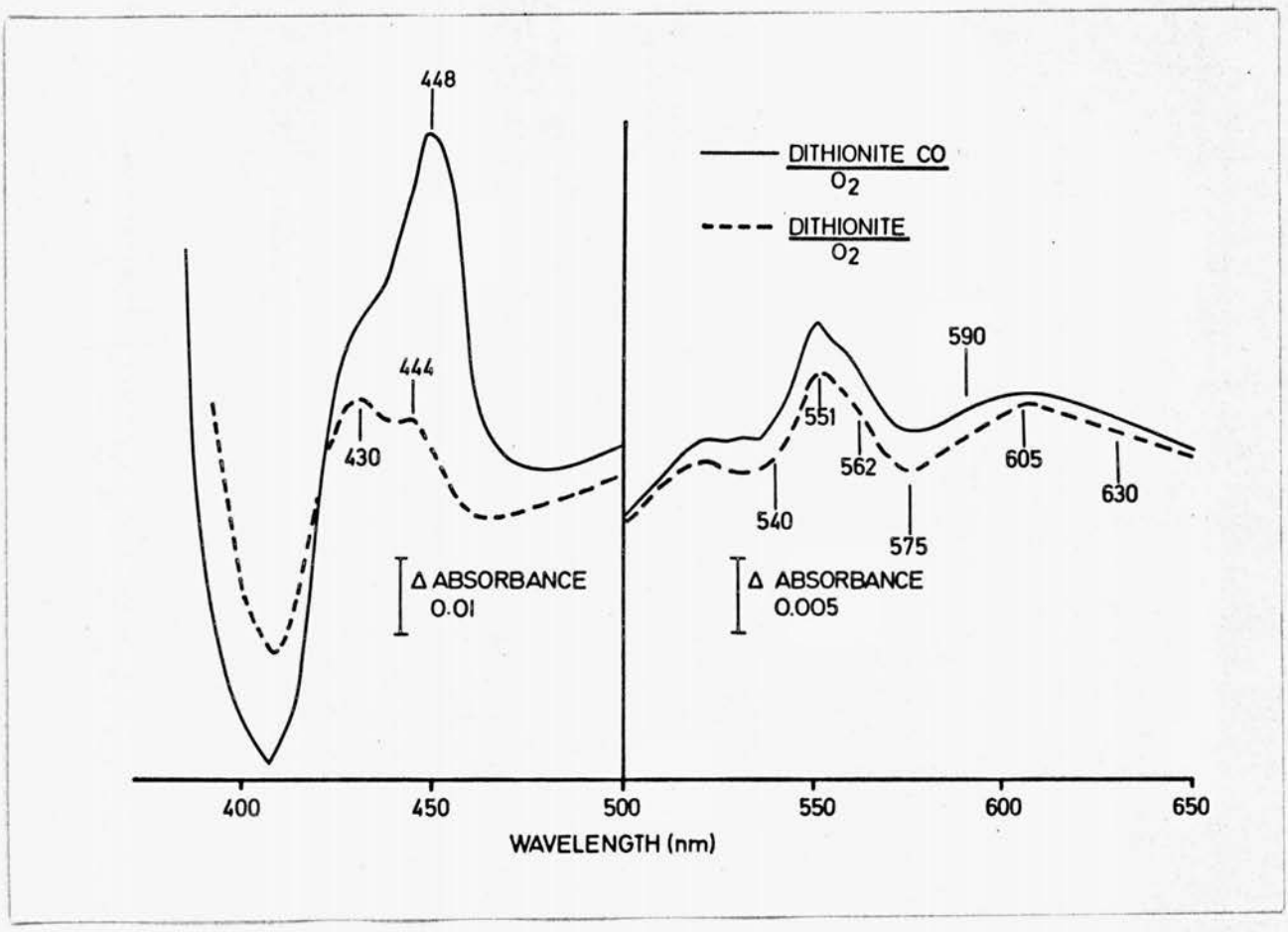


Figure 5.2. Difference spectra showing the respiratory chain cytochromes of rat luteal mitochondria. Mitochondrial protein concentration was 1 mg/ml. Using the extinction coefficients of Estabrook and Holowinsky (1961) the cytochrome contents of luteal mitochondria were calculated as a(a_3) 0.13 nmoles/mg protein, b 0.18 nmoles/mg protein and c(c_1) 0.32 nmoles mg/ protein.

cytochrome P450 peak is probably due to a CO complex with reduced cytochrome a_3 (Yonetani (1960)).

Respiratory chain cytochromes in rat luteal mitochondria

As well as the cytochrome P450 containing electron transport hydroxylating chain, luteal, adrenal cortical and testicular mitochondria contain the normal respiratory chain (Cammer and Estabrook (1967b); McIntosh et al (1971); Cooper and Thomas (1970); Mason et al (1973)). Figure 5.2 shows the dithionite reduced minus aerobic difference spectrum of rat luteal mitochondria. The peaks and shoulders at 551, 562 and 605 nms are the α bands of cytochrome cc_1, b and aa_3. The respiratory chain cytochrome content was measured (see chapter 2) and the results are shown in table 5.1. The values obtained for the amounts of respiratory chain cytochrome compare well with those found by other workers (Table 5.1). Also shown in figure 5.2 is the reduced-CO minus aerobic difference spectrum of rat luteal mitochondria. The appearance of bands at 430 and 590 nm were due to a CO complex of reduced cytochrome a_3 (Yonetani (1960)). The band appearing at 448 nM was due to cytochrome P450 and only appears in tissues containing this pigment. When cytochrome a_3 is present in a greater than 1 to 1 ratio in comparison to cytochrome P450, it shifts the peak of the P450 spectrum towards 460 nm. This is due to a trough in the reduced cytochrome a_3 - CO spectrum at 446 nm (Mason et al (1973)). There is no interference in the luteal cytochrome P450 spectrum from cytochrome a_3. Apart from the demonstration of its presence, little is known of the properties of luteal mitochondrial cytochrome P450. The steroid and nitrogenous ligand binding properties of the cytochrome were therefore investigated.

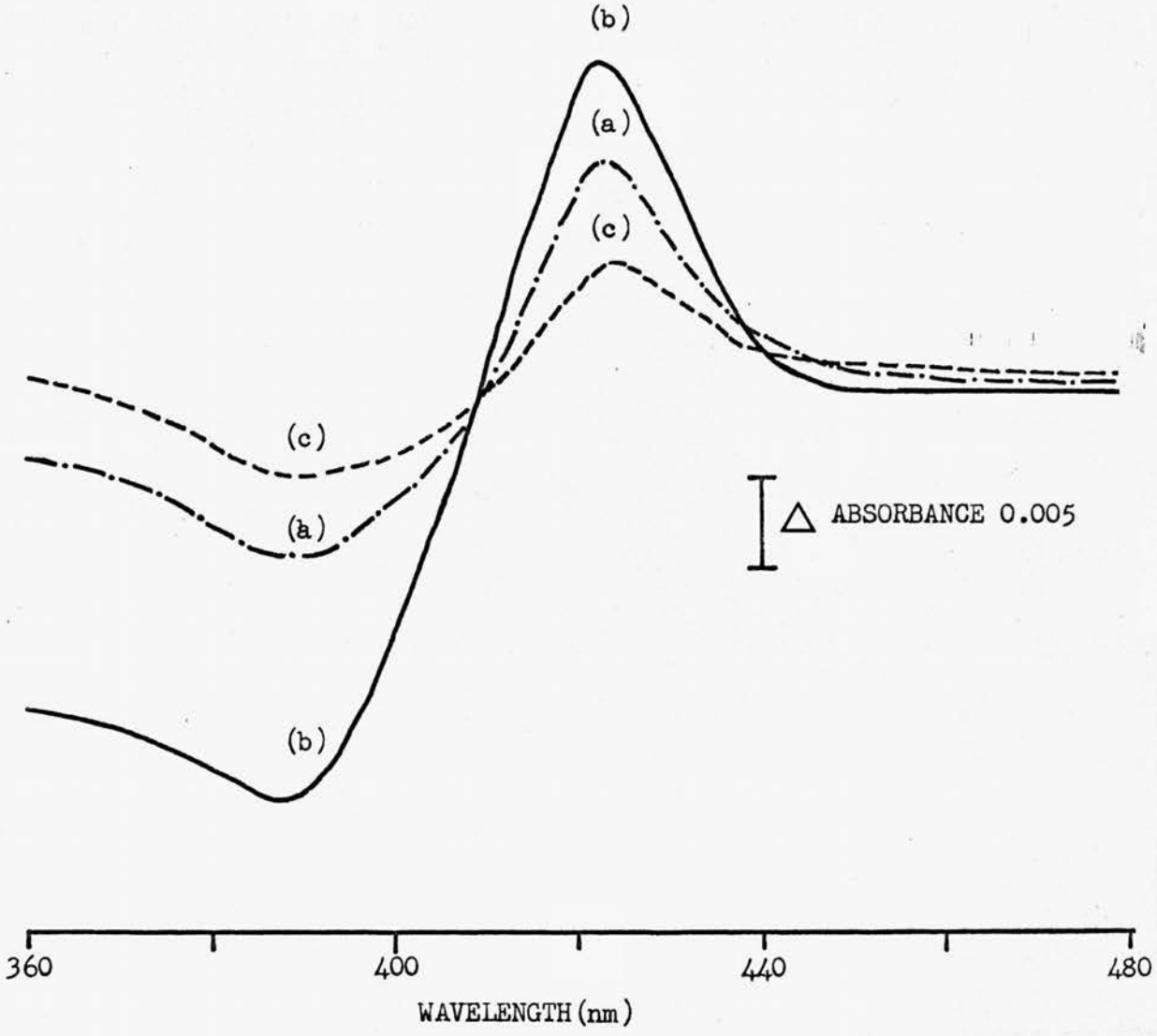


Figure 5.3. Pregnenolone induced difference spectra of rat luteal mitochondria. Spectra were obtained by the addition of saturating amounts (final concentration 12 μM) of pregnenolone in ethanol to mitochondria, suspended in medium A, in the sample cuvette of a dual beam spectrophotometer. Equal volumes of solvent were added to the mitochondria in the reference cuvette. The figure shows the pregnenolone induced spectra obtained at three different pH values. Protein concentrations were 1.2 mg/ml in each case ((a) pH 7.2, (b) 6 and (c) pH 8).

Pregnenolone binding spectra of rat luteal mitochondrial cytochrome P450

Pregnenolone, dissolved in a small volume ($< 10/\mu l$) of ethanol, was added to a suspension of luteal mitochondria, in the sample cuvette of a dual beam spectrophotometer. A similar volume of ethanol was added to the reference cuvette. The resultant difference spectrum is shown in figure 5.3(a). This has a peak at 424 nm and a trough at 385 to 390 nm. Steroid induced changes of this type are generally called inverted type I spectra. Pregnenolone induced inverted type I difference spectra displayed by adrenal cortex mitochondrial cholesterol side chain cleavage cytochrome P450 have been interpreted as representing a transition of the substrate bound form of the enzyme to that in which the substrate has been displaced (Jefcoate and Boyd (1971)). It has been demonstrated using EPR spectroscopy that this type of change is associated with a transition from a high spin state to a low spin state of the cytochrome P450 (Whysner et al (1970)). The binding constant for the pregnenolone induced spectral change, in luteal mitochondria, was about $1/\mu$ M (see figure 7.6). Changes in pH have been shown to affect the magnitude of pregnenolone induced difference spectra in bovine and rat adrenal mitochondria (Mitani and Horie (1969b); Jefcoate and Boyd (1971); Jefcoate et al (1973), (1974)). Figure 5.3(b)(a) and (c) shows the pregnenolone induced difference spectra, of rat luteal mitochondrial cytochrome P450, at pH values of 6, 7.2 and 8. These spectra were recorded in the presence of $6.4/\mu m$ cyanoketone in the sample cuvette. The cyanoketone caused a small inverted type 1 spectrum which was not observed when the pregnenolone was added before it. This small change was therefore included in the total inverted type I difference spectrum. The magnitude of the pregnenolone induced difference spectrum

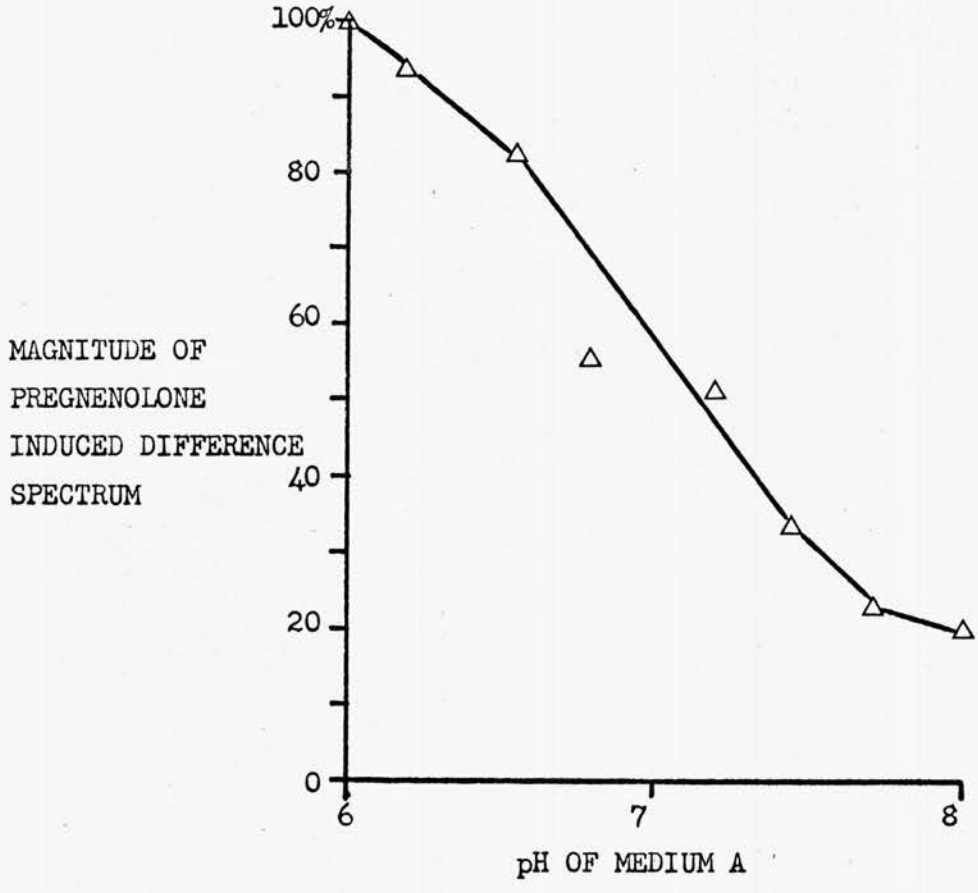
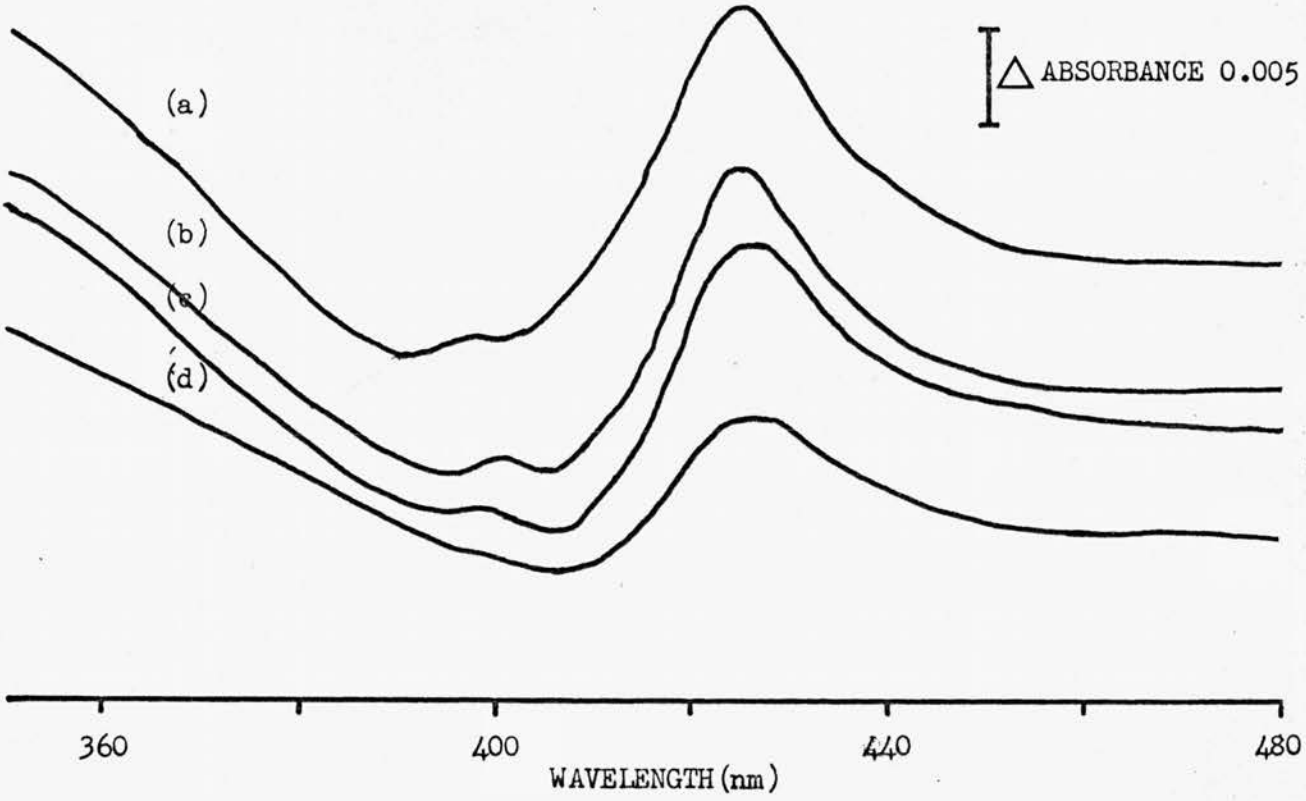


Figure 5.4. The effect of pH on the magnitude of the pregnenolone induced difference spectrum of rat luteal mitochondria. Pregnenolone induced difference spectra were measured in luteal mitochondria suspended in buffer in the pH range 6 to 8. The magnitudes of the spectra (Δ Absorbance 424 nm - 385 nm/mg protein) were then expressed as a percentage of the largest spectrum which was obtained at pH 6.

($\triangle$ Absorbance 424-385 nm) at pH 8 was about 20% to 30% of the value at pH 6 (figures 5.3 and 5.4). Total cytochrome P450 measurements did not vary over this pH range. This pH dependent change in the pregnenolone binding spectrum of luteal cytochrome P450 is similar to the pH dependent changes observed with adrenal cortex mitochondrial cholesterol side chain cleavage cytochrome P450. As the magnitude of the pregnenolone binding spectrum is thought to depend on the amount of cholesterol bound to cytochrome P450 (Jefcoate and Boyd (1971)), localised changes of pH in the cell could change the amount of high spin cholesterol side chain cleavage cytochrome P450. Changes in the amount of cholesterol bound to cytochrome P450 as a high spin complex would change the rate of cholesterol side chain cleavage (Simpson et al (1972)). The pH dependent spin state changes of cytochrome P450 may play an important role in the control of cholesterol side chain cleavage activity by trophic hormones. This property is unique to cholesterol side chain cleavage P450, it is not displayed by other forms of the cytochrome (Jefcoate et al (1973), (1974)). The pH activity curve of cholesterol side chain cleavage (Figure 3.6) does not however show a fall in activity from pH6 to 8 following the changes in the magnitude of the pregnenolone induced spectrum. This may be due to the pH changes altering the activity of the other components of the cholesterol side chain cleavage system. It is possible the changes in pH each side of 7.4 could have decreased the synthesis of NADPH or the transfer of reducing power to the cytochrome P450. However pH changes in the immediate vicinity of the cholesterol side chain cleavage cytochrome P450 could still be important in activation and inhibition of pregnenolone production.

The addition of deoxycorticosterone did not produce a difference



(a), (b) AND (c) RECORDED 15, 5 AND 2 MINUTES AFTER ADDITION OF MALATE TO THE SAMPLE CUVETTE . (d) RECORDED AFTER ADDITION OF PREGNENOLONE (12 µM) TO BOTH CUVETTES AFTER (a) RECORDED

Figure 5.5. The malate induced difference spectrum of rat luteal mitochondria. Mitochondria were suspended in medium A (1.1 mg protein/ml final concentration in the reference and sample cuvettes of a dual beam spectrophotometer). Rotenone 3 µg/ml was present in both cuvettes to prevent the reduction of respiratory chain cytochromes. 5 mM Malate was added to the sample cuvette and spectra were then recorded after 2, 5, 10 and 15 minutes.

spectrum with rat luteal mitochondria, unlike adrenal cortex mitochondria. This reflects the absence of 11 β-hydroxylase cytochrome P450 from luteal mitochondria. With adrenal cortex mitochondria deoxycorticosterone causes a transition from oxidised low spin cytochrome P450 (λ max 420 nm) to oxidised high spin substrate bound cytochrome P450 (λ max 390 nm) (Whysner et al (1970)). This results in a type I difference spectrum (λ max 390 nm λ min 420 nm).

McIntosh et al (1973) have reported that bovine luteal mitochondrial cytochrome P450 will produce an inverted type 1 difference spectrum with deoxycorticosterone the Ks for this interaction being 300 μM; 300 times greater than the Ks for the interaction of the steroid with adrenal cortex mitochondrial cytochrome P450.

No type I difference spectrum could be observed on the addition of cholesterol to luteal mitochondria. This presumably reflects the lack of effect of added cholesterol on the initial rate of cholesterol side chain cleavage in luteal mitochondria (figure 4.1). Difficulties were encountered when attempts were made to record cholesterol induced difference spectra. Large turbidity changes were observed on addition of the steroid to mitochondrial suspensions.

Malate induced difference spectra with rat luteal mitochondrial cytochrome P450

When malate was added to luteal mitochondria an inverted type I difference spectrum developed over fifteen minutes (Figure 5.5). Rotenone was added to prevent the reduction of respiratory chain cytochromes. Thus malate appears to have produced a high to low

spin transition in luteal mitochondrial cytochrome P450. This spectrum was produced when the malate was added in the presence or absence of cyanoketone. Hence it was probably not due to the accumulation of pregnenolone. Progesterone, which is formed from pregnenolone, does not give a difference spectrum with luteal mitochondrial cytochrome P450. The malate induced spectrum took ten to fifteen minutes to develop a similar time course to the depletion of cholesterol in luteal mitochondria (figures 3.1 and 3.4). It is probable therefore that the spectrum was due to the metabolism of cholesterol, from the cytochrome P450, causing a high to low spin transition. Immediately after malate addition a difference spectrum appeared (λ max 428 nm λ min 406 nm). On the subsequent development of the spectrum the peak and trough shifted to 424 and 390 nm respectively. When pregnenolone was added to the reference cuvette fifteen minutes after the addition of the malate most of the malate induced spectrum was cancelled out apart from the contribution, λ max 428 nm λ min 406 nm, observed immediately after the malate addition. Pregnenolone addition to the sample cuvette after it had been added to the reference cuvette did not then cause any change in the spectrum. Thus malate and pregnenolone difference spectra both measure the amount of cholesterol bound to cytochrome P450 in luteal mitochondria. Pregnenolone induced inverted type I difference spectra are probably a better method than malate induced spectra for measuring high spin luteal mitochondrial cytochrome P450, as they do not contain the contribution from the component λ max 428 λ min 406 nm. A similar component has demonstrated in the malate induced inverted type 1 difference spectrum of bovine cholesterol side chain

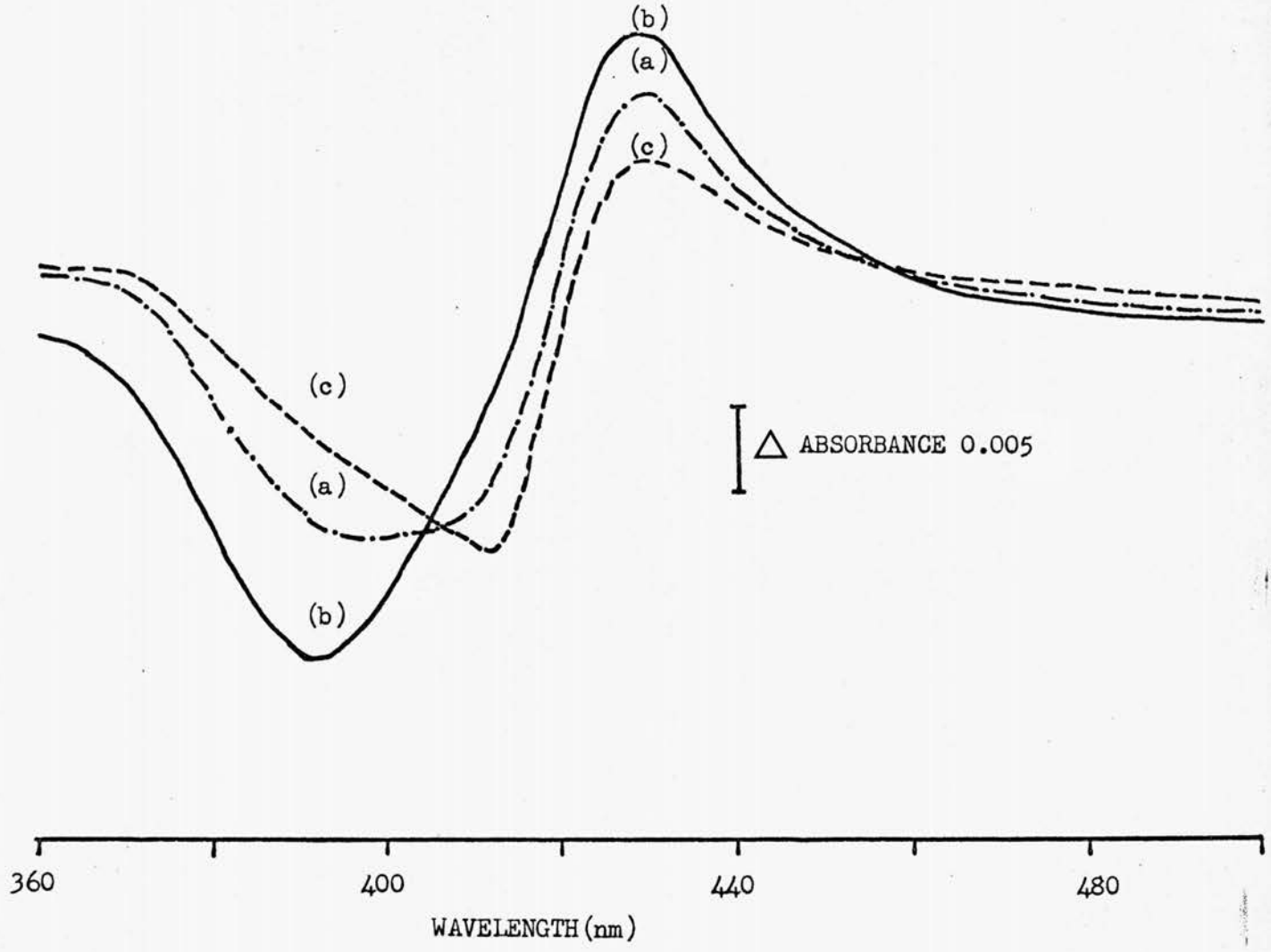


Figure 5.6. The amino-glutethimide induced difference spectrum of rat luteal mitochondria. Saturating acounts of amino-glutethimide (final concentration 33 μM) produced the spectra when added to suspensions of luteal mitochondria in the sample cuvette of a dual beam spectrophotometer. Shown are the difference spectra produced at pH6, 7.2 and 8. Protein concentrations were 1.5 mg/ml in each case ((a) pH 7.2, (b) pH 6 and (c) pH 8).

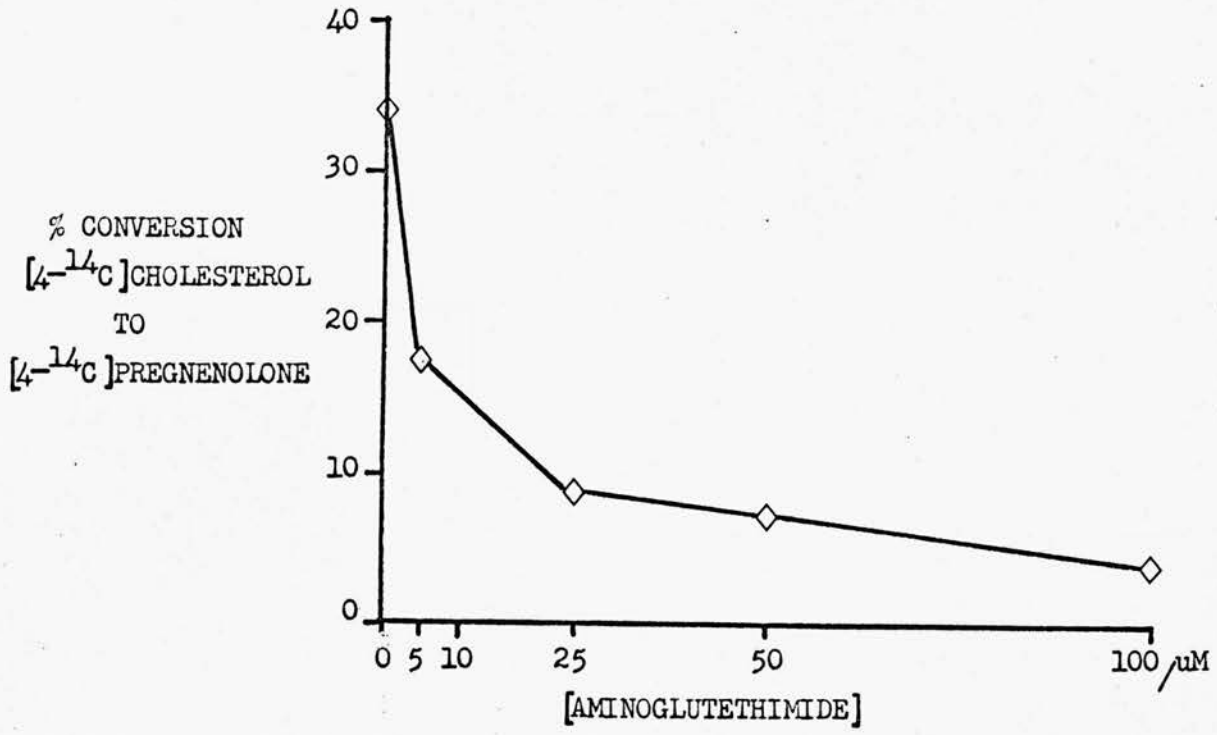


Figure 5.7. The inhibition of cholesterol side chain cleavage in luteal mitochondria by aminoglutethimide. Cholesterol side chain cleavage activity was measured as the percentage conversion of [4-^{14}C] cholesterol to [4-^{14}C] pregnenolone in ten minute incubations. The method used is described in chapter 2.

cleavage cytochrome P450 (Simpson et al (1971)). The difference spectrum of the component (λ max 428 λ min 406) induced by malate is similar to the reduced minus oxidised difference spectrum of microsomal cytochrome b_5 (Omura and Sato (1964a,b)). This could indicate microsomal contamination of the luteal mitochondria. The spectrum could also have been due to a mitochondrial cytochrome whose reduction is insensitive to rotenone.

Spectra induced by aminoglutethimide with luteal mitochondrial cytochrome P450

The binding of certain amines to cytochrome P450 of adrenal cortex mitochondria, to give a type II difference spectrum, is well documented (Jefcoate and Gaylor (1970); Jefcoate and Boyd (1971); Jefcoate et al (1973), (1974)). Figure 5.6(a) shows the aminoglutethimide induced difference spectrum with the cytochrome P450 of rat luteal mitochondria. This shows a peak at 430 nm and a very broad minimum at 390 nm to 410 nm. The binding constant for this interaction was 1 μM, taking Δ absorbance 430 nm - 410 nm as a measure of binding. This binding constant is similar to the concentration of aminoglutethimide (2 μM) which causes 50% inhibition of cholesterol side chain cleavage activity in incubations of luteal mitochondria (figure 5.7). The inhibitory effect of aminoglutethimide is probably caused by its reported ability to prevent the reduction of cytochrome P450 (McIntosh et al (1973)). This is a necessary step in the hydroxylations catalysed by the cytochrome (Estabrook et al (1973a)).

The broad trough in the aminoglutethimide induced type II difference spectrum (figure 5.6) suggested that this may be a complex

interaction of the drug with more than one form of cytochrome P450. In adrenal cortex mitochondria the difference spectra between the amine complex and high and low spin cytochrome P450 have λ max 425 nm, λ min 390 nm and λ max 433 nm, λ min 413 nm respectively (Jefcoate et al (1974)). Changes in pH alter the spin state equilibrium of luteal mitochondrial cytochrome P450 (figures 5.3, 5.4). Figure 5.6 shows aminoglutethimide induced difference spectra of rat luteal mitochondria at different pH values. At pH6 the cytochrome P450 was predominantly in the high spin form (figure 5.4) the aminoglutethimide induced difference spectrum showing λ max 427 nm λ min 394 nm (figure 5.6(b)). As the pH of the medium is raised the amount of low spin cytochrome P450 increases (figure 5.4). This caused a change in the aminoglutethimide difference spectrum of luteal mitochondrial cytochrome P450 (figure 5.6). The absorbance at 394 nm probably decreased due to the fall in the amount of high spin cytochrome P450. At the same time λ max of the difference spectrum shifted from 426 nm towards 434 nm. The aminoglutethimide induced difference spectrum of rat luteal mitochondria appears to be sensitive to the spin state of the mitochondrial cytochrome P450. High spin P450 causing λ min 394 nm and low spin P450 causing λ min 410 nm. The ratio of absorbances at 390 and 410 nm of aminoglutethimide induced difference spectra have been used to quantitate the amounts of high and low spin cytochrome P450 in adrenal cortex mitochondria (Jefcoate et al (1973), (1974)). Such a method would seem to be applicable to luteal mitochondrial cytochrome P450.

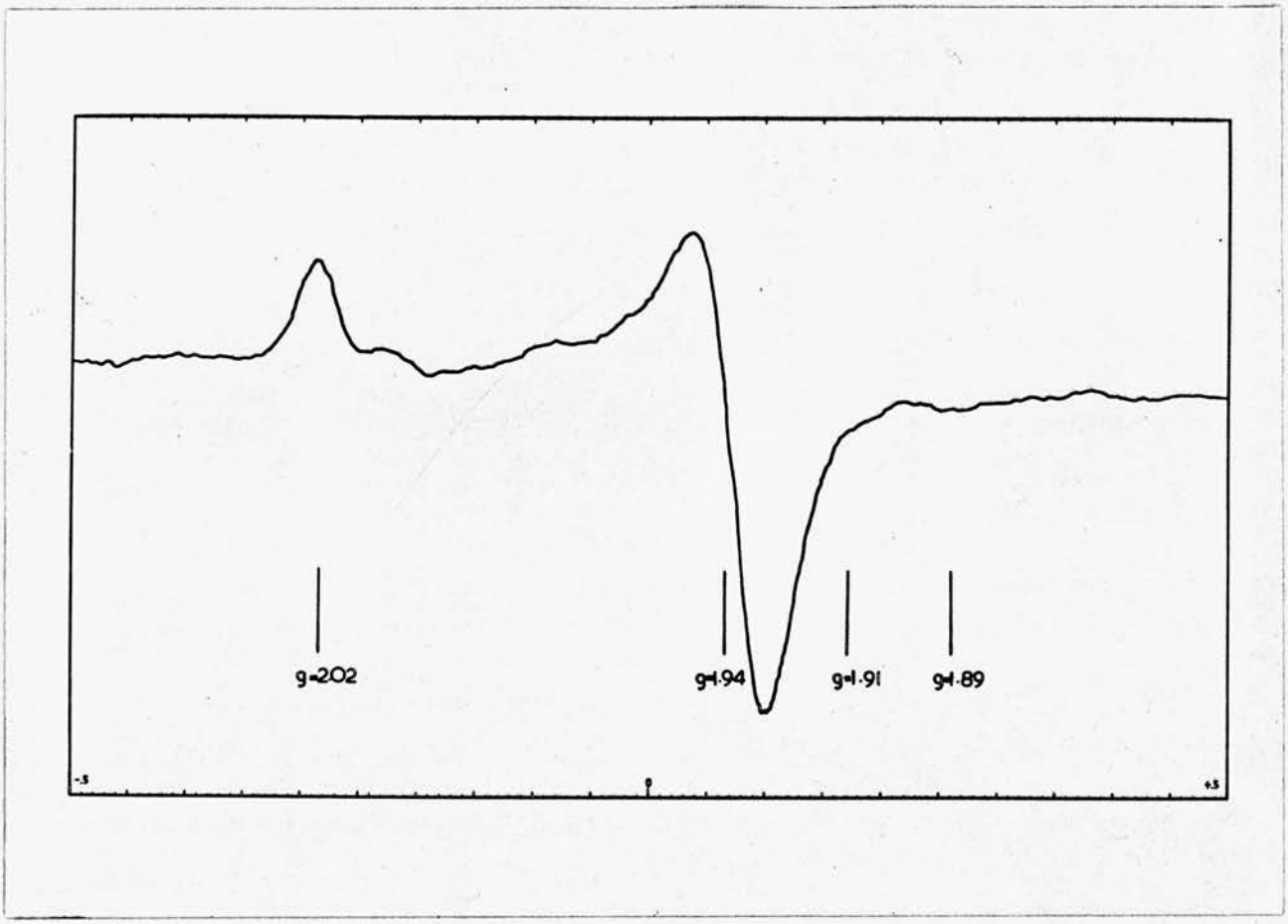


Figure 5.8. The E.P.R. spectrum of ovarian ferredoxin in rat luteal mitochondria. Mitochondria 3 mg protein/ml in medium A were reduced with dithionite.

Cytochrome P450 in microsomes from rat corpus luteum

Cytochrome P450 (0.07 n moles/mg protein) was detected in the microsomal fraction of rat corpus luteum. The function of this cytochrome P450 is uncertain. It did not display any binding spectrum with pregnenolone nor was cholesterol side chain cleavage activity detected in the microsomes (table 3.4). The microsomal cytochrome P450 may represent a contamination of the fraction with particles of disrupted mitochondria or newly synthesised P450 which will be incorporated into the mitochondria. The lack of cholesterol side chain cleavage activity in the microsomes may be because all the components of the system are not present.

Iron sulphur proteins in rat luteal mitochondria

The iron sulphur protein, of the cholesterol side chain cleavage system, of luteal mitochondria has been less well studied than the corresponding protein from adrenal cortex mitochondria (review Estabrook et al (1973b)). Adrenal ferredoxin (adrenodoxin) has an electron paramagnetic resonance (EPR) spectrum detectable at liquid nitrogen temperatures, when it has been reduced either enzymically or with dithionite. The first derivative of the spectrum has characteristic signals at $g = 1.94$ and $g = 2.03$ probably due to the association of a high spin ferrous or ferric ion with a sulphur ligand or ligands (see Estabrook et al (1973b)). The height of the signal at $g = 1.94$ is proportional to the amount of reduced adrenal ferredoxin present (Estabrook et al (1973b)). Concentrations of the iron sulphur protein down to 1 nmole/ml can be detected. Figure 5.8

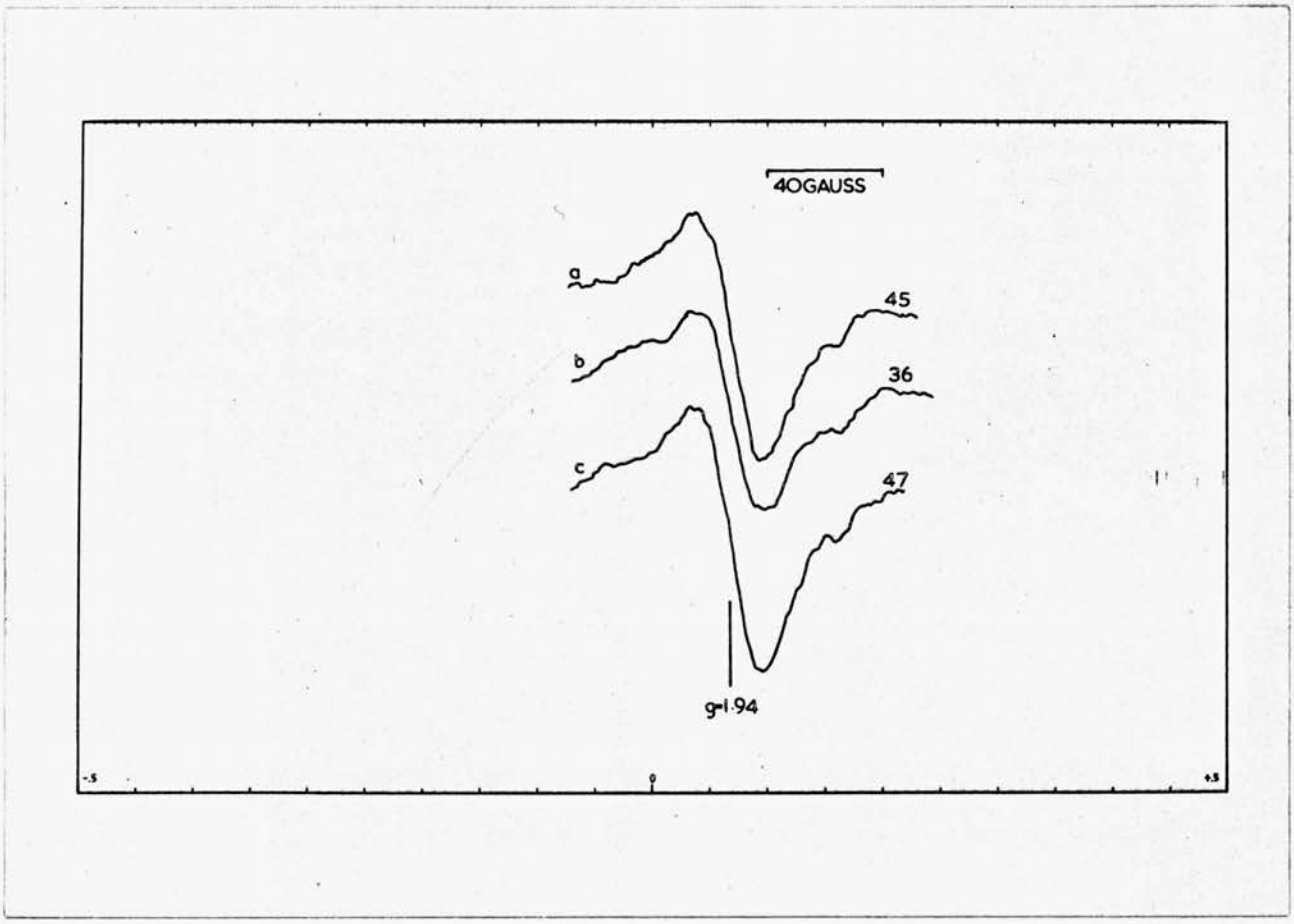


Figure 5.9. The E.P.R. spectra (g=1.94 signal only) of ovarian ferredoxin during incubations of luteal mitochondria in the presence of malate. Signal (a) was recorded after dithionite reduction of an aliquot of the mitochondrial suspension; (b) recorded 2 minutes after addition of malate to the incubation; (c) recorded 15 minutes after the addition of malate. Other conditions for recording the spectra are shown on the figure. Mitochondrial protein concentration in incubations was 2.0 mg/ml.

shows the demonstration of the presence of iron sulphur protein in rat luteal mitochondria. The signals at $g = 1.94$ and 2.02 are characteristic of an adrenal ferredoxin type iron sulphur protein. Signals at $g = 1.89$ to 1.91, characteristic of respiratory chain iron sulphur proteins, rather than adrenal ferredoxin like proteins were not present (Mason et al (1973)). High concentrations of respiratory chain iron sulphur proteins could have masked the signal from the ovarian ferredoxin. The spectral evidence shown in figure 5.8 confirms the observations of Kimura and Ohono (1968) and Baron (1973) who demonstrated an adrenal ferredoxin like protein, in luteal mitochondria, by partial purification and immunological techniques. The amount of ovarian ferredoxin could not be quantitated, as a purified preparation was not available to construct a calibration curve using the height of the $g = 1.94$ signal. However in mitochondrial preparations it was possible to compare the amounts of reduced iron sulphur protein, under varying incubation conditions, to the total amount present, expressed as the height of the $g = 1.94$ dithionite reduced signal.

Mitochondria were incubated in medium A and cholesterol side chain cleavage activity initiated with malate. A 0.5 ml sample was withdrawn from the incubation 90 seconds later then transferred into a quartz EPR tube and frozen in liquid nitrogen. After fifteen minutes incubation another sample was taken and frozen in a quartz EPR tube in liquid nitrogen. A third 0.5 ml sample from the incubation was reduced with dithionite before being frozen like the other samples. The derivative EPR signals of these samples are shown in figure 5.9. The size of the $g = 1.94$ signal was similar in the 15 minute incubated and dithionite reduced samples. The

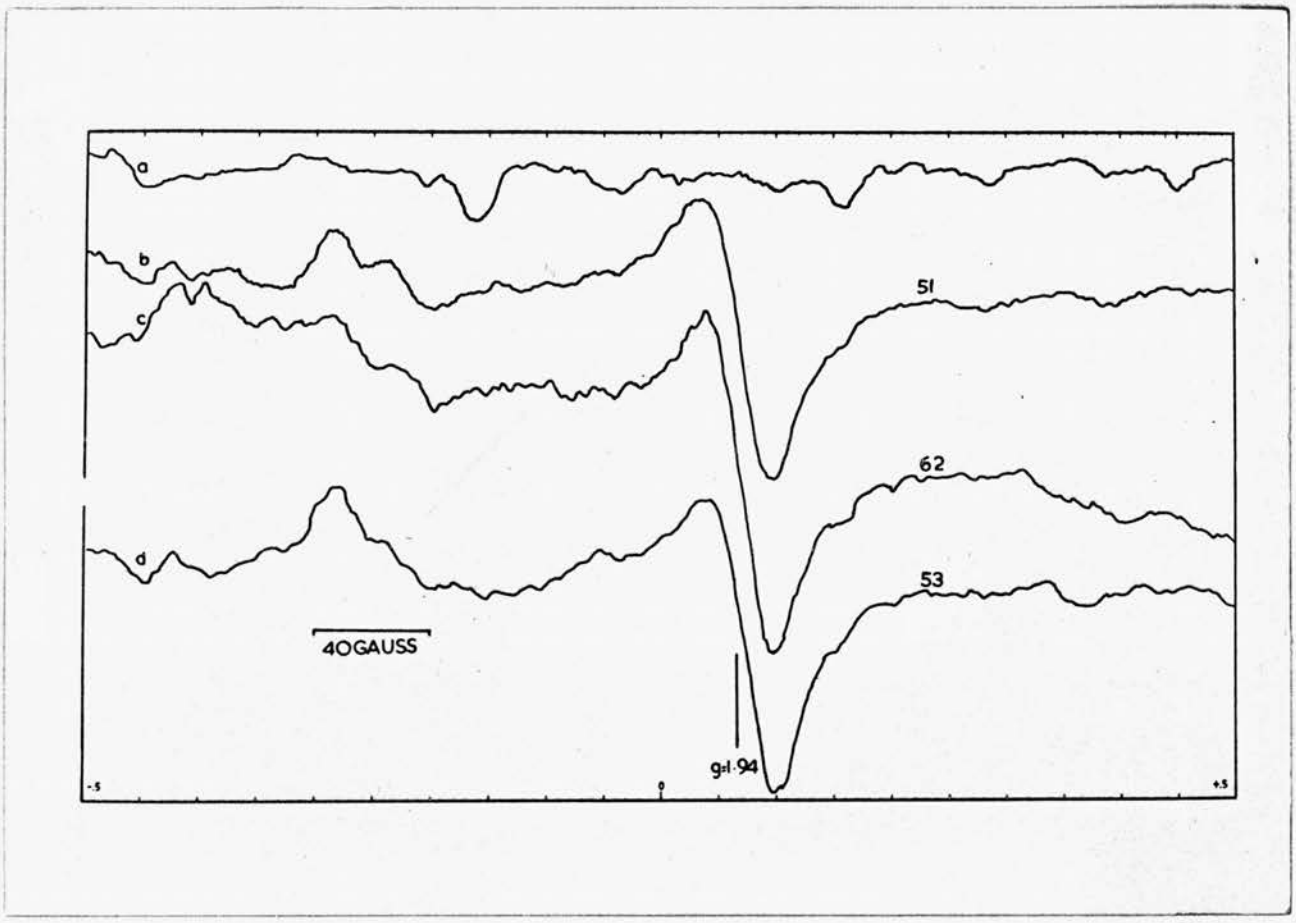


Figure 5.10. The E.P.R. spectra of ovarian ferredoxin during incubations of luteal mitochondria in the presence of malate and cholesterol. Signal (a) was recorded before the addition of malate; (b) and (c) were recorded as in Figure 5.9; (d) was recorded 20 minutes after the addition of malate in the presence of cholesterol (final concentration 260 μM). The numbers on the trace refer to the amplitude of the g = 1.94 signal. Mitochondrial protein concentration in incubations was 2.5 mg/ml.

$g = 1.94$ signal in the sample withdrawn after 90 seconds was 45 divisions which was 66% of the maximum signal height obtained in the dithionite reduced sample (68 divisions). Thus in an incubation where steroidogenesis was continuing at the maximum rate the ovarian ferredoxin was 66% reduced. When the rate of steroidogenesis began to decrease (15 minute sample) the ovarian ferredoxin became almost fully reduced. In incubations, even when steroidogenesis is continuing at the maximum rate, there is a large excess of reduced iron sulphur protein to supply electrons for the cholesterol side chain cleavage cytochrome P450. It has previously been shown that when the rate of cholesterol side chain cleavage begins to slow down in in vitro incubations, pregnenolone production can be accelerated by addition of cholesterol (figures 4.4 and 4.7). The effect of addition of cholesterol on the levels of reduced ovarian ferredoxin in incubation is shown in figure 5.10. Samples, from the incubation of luteal mitochondria, were taken for EPR analysis before the addition of malate and two and twenty minutes afterwards. 200 μM cholesterol was then added to the incubation and another sample taken for EPR analysis five minutes later. When no malate had been added there was no signal in the range 3350 ± 200 G . This showed all the endogenous substrate had been washed from the mitochondria during their preparation. The $g = 1.94$ signal of the 2 minute sample was 75% of the size of the twenty minute sample. After addition of the extra cholesterol the $g = 1.94$ signal again fell to 75% of that of the twenty minute sample. Thus when steroidogenesis is continuing at maximum rates there is a large excess of reduced ovarian ferredoxin. The fact that the $g = 1.94$ signal alters in size, depending on the rate of steroidogenesis in incubations, is further evidence that it

is caused by ovarian ferredoxin. Absolute proof of this will only come with purification of the components of the cholesterol side chain cleaving system and spectral analysis of the iron sulphur protein component.

As there appears to be an excess of reduced ovarian ferredoxin during steroidogenesis in these in vitro incubations, the rate of cholesterol side chain cleavage is unlikely to be regulated by the supply of electrons. A similar conclusion has been reached concerning the rate limiting step in the 11 β-hydroxylation of deoxycorticosterone in bovine adrenal cortex mitochondria (Estabrook et al (1973a)).

Summary

(a) The concentrations of cytochrome P450 and respiratory chain cytochromes have been determined in the rat luteal mitochondria.

(b) Malate and pregnenolone have been shown to induce inverted type 1 difference spectra with rat luteal mitochondria. These have been interpreted as a high to low spin transition in the luteal mitochondrial cytochrome P450. The high spin cholesterol complex of luteal mitochondrial cytochrome P450 appears to be sensitive to changes of pH.

(c) Aminoglutethimide binds to luteal mitochondrial cytochrome P450 to give a typical type II amine binding spectrum, λ max 425-433 nm λ min 390-410 nm, dependent on the spin state of the cytochrome P450.

(d) An adrenal ferredoxin like iron sulphur protein (ovarian ferredoxin) has been shown by EPR spectroscopy to be present in luteal mitochondria. The ovarian ferredoxin remains over 65% reduced during maximum rates of cholesterol side chain cleavage in vitro. This suggests that electron supply may not be important as a rate limiting step in luteal steroidogenesis in vitro.

CHAPTER 6

THE EFFECT OF IN VIVO TREATMENT OF RATS WITH LUTEINIZING HORMONE ON CHOLESTEROL SIDE CHAIN CLEAVAGE ACTIVITY IN LUTEAL MITOCHONDRIA

Introduction

It is now well established that LH treatment in vivo and in intact cells in vitro stimulates ovarian progesterone production (Chapter 1). This stimulation of steroidogenesis in luteal tissue probably involves an increase in the activity of the cholesterol side chain cleavage enzyme which converts cholesterol to pregnenolone (Hall and Koritz (1965); Channing and Villee (1966); Major et al (1966); Hall and Young (1968)). Most of these studies have not provided direct evidence that LH can stimulate conversion of cholesterol to pregnenolone as they were carried out with tissue slices. The only work so far reported to show the effect of in vivo HCG treatment on cholesterol metabolism in isolated luteal mitochondria has been performed in the rat. HCG treatment of superovulated rats one hour before killing was reported to cause a stimulation of the cholesterol side chain cleavage activity in isolated luteal mitochondria (Stevenson et al (1973); Robinson and Stevenson (1973)). In this work, a radioactive assay was used for cholesterol side chain cleavage, which was measured as the percentage conversion of $[4\text{-}^{14}C]$ cholesterol to products. This assumed that the total cholesterol pool sizes in mitochondria from HCG treated and untreated rat corpora lutea were the same. However if the pool sizes had changed, these results would have been misleading. It was therefore decided to compare the actual formation of pregnenolone

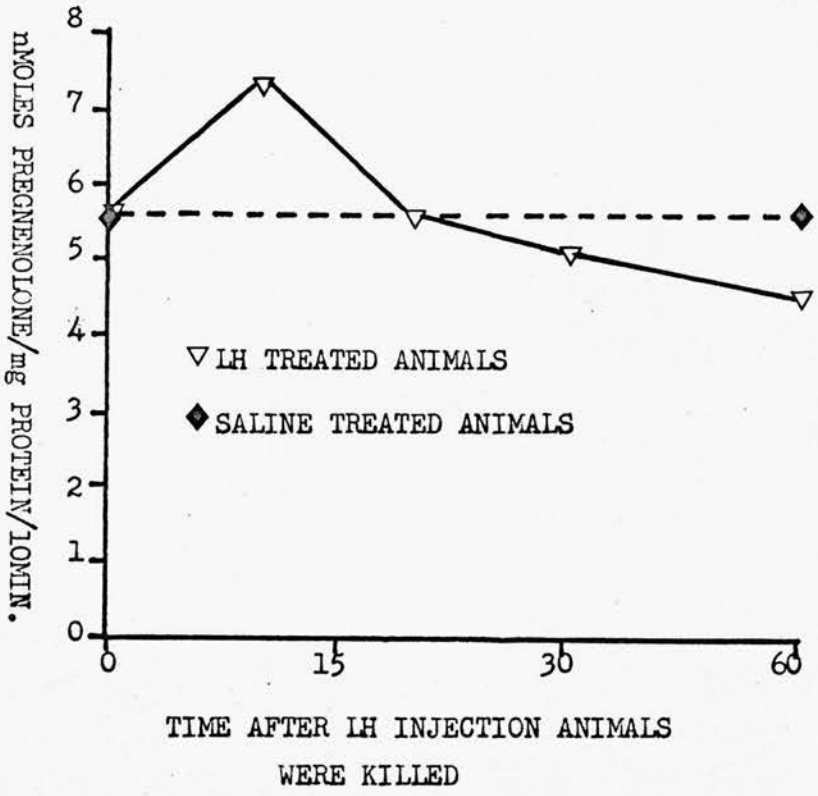


Figure 6.1. The effect of prior injections of rats with LH (20 µg / rat) on the pregnenolone production in isolated luteal mitochondria. Pregnenolone production, supported by malate in ten minute incubations, was measured using the methods described in chapter 2.

in luteal mitochondria from superovulated rats treated with either LH or HCG with those from animals which had been treated with 0.9% saline. LH from several different species and HCG have been shown to have very similar amino acid sequences. They have also been shown to have similar biological properties. They are immunologically very similar and cross react in radio-immuno assays. The two hormones also share the same receptors in luteal tissue (see review by Channing and Kammerman (1974)). Therefore HCG was sometimes used in place of LH during investigations of cholesterol side chain cleavage activity in rat corpora lutea.

The effect of LH or HCG injection on pregnenolone production in rat luteal mitochondria

Superovulated rats were injected subcutaneously with 20 µg of LH (N.I.H.S-18) dissolved in 0.2 mls of 0.9% saline. This dose of LH is known to stimulate steroidogenesis in the rat corpus luteum (Flint and Denton (1969)). The rats (groups of 8) were killed 10, 20, 30 and 45 minutes after the injections of LH. Control animals were killed at time zero and 45 minutes after injection with 0.9% saline. Mitochondria were prepared from the ovaries of each group of rats and incubated for 10 minutes in the presence of malate. The results of this experiment are shown on figure 6.1. The LH treatment initially seemed to increase the luteal mitochondrial pregnenolone production from 5.6 to 7.4 n moles/mg protein/10 minutes. However, twenty minutes after the injection of LH, the production of pregnenolone by the luteal mitochondria had fallen to the initial value of 5.6 n moles/mg protein/10 minutes. This fall continued

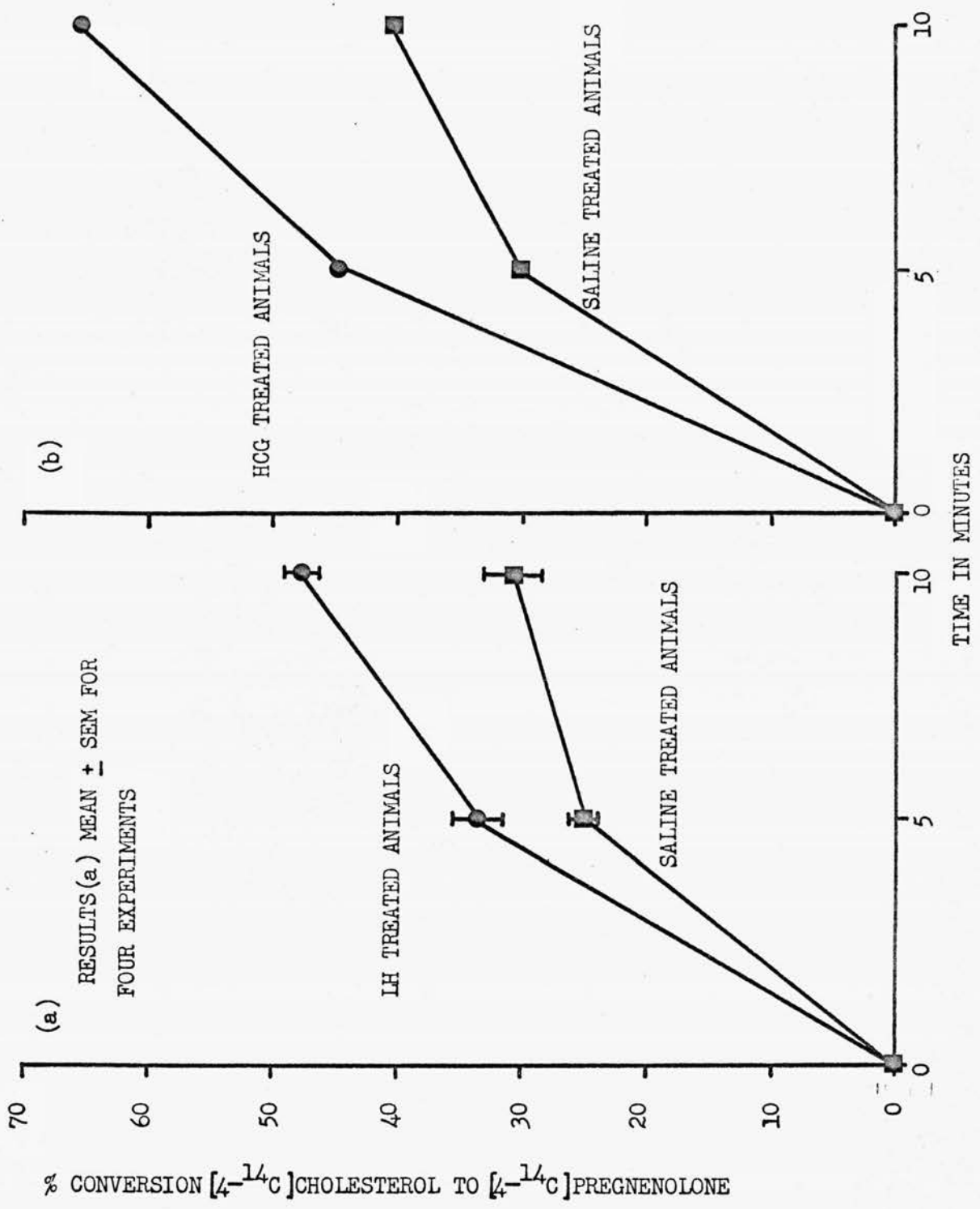


Figure 6.2. The effect of injections with (a) LH (20 µg/rat) and (b) HCG (25 iu) one hour before killing on the conversion of [4-^{14}C] cholesterol to [4-^{14}C] pregnenolone in rat luteal mitochondria. [4-^{14}C] cholesterol was preincubated with the mitochondria for ten minutes before initiation of side chain cleavage with malate.

after 30 and 45 minutes LH treatment. Luteal mitochondria from rats which had received a saline injection 45 minutes before killing had the same cholesterol side chain cleavage activity as those killed at time zero. LH treatment *in vivo*, therefore, appeared to cause a transitory stimulation of pregnenolone production, from endogenous cholesterol, measured in isolated luteal mitochondria. This stimulation lasted for twenty minutes and then pregnenolone production declined below control values. This is an apparent contradiction of the results of Robinson and Stevenson (1973) who found a stimulation of luteal mitochondrial cholesterol side chain cleavage activity one hour after treatment of superovulated rats with LH or HCG. They however used a radioactive assay for measuring cholesterol metabolism. It was therefore of interest to establish how a treatment with LH or HCG one hour before killing affected the percentage conversion of [4 -^{14}C] cholesterol to [4 -^{14}C] pregnenolone in the luteal mitochondria of the rats used in this study. Figure 6.2 shows the results of experiments in which rats received a subcutaneous injection of (a) 20 µg of LH or (b) 25 (iu) of HCG, dissolved in 0.2 ml 0.9% saline, one hour before being killed. Control animals received injections of 0.9% saline. The treatment with LH or HCG stimulated the percentage conversion of [4 -^{14}C] cholesterol to [4 -^{14}C] pregnenolone supported by malate in the luteal mitochondria. This also occurs when isocitrate or NADPH are used as electron donors for cholesterol side chain cleavage. Malate in the presence of $NADP^+$ also supports increased production of [4 -^{14}C] pregnenolone in luteal mitochondria of animals treated with LH compared to those from animals given injections of 0.9% saline

Table 6.1

The effect of injection of rats with HCG, one hour before killing, on cholesterol side chain cleavage supported by different electron donors in rat luteal mitochondria.

electron donor	% conversion	
	HCG mitochondria	Saline mitochondria
5mM malate	34	25.6
5mM malate + 1mM $NADP^+$	37.5	27.7
5mM malate + 1mM NADPH	39.2	28.6
1mM NADPH	13.7	10.3
10mM D.L. isocitrate	41.2	31.6
10mM D.L. isocitrate + 1mM $NADP^+$	41.5	29.5

Rats were injected with HCG (25iu) or 0.9% saline 1 hour before being killed. Luteal mitochondria were prepared from the ovaries of these animals. The mitochondria were then preincubated with $[4\text{-}^{14}C]$ cholesterol before the addition of an electron donor. The percentage conversion of $[4\text{-}^{14}C]$ cholesterol to $[4\text{-}^{14}C]$ pregnenolone was then determined in ten minute incubations as described in chapter 2. Mitochondrial protein concentrations were 0.8 and 1.0 mg/ml in the incubations of HCG and saline mitochondria respectively.

Table 6.2

The effect of HCG injection of rats, one hour before killing, on cholesterol content, cytochrome P450 content and pregnenolone production of luteal mitochondria

	HCG treated animals	Saline treated animals
mitochondrial cytochrome P450 n moles/mg protein	0.47 ± 0.06(5)	0.49 ± 0.05(5)
mitochondrial pregnenolone production n moles/mg protein/10 minutes	2.27 ± 0.15(6)	4.22 ± 0.16(6)
mitochondrial cholesterol n moles/mg protein	11.67 ± 0.63(8)	20.82 ± 0.55(8)

Luteal mitochondria were isolated from rats which had previously been treated with HCG or 0.9% saline one hour before being killed. Experimental procedure was as described in Table 7.4 (All results are expressed as mean ± SEM.; number of determinations in parenthesis).

(table 6.1). This confirms the findings of Robinson and Stevenson (1973) that LH or HCG treatment of superovulated rats one hour before killing can increase the luteal mitochondrial conversion of [4 -^{14}C] cholesterol to products. This observation applied when several different electron donors were used to support cholesterol side chain cleavage. However, as shown in figure 6.1, the luteal mitochondrial pregnenolone production from endogenous cholesterol falls below control values 45 minutes after treatment of the rat with LH. These contradictory results can be explained by comparison of the total amounts of cholesterol present in the luteal mitochondria of HCG treated and saline treated rats (table 6.2). The isocitrate supported production of pregnenolone from endogenous cholesterol is also shown for these mitochondria. Table 6.2 shows results from the luteal mitochondria from HCG treated animals, the production of pregnenolone was 2.27 n moles/mg protein/5 mins. This is a reduction of 45% compared to the 4.22 n moles pregnenolone mg protein/5 mins produced by the mitochondria of saline treated animals. The cholesterol content of the mitochondria was 20.82 n moles/mg protein in the saline injected animals and 11.67 n moles/mg protein in the HCG injected animals. Thus HCG treatment which is known to stimulate steroidogenesis in the rat corpus luteum (Rao and Saxena (1973)) has reduced the cholesterol content and pregnenolone production in isolated luteal mitochondria. The fall in the cholesterol pool size explains why the percentage conversion of [4 -^{14}C] cholesterol to [4 -^{14}C] pregnenolone is stimulated in these mitochondria by LH treatment whereas actual production of pregnenolone is apparently inhibited. An increased percentage conversion of [4 -^{14}C] cholesterol to products of a reduced total cholesterol pool size represents

less actual formation of product than a smaller percentage conversion of a larger pool size.

In studies on the sheep corpus luteum, Collett et al (1973) have reported that infusion of ovine LH causes a stimulation of progesterone production in the gland. This stimulation lasts for about half an hour after which time progesterone production returns to control values. The progesterone production can not then be stimulated again despite repeated infusions of LH. Collet et al (1973) suggest that this fall off of progesterone secretion after the initial stimulation by LH is due to the supplies of cholesterol for steroidogenesis becoming depleted. The reduced levels of cholesterol in luteal mitochondria from LH or HCG treated rats could have been due to a similar phenomenon to that reported by Collet et al (1973) in the sheep corpus luteum. The time course of changes in blood concentrations of progesterone in response to LH or HCG treatment have not been measured in these superovulated rats. Thus it is not known whether the rat corpora lutea became unable to respond to LH stimulation, like the corpus luteum of the sheep. In chapter 4 the ability of the luteal mitochondria to rapidly deplete their endogenous cholesterol has been discussed. If LH treatment had activated the cholesterol side chain cleavage enzymes, this would have caused increased metabolism of luteal mitochondrial cholesterol and possibly its depletion. It was therefore decided to investigate the addition of cholesterol to incubations of luteal mitochondria, from LH or HCG treated and saline treated animals.

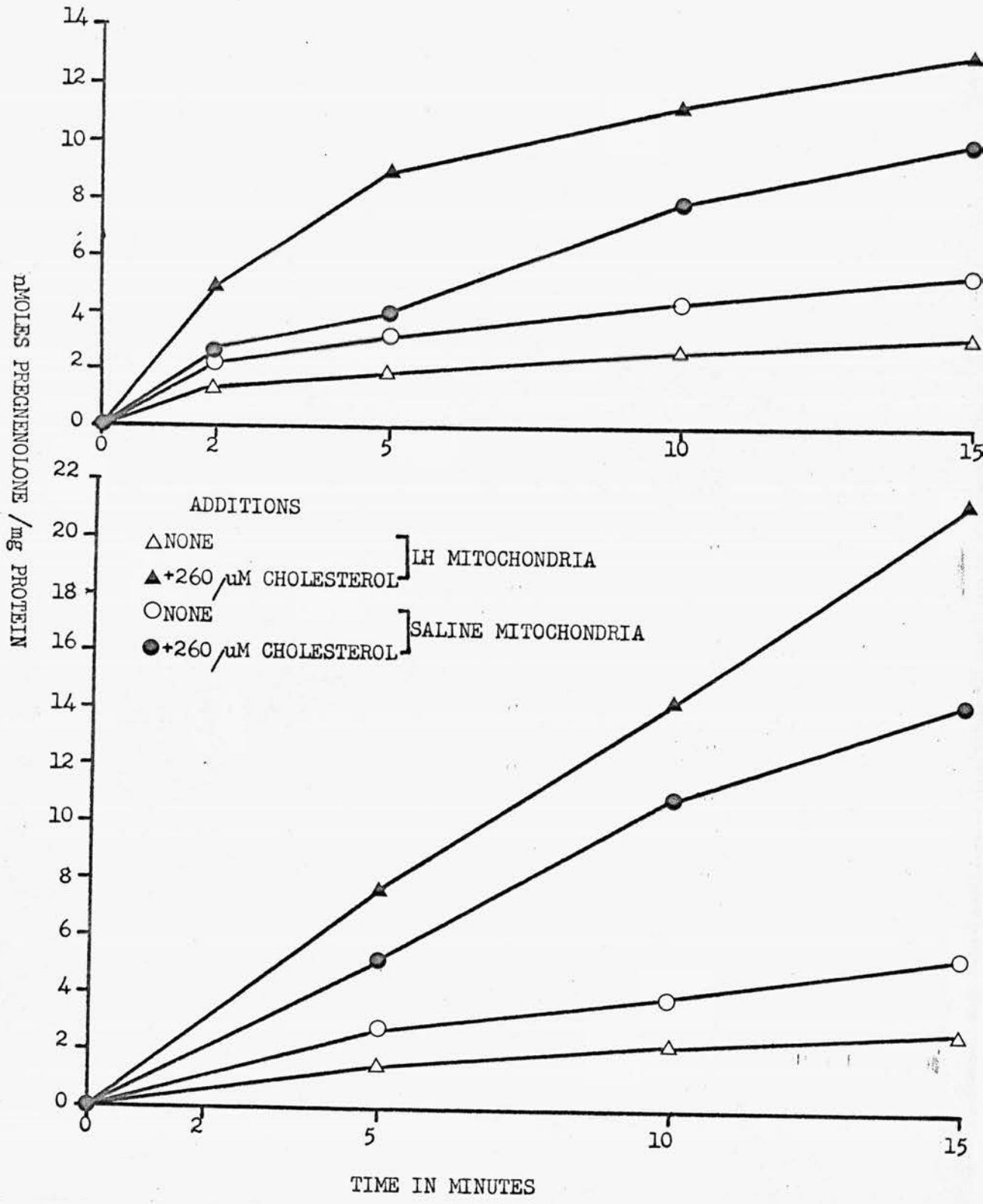


Figure 6.3. The time course of metabolism of endogenous and added cholesterol by luteal mitochondria from rats injected with LH (20 /ug/animal) one hour before killing. Shown in (a) and (b) are two experiments in which the malate supported mitochondrial pregnenolone production was measured using the methods described in chapter 2.

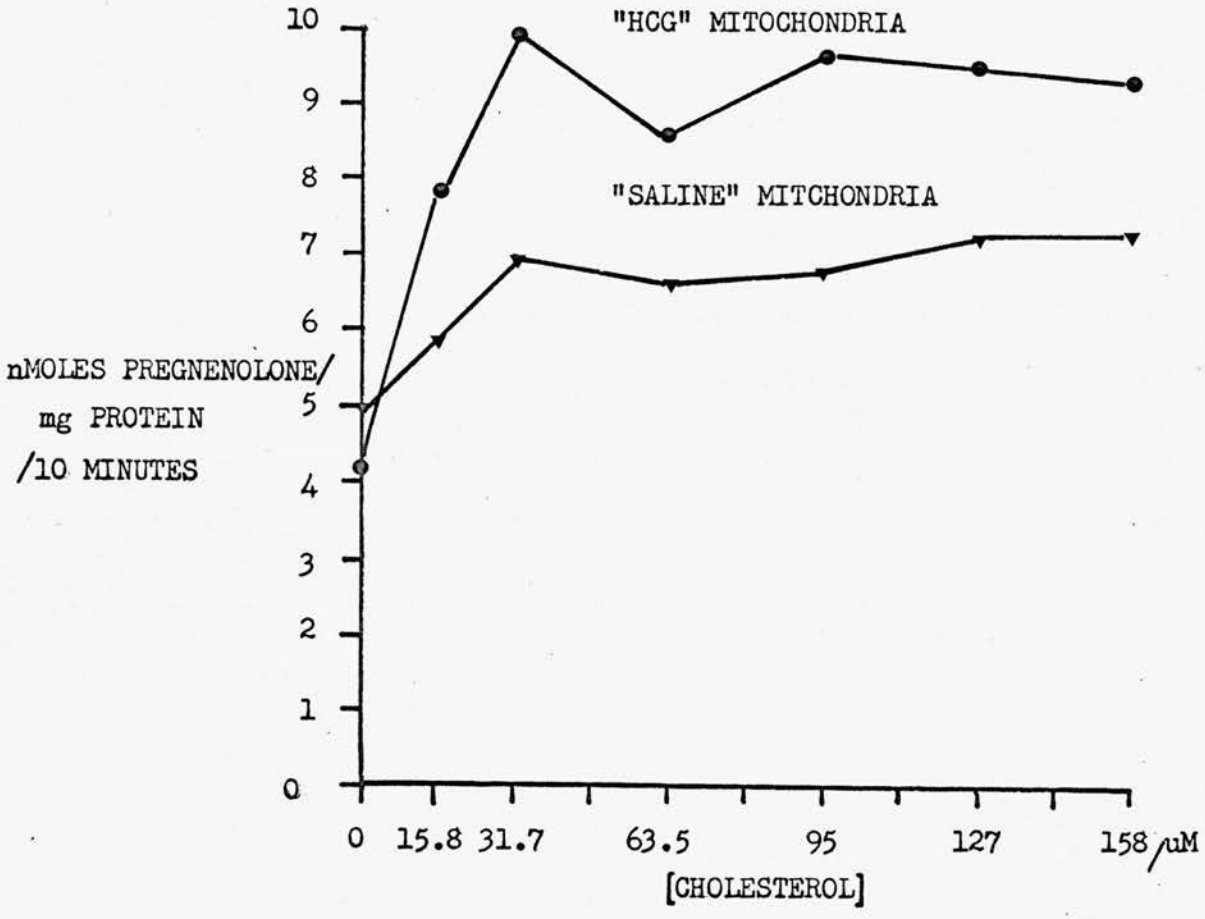


Figure 6.4. The effect on pregnenolone production of adding different amounts of cholesterol to incubations of luteal mitochondria from rats injected with HCG (25 iu) or 0.9% saline one hour before being killed. Malate supported pregnenolone production was measured in ten minute incubations as described in chapter 2.

The effect of added cholesterol on the cholesterol side chain cleavage activity of luteal mitochondria from rats which had been injected with LH or 0.9% saline one hour before killing

Figure 6.3 shows the time course of pregnenolone production, in the presence and absence of cholesterol, in luteal mitochondria from superovulated rats treated with LH or 0.9% saline one hour before being killed. Two experiments performed on different groups of rats are shown in the figure. In both cases it can be seen that LH treatment has decreased the pregnenolone formed from endogenous mitochondrial cholesterol. In the luteal mitochondria from saline injected animals 3.2 and 2.8 n moles of pregnenolone/mg protein were formed in five minutes, whereas 1.8 and 1.4 n moles/mg protein/5 minutes were formed in the luteal mitochondria of the LH injected animals. When 260 µM cholesterol, in acetone, was added to the incubations the luteal mitochondria from the LH injected animals now produced more pregnenolone than those from saline injected animals; 9.0 and 7.5 n moles pregnenolone/mg protein/5 minutes against 5 and 3.8 n moles pregnenolone/mg protein/5 minutes. Thus the luteal mitochondria from LH treated rats appear to be able to metabolise cholesterol at a faster rate than the mitochondria from saline treated animals. Addition of 260 µM cholesterol caused 22% and 78% more pregnenolone to be produced in the mitochondria from saline treated animals in five minutes. The stimulation was 400% and 440% in the mitochondria from LH treated animals. Figure 6.4 shows the effect of adding different amounts of cholesterol to luteal mitochondria from HCG and saline treated animals. Production of pregnenolone was again greater in luteal mitochondria from the saline treated rats compared to those

which were from HCG treated animals. The addition of cholesterol, above concentrations of 15.8 μM, caused a greater increase in the pregnenolone production of the luteal mitochondria from the HCG treated animals compared to those from the saline treated animals. With extra cholesterol, the mitochondria from the HCG treated animals produced more pregnenolone than those from saline treated animals. This increased pregnenolone production (43%) in mitochondria from HCG treated animals was maximal after addition of 31.7 μM cholesterol and did not change as the cholesterol concentration increased up to 158 μM. No attempt was made to work out Kms for the cholesterol side chain cleavage reaction as this would involve several problems. The mitochondria from HCG and saline treated animals had differing amounts of endogenous substrate cholesterol. Cholesterol is very insoluble in aqueous solutions (0.2 mg/100 mls; 5.2 μM) therefore when it is added to incubations at concentrations greater than this it will be selectively taken up by the lipophilic membranes of the mitochondria. Thus the actual concentration of the cholesterol at the active site of the cholesterol side chain cleavage cytochrome P450 in the inner mitochondrial membrane would not be known. The method of addition of the cholesterol to the incubations would also make a difference to any Km values obtained. Cholesterol could be added in lipid micelles (Bell and Harding (1974)), dissolved in detergent or in a solution of organic solvent (acetone or ethanol). The lipid micelles and the detergent would compete with the mitochondrial membrane for the binding of the cholesterol. The phospholipids used to make lipid micelles have been shown to have an effect on cytochrome P450 dependent reactions (Williamson and O'Donnell (1969); Strobel et al (1970); Mason and Boyd (1971)). Thus different Km values may be obtained depending on the method of addition of cholesterol to incubations.

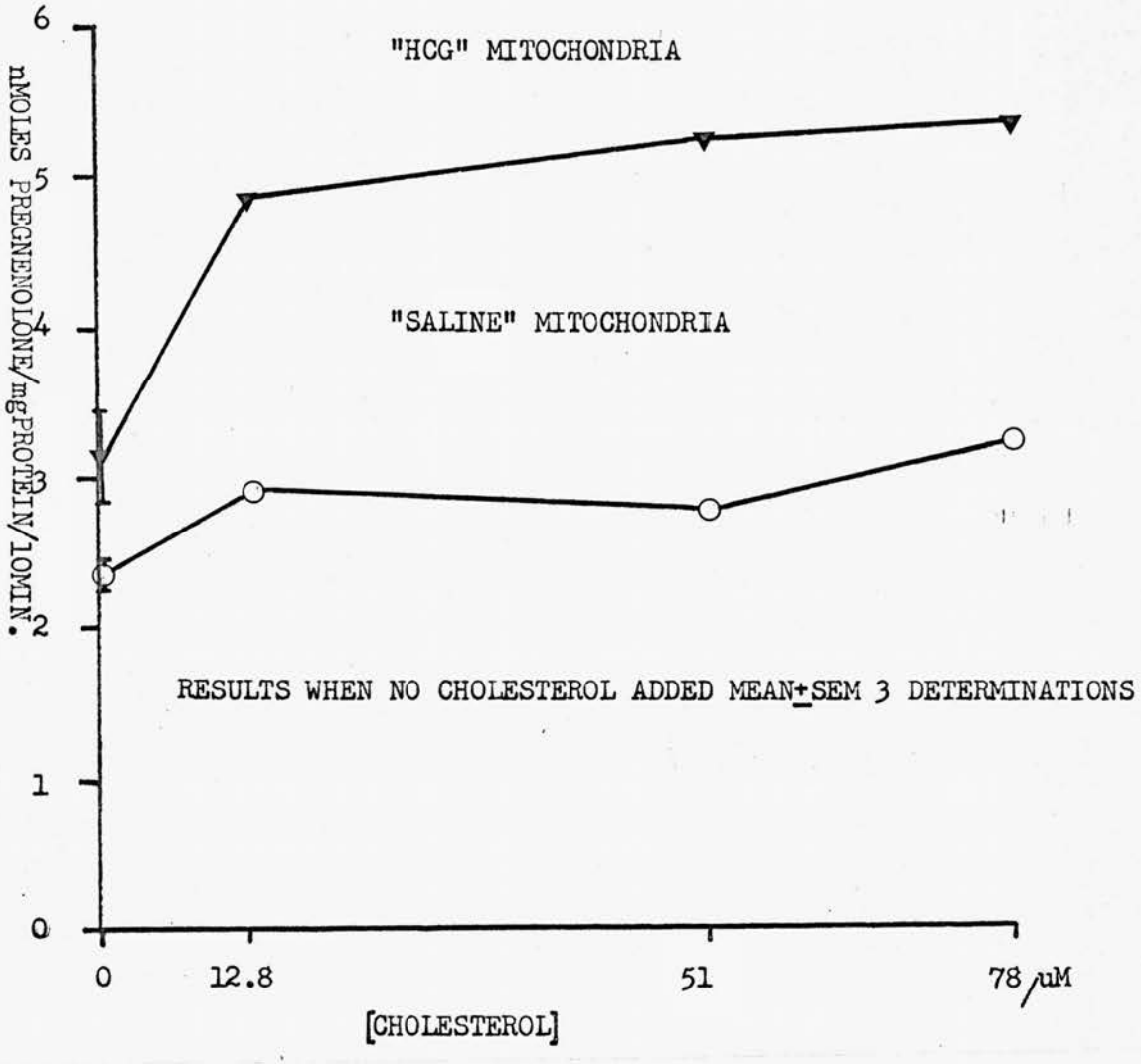


Figure 6.5. The effect on pregnenolone production of adding different amounts of cholesterol to incubations of luteal mitochondria from rats injected with HCG (25 iu) or 0.9% saline fifteen minutes before being killed. The incubations were carried out as described for figure 6.4.

Table 6.3

The effect of HCG injection of rats, fifteen minutes before killing, on cholesterol content, cytochrome P450 content and pregnenolone production of luteal mitochondria

	HCG treated animals	Saline treated animals
mitochondrial cytochrome P450 n moles/mg protein	0.42	0.40
mitochondrial pregnenolone production n moles/mg protein/10 minutes	3.81 ± 0.49(6)	2.56 ± 0.25(6)
mitochondrial cholesterol n moles/mg protein	14.3 ± 0.8 (4)	14.7 ± 0.5 (4)

Luteal mitochondria were isolated from rats which had previously been treated with HCG or 0.9% saline fifteen minutes before being killed. Experimental procedure was as described in Table 7.4 (All results are expressed as mean ± SEM; number of determinations in parenthesis).

The effect of added cholesterol on the cholesterol side chain cleavage activity of luteal mitochondria from rats which have been injected subcutaneously with HCG or 0.9% saline fifteen minutes before killing

LH caused a small rise in luteal mitochondrial pregnenolone production from endogenous cholesterol within the first twenty minutes of being injected subcutaneously into rats (figure 6.1). It was therefore decided to investigate whether a short term treatment with LH or HCG would stimulate the metabolism of exogenous cholesterol by the luteal mitochondria. Figure 6.5 shows the effect on pregnenolone production of adding differing amounts of cholesterol to luteal mitochondria from rats treated for fifteen minutes with HCG or 0.9% saline before being killed. The fifteen minutes of treatment with HCG had caused a small stimulation of pregnenolone production from endogenous cholesterol in the luteal mitochondria in comparison to the production in saline injected controls. This stimulation was increased to 50% by the addition of cholesterol. The cholesterol had little effect on pregnenolone production in the mitochondria from saline injected rats whereas a 40% stimulation of production was caused in the mitochondria from HCG injected rats. This is similar to the phenomenon observed when cholesterol is added to luteal mitochondria of rats which had been treated for one hour with HCG or 0.9% saline before being killed (figure 6.4). The cholesterol content of the isolated luteal mitochondria from rats treated for 15 mins with HCG or saline is shown in table 6.3. There did not appear to be any difference in content between the two groups.

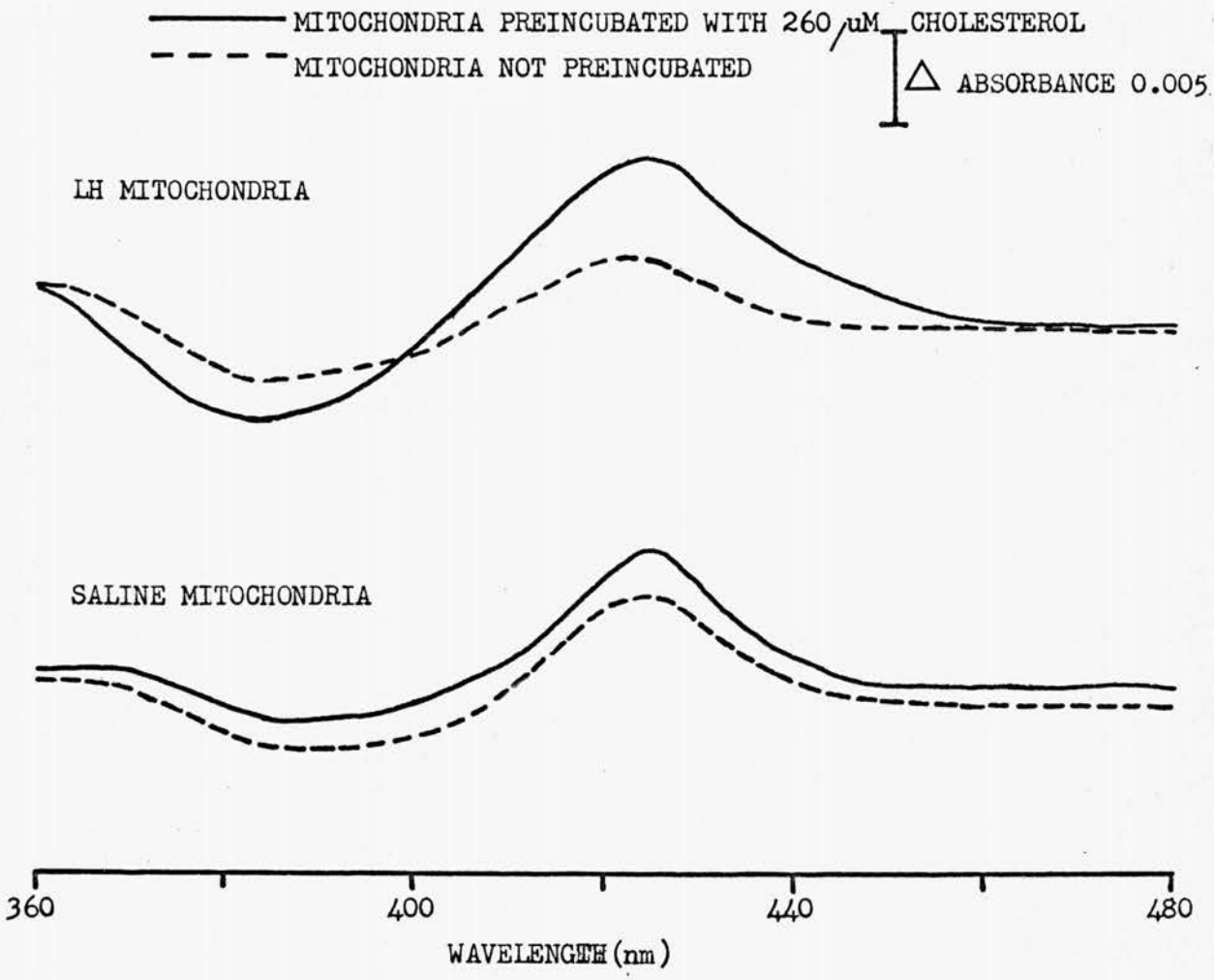


Figure 6.6. Pregnenolone induced difference spectra of luteal mitochondria from rats injected with LH or 0.9% saline one hour before being killed. Also shown are the difference spectra induced by pregnenolone with the mitochondria after they had been preincubated with 260 µM cholesterol. Protein concentrations 0.7 mg/ml LH mitochondria, 0.6 mg/ml saline mitochondria.

Effects of treatment with HCG or LH on the spectral properties of cytochrome P450 in rat luteal mitochondria

Treatment with HCG over a number of days is known to stimulate the formation of cytochrome P450 in luteal and testicular mitochondria in the rat (Cooper and Thomas (1970); Mason et al (1973)). Subcutaneous injections of HCG, fifteen minutes or one hour before killing did not however increase the levels of cytochrome P450 in luteal mitochondria above values obtained from mitochondria of saline injected controls (table 6.2). The effects of HCG on steroidogenesis were therefore not mediated by altering the total amounts of cholesterol side chain cleavage cytochrome P450 in the mitochondria. The initial rates of cholesterol side chain cleavage in incubations of adrenal cortex mitochondria have been correlated to the levels of cholesterol bound as a high spin complex of cytochrome P450 (Simpson et al (1972)). This complex can be measured by the magnitude of the inverted type I difference spectrum induced by addition of pregnenolone to the mitochondria (Simpson et al (1972)).

Figure 6.6 shows the pregnenolone induced inverted type 1 difference spectra of luteal mitochondria from rats which had been treated with HCG or 0.9% saline one hour before killing. The magnitude of this spectrum can be seen to be reduced from 13(Δ Absorbance 424 - 385 nm x 10^3/mg protein) in the mitochondria from saline treated animals to 7(ΔAbsorbance 424 - 385 nm x 10^3/mg protein) in the mitochondria from HCG treated animals. This reflects the HCG induced fall in mitochondrial pregnenolone production (table 6.1). The pregnenolone induced inverted type 1 spectra were then determined in the mitochondria after they had been preincubated for

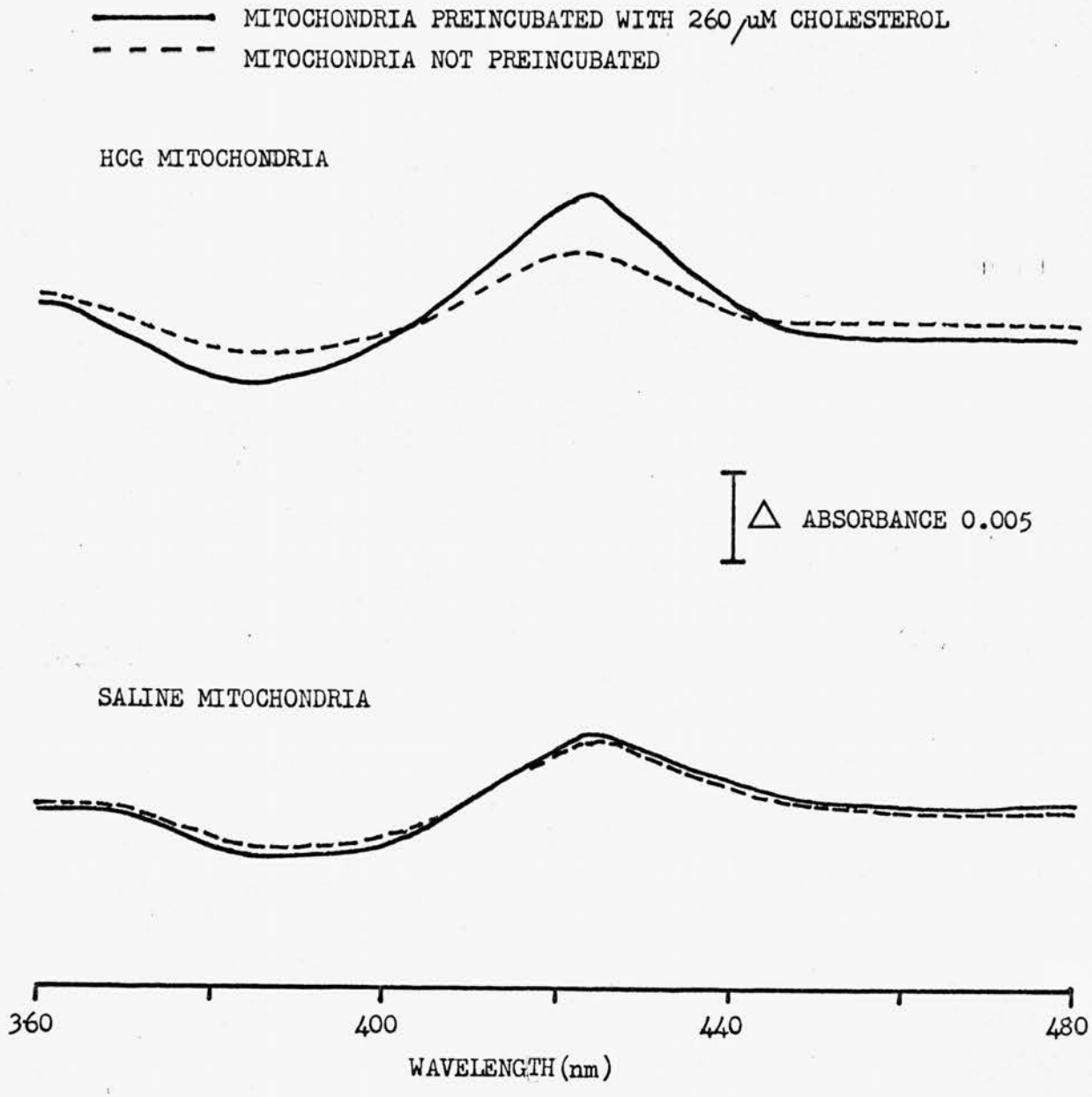


Figure 6.7. Pregnenolone induced difference spectra of luteal mitochondria from rats injected with HCG or 0.9% saline fifteen minutes before being killed. Also shown are the difference spectra induced by pregnenolone with the mitochondria after they had been preincubated with 260 µM cholesterol. Protein concentrations 0.6 mg/ml HCG mitochondria, 0.7 mg/ml saline mitochondria.

10 minutes with 158 /uM cholesterol. The magnitudes of the spectra were now 15 and 20 ($\triangle$ Absorbance 424 - 385 nm x 10^3/mg protein) for the mitochondria from the saline treated and HCG treated animals. The spectral change for the mitochondria from HCG treated animals was now greater than the spectral change observed with the mitochondria from saline treated animals. Thus the addition of cholesterol to mitochondria from HCG treated animals results in a large increase in pregnenolone formation in incubations as well as the pregnenolone induced difference spectrum. Cholesterol had little effect however on pregnenolone production or spectra in the mitochondria from saline injected animals. This suggests the cytochrome P450 of these mitochondria could bind very little extra cholesterol unlike those from HCG treated animals.

Treatment of superovulated rats, fifteen minutes before killing, with HCG increased the pregnenolone binding spectrum in comparison to mitochondria from saline injected controls. This increase was very small being 8.5 to 9.5 ($\triangle$ Absorbance 424 - 385 nm x 10^3/mg protein ,figure 6.7). The 15 minute treatment with HCG also produced a small increase in the pregnenolone production from endogenous cholesterol in the mitochondria (figure 6.5). When mitochondria were preincubated with 158 /uM cholesterol, ten minutes before recording of the pregnenolone spectra; they were 18.5 and 10 ($\triangle$ Absorbance 424 - 385 nm x 10^3/mg protein) respectively for HCG and saline treated animals. Again the mitochondria from HCG treated animals had a greater ability to bind and metabolise exogenously added cholesterol in comparison to those from saline treated animals (figure 6.7 and 6.5).

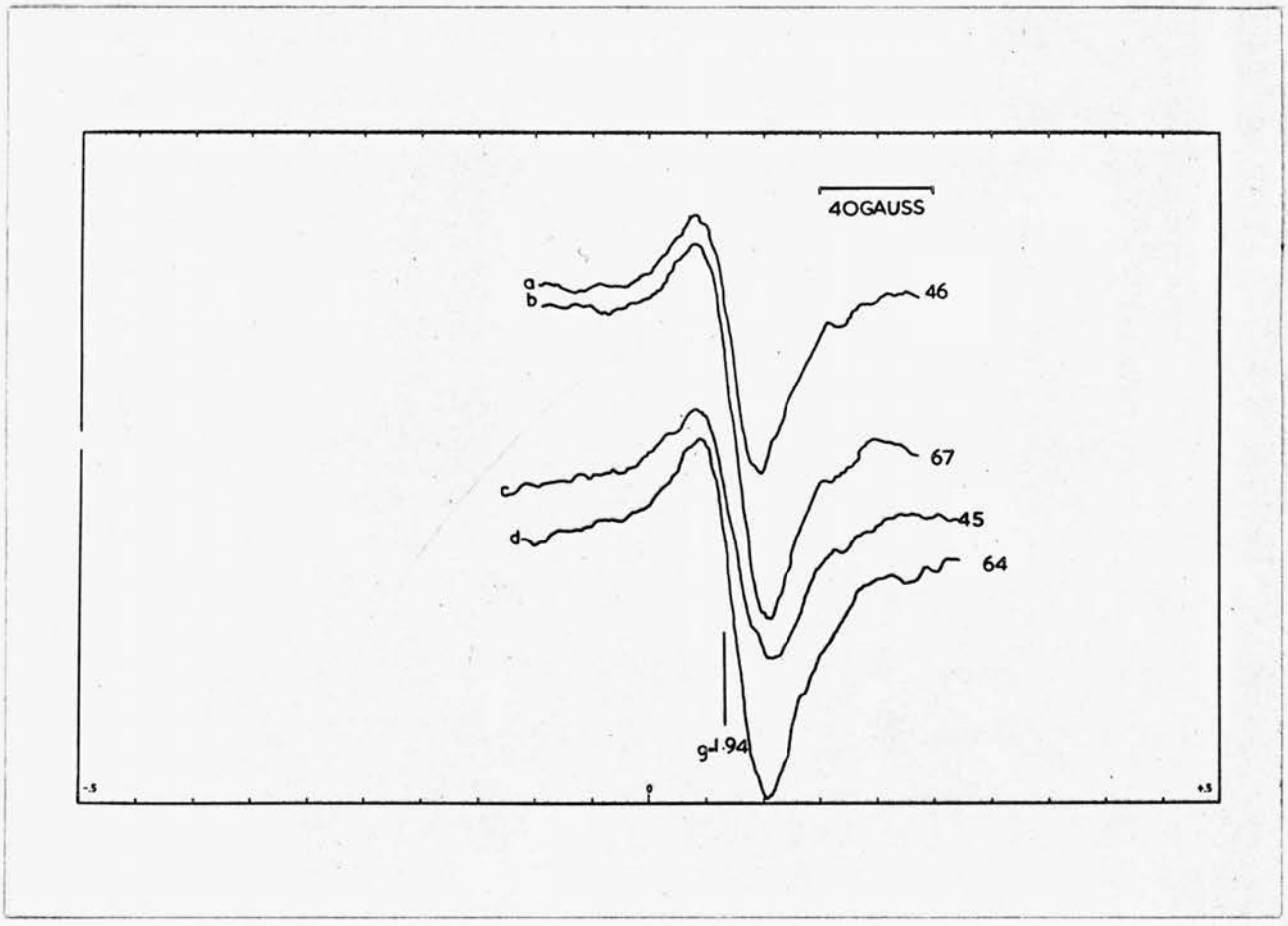


Figure 6.8. The E.P.R. spectra of luteal mitochondria from rats treated with LH or 0.9% saline one hour before being killed. The spectra were recorded under similar conditions to those described for figure 5.8. Protein concentrations were 2.3 mg/ml in each case. Cholesterol side chain cleavage activity was initiated in the mitochondria by the addition of malate. Signals a and b were recorded from the mitochondria of LH treated animals; c and d from mitochondria of saline treated animals (b and d are dithionite reduced samples, a and c are signals recorded in samples taken two minutes after the addition of malate to incubations). Numbers at the side of the trace refer to the amplitude of the signals.

The effects of subcutaneous injections of LH one hour before killing on ovarian ferredoxin in rat luteal mitochondria

It has been postulated that the effects of LH or HCG treatment on steroidogenesis in rat luteal mitochondria could be mediated by alteration of the supply of electrons to the cholesterol side chain cleavage cytochrome P450 (Robinson and Stevenson (1973)). Investigations of the steady state level of reduced ovarian ferredoxin, during steroidogenesis, were made to see if this was changed by LH treatment. Luteal mitochondria were prepared from rats treated for one hour with LH or 0.9% saline before killing. These mitochondria were incubated with malate and samples withdrawn for EPR spectroscopy after two minutes. Other samples of the mitochondria were reduced with dithionite. The EPR signals of these samples are shown in figure 6.8. The similarity in the size of the signals on dithionite reduction, shows that synthesis of iron sulphur protein had not been stimulated by one hours treatment with LH. In both incubations in the initial rapid burst of pregnenolone production the iron sulphur protein was 75% reduced. If LH treatment had increased the flow of electrons to cytochrome P450 the steady state levels of reduced iron sulphur protein would probably have been increased over control values. This would have increased the "pressure" of electrons on the cytochrome P450. It is therefore unlikely that the hormone treatment increases steroidogenesis by increasing the supply of electrons to cytochrome P450.

Summary of the effects of HCG/LH treatment on luteal mitochondrial cholesterol side chain cleavage activity

LH treatment appeared to cause a transient stimulation of cholesterol side chain cleavage activity in rat luteal mitochondria. This stimulation of pregnenolone production from endogenous cholesterol lasted for about twenty minutes before the production of steroid fell below control values (figure 6.1). This fall in pregnenolone production was probably due to a lack of cholesterol substrate in the luteal mitochondria (table 6.2). In fact in the presence of extra cholesterol the mitochondria from LH/HCG (1hr) treated animals could produce pregnenolone at a greater rate than those from saline injected controls (figures 6.3 and 6.4). Luteal mitochondria from animals treated for 15 minutes with HCG could also produce more pregnenolone than those from saline injected controls (figure 6.5). In this case HCG treatment had caused a small stimulation of pregnenolone production from endogenous cholesterol which had not yet fallen below control values (table 6.3). Thus LH/HCG treatment *in vivo* of rats causes an activation of the cholesterol side chain cleavage enzymes as subsequently assayed in isolated luteal mitochondria. This activation can only be seen on provision of the mitochondria with adequate supplies of cholesterol.

The increase in activity of the cholesterol side chain cleavage enzymes is associated with an increase of the inverted type 1 pregnenolone binding spectrum of cytochrome P450 (figures 6.6 and 6.7). Thus activation of cholesterol metabolism by LH/HCG may be due to an increase in the binding of cholesterol to cytochrome P450 in luteal mitochondria. After one hours treatment with LH/HCG although the mitochondrial cholesterol side chain cleavage enzymes were more

active than those of saline injected controls they produced less pregnenolone from endogenous cholesterol. This decrease in endogenous mitochondrial cholesterol could have been due to its increased metabolism to pregnenolone not being fully compensated for by supplies of cholesterol from the cytoplasmic lipid droplets (Armstrong and Flint (1973)). Thus *in vivo* after LH/HCG treatment there could be an increased turnover of cholesterol in the luteal mitochondria, causing an increase in luteal progesterone output. When the mitochondria are isolated they are cut off from a supply of cholesterol and unless this is added back they appear to be less active than those from saline injected animals.

The decrease in pregnenolone production in LH/HCG treated rat luteal mitochondria could also represent a decrease in the steroid output of the corpus luteum similar to the phenomenon reported to occur in the sheep corpus luteum on repeated stimulation with LH (Collet *et al* (1973)). Thus the decreased pregnenolone in incubations of rat luteal mitochondria from animals treated for one hour with LH/HCG could represent stimulated or inhibited *in vivo* steroid output in the corpus luteum. This could be resolved by measurement of changes in blood concentration of progesterone after LH stimulation of the rat. It is probable that after one hour's treatment with LH the rat corpus luteum is producing increased levels of progesterone. Depletion of cholesterol in the corpora lutea of superovulated rats occurs for at least four hours after LH treatment (Bell *et al* (1964)). In *in vitro* incubations, LH can stimulate progesterone production in slices of rat corpora lutea for up to three hours. (Hermier *et al* (1971)). Hence the decreased levels of cholesterol in luteal mitochondria after one hour of LH or HCG treatment are probably due to increased turnover of steroid.

RESTRAINING CAGE FOR TAIL VEIN INJECTION OF RATS

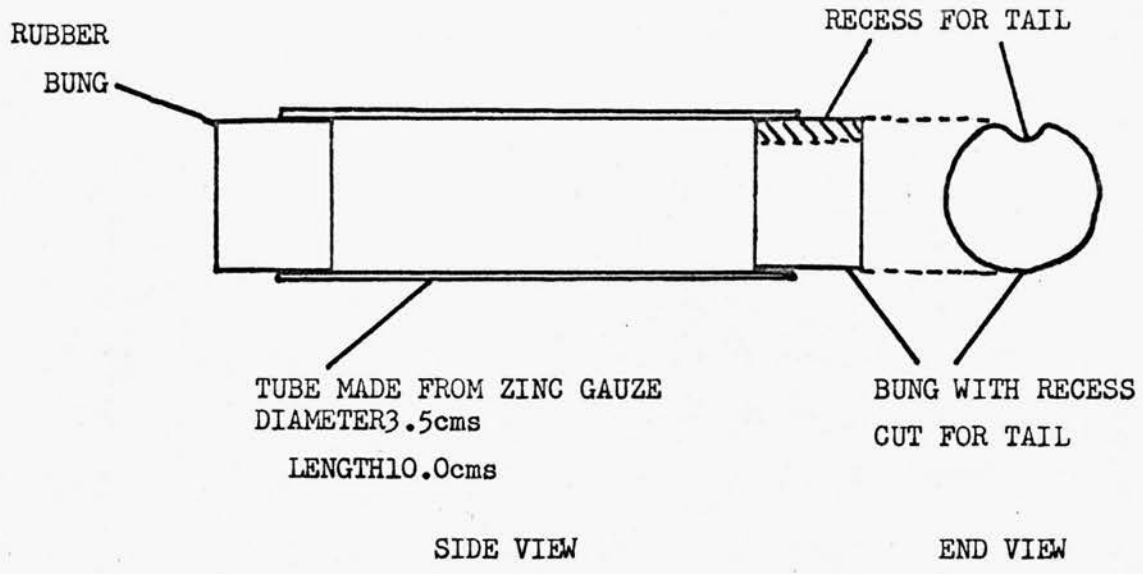


Figure 6.9. Diagram of the restraining cage used during tail vein injection of rats.

The effect of injections of LH, HCG and saline into the tail vein of rats on cholesterol side chain cleavage in luteal mitochondria

In the experiments already described in this chapter the rats were injected subcutaneously with gonadotrophins. Previously LH had been injected into the rats through the tail vein. For these injections the rats were placed in the restraining cage shown in figure 6.9. The rats are placed in the tube with their tails projecting through a hole in the rubber bung. Hormones, dissolved in 0.1 ml of 0.9% saline , could then be injected into the tail vein. This process could take up to three minutes and appeared to cause the rats some distress.

Experiments were carried out in which superovulated rats were injected in the tail vein with 20 µg of LH in 0.1 ml 0.9% saline or the saline alone. In a number of experiments this treatment had little or no effect on luteal mitochondrial cholesterol side chain cleavage activity. Any stimulation of cholesterol side chain cleavage activity, measured as the percentage conversion of [4 -^{14}C] cholesterol to [4 -^{14}C] pregnenolone could not be obtained consistently. Similarly tail vein injections of HCG did not consistently affect luteal mitochondrial cholesterol side chain cleavage.

This lack of effect of LH or HCG administration by tail vein injection could have been due to several factors: (a) The hormones may have been inactive, although they were active when injected subcutaneously. (b) The hormones may not have been successfully injected into the tail vein. (c) Endogenous gonadotrophins may have been maximally stimulating the steroid producing enzymes. (d) Cholesterol side chain cleavage activity may not be affected by LH treatment. (a) and (d) are unlikely reasons as the HCG and LH

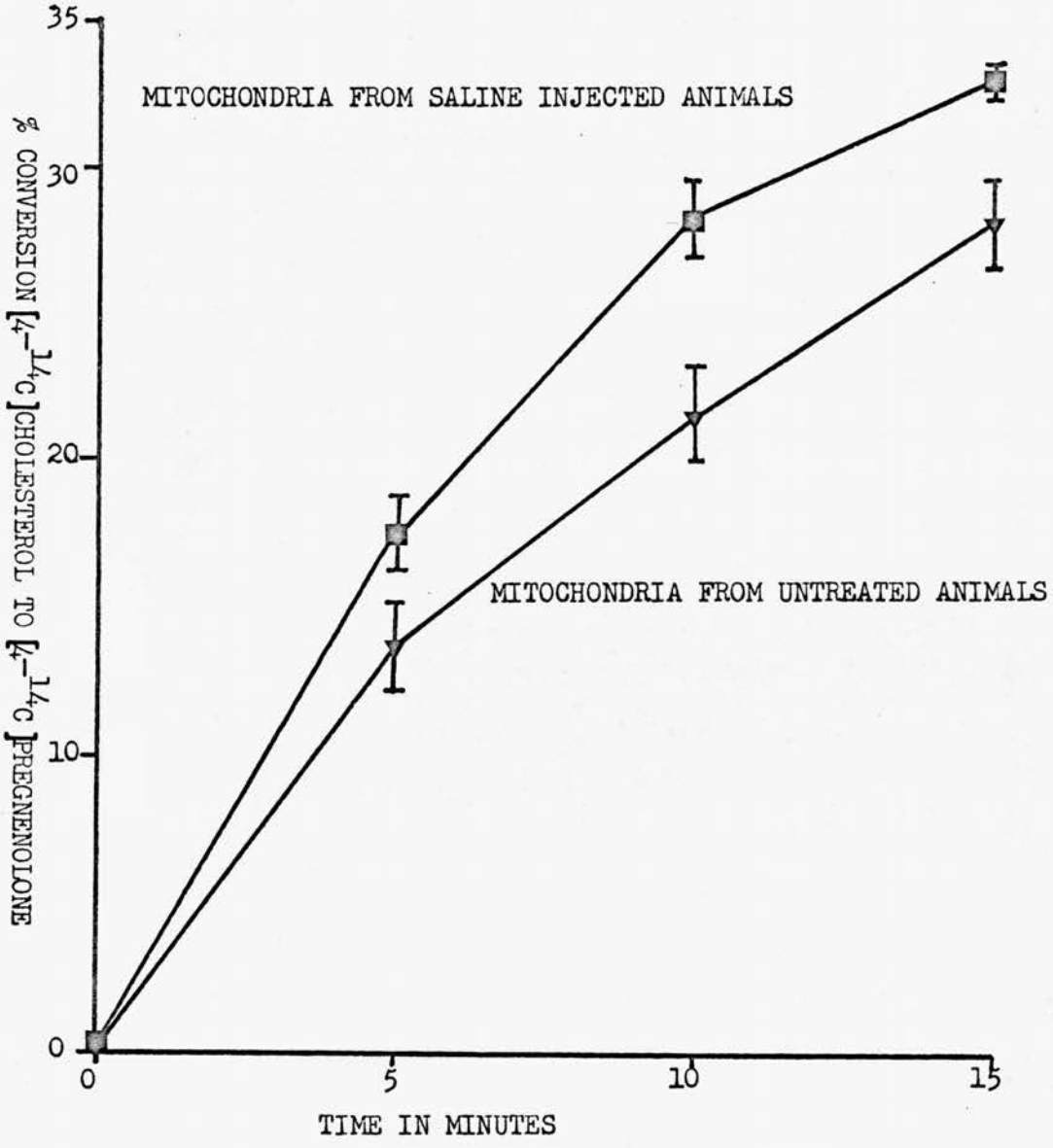


Figure 6.10. The effect of tail vein injection with 0.9% saline thirty minutes before killing on the conversion of [4-^{14}C] cholesterol to [4-^{14}C] pregnenolone in rat luteal mitochondria. Side chain cleavage, supported by malate, was measured using the methods described in chapter 2. Protein concentrations were 1.1 mg/ml in each set of incubations. Results expressed as mean $\pm$ SEM for four determinations.

were shown to affect cholesterol side chain cleavage when administered subcutaneously.

Ajika et al (1972) have demonstrated that stress, such as ether anaesthesia, causes an elevation of plasma LH levels in the rat. The ovarian cholesterol depletion assay for LH, described by Bell et al (1964), has been shown to be affected by a stress effect. Leonardelli and Poulain (1970) showed that intraventricular injections of physiological saline produced a depletion of ovarian cholesterol in the superovulated rat. Rats were, therefore, injected with 0.1 ml of 0.9% saline in the tail vein and luteal mitochondrial cholesterol side chain cleavage activity compared to that from uninjected controls. The results of this experiment are shown in figure 6.10. The saline injection can be seen to have caused a 25% stimulation of /4 -^{14}C/ pregnenolone production in incubations. Thus when hormones were injected into the tail vein of the rat the stimulation of pregnenolone production by the injection may have masked any effect of the hormone. Subcutaneous injections of saline had no effect on mitochondrial cholesterol metabolism (figure 6.1). This form of injection is very rapid taking no more than five seconds and causes little distress to the rats. Whether the stress of tail vein injection causes a release of pituitary hormones in the rat could be investigated using radioimmunoassays for LH in the plasma.

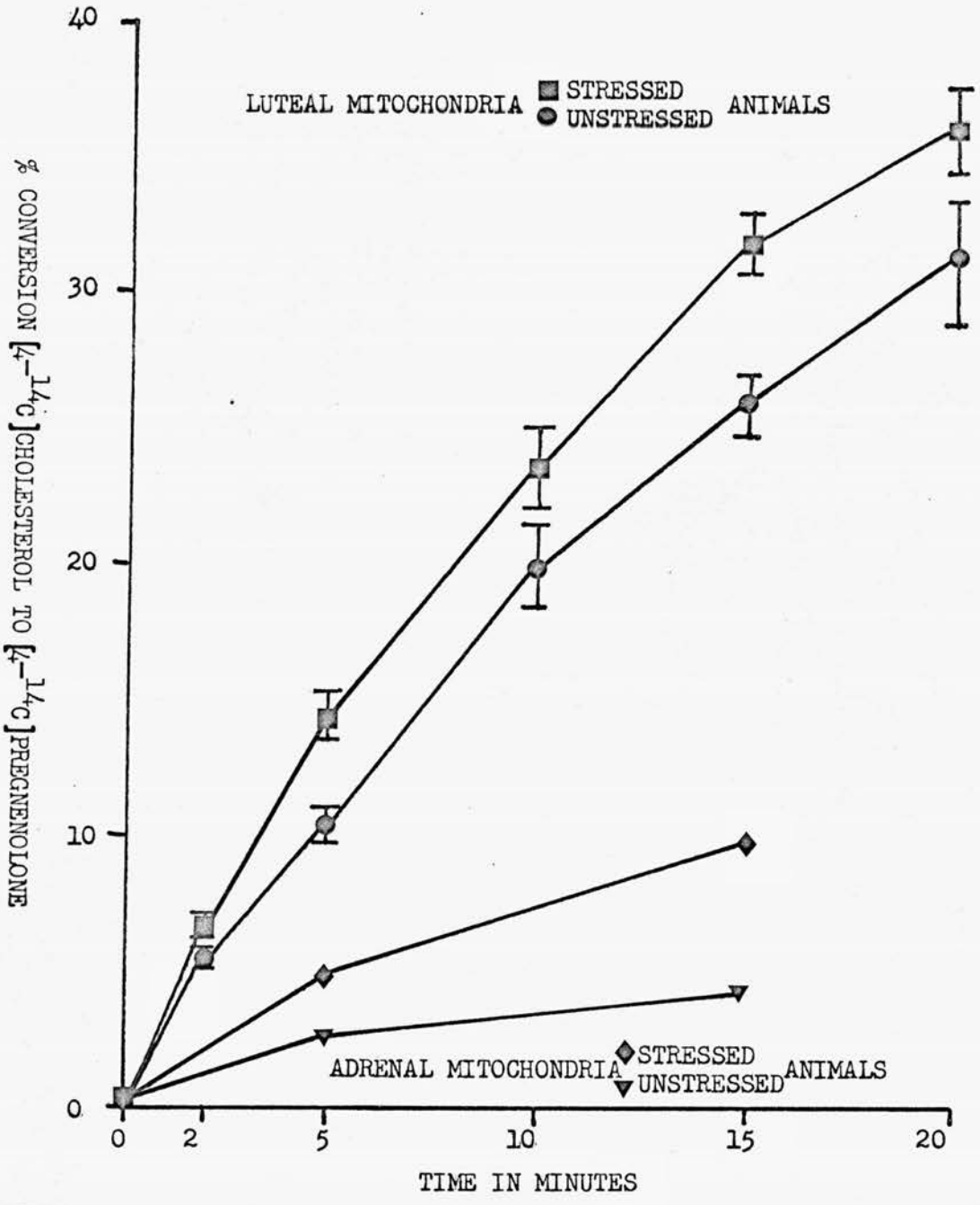


Figure 6.11. The effect of ten minute ether anaesthesia stress on the percentage conversion of [4-^{14}C] cholesterol to [4-^{14}C] pregnenolone measured in the mitochondria of rat adrenal and luteal tissue. Side chain cleavage activity was measured as described for figure 6.10. Adrenal and luteal mitochondrial protein concentrations were 0.8 mg/ml and 0.9 mg/ml in both sets of incubations respectively. Results for luteal mitochondria are mean $\pm$ SEM of 4 incubations.

The effect of ether anaesthesia stress on cholesterol side chain cleavage activity in rat luteal mitochondria

Ether anaesthesia stress can cause a stimulation of cholesterol side chain cleavage activity in rat adrenal cortex mitochondria (Simpson et al (1972)). Ether anaesthesia causes a twofold increase of serum LH concentrations in the rat within two minutes of administration (Ajika et al (1972)). Increases also occur in serum prolactin and to a lesser extent FSH. Figure 6.11 shows the percentage conversion of $[4\text{-}^{14}C]$ cholesterol to $[4\text{-}^{14}C]$ pregnenolone in luteal mitochondria from superovulated rats which had been anaethetised for ten minutes or left untreated. The ether anaesthesia can be seen to have stimulated the production of $[4\text{-}^{14}C]$ pregnenolone by 50% in incubations of the luteal mitochondria. The ether stress also increased $[4\text{-}^{14}C]$ pregnenolone production in the adrenal mitochondria of the rats (figure 6.11). This agrees with the findings of Simpson et al (1972) on the effects of ether stress on adrenal mitochondrial cholesterol side chain cleavage activity. Thus, as in the adrenal cortex, steroidogenic activity in the mitochondria of the corpus luteum is affected by stress to the animal, due presumably to the stress causing release of endogenous gonadotrophins.

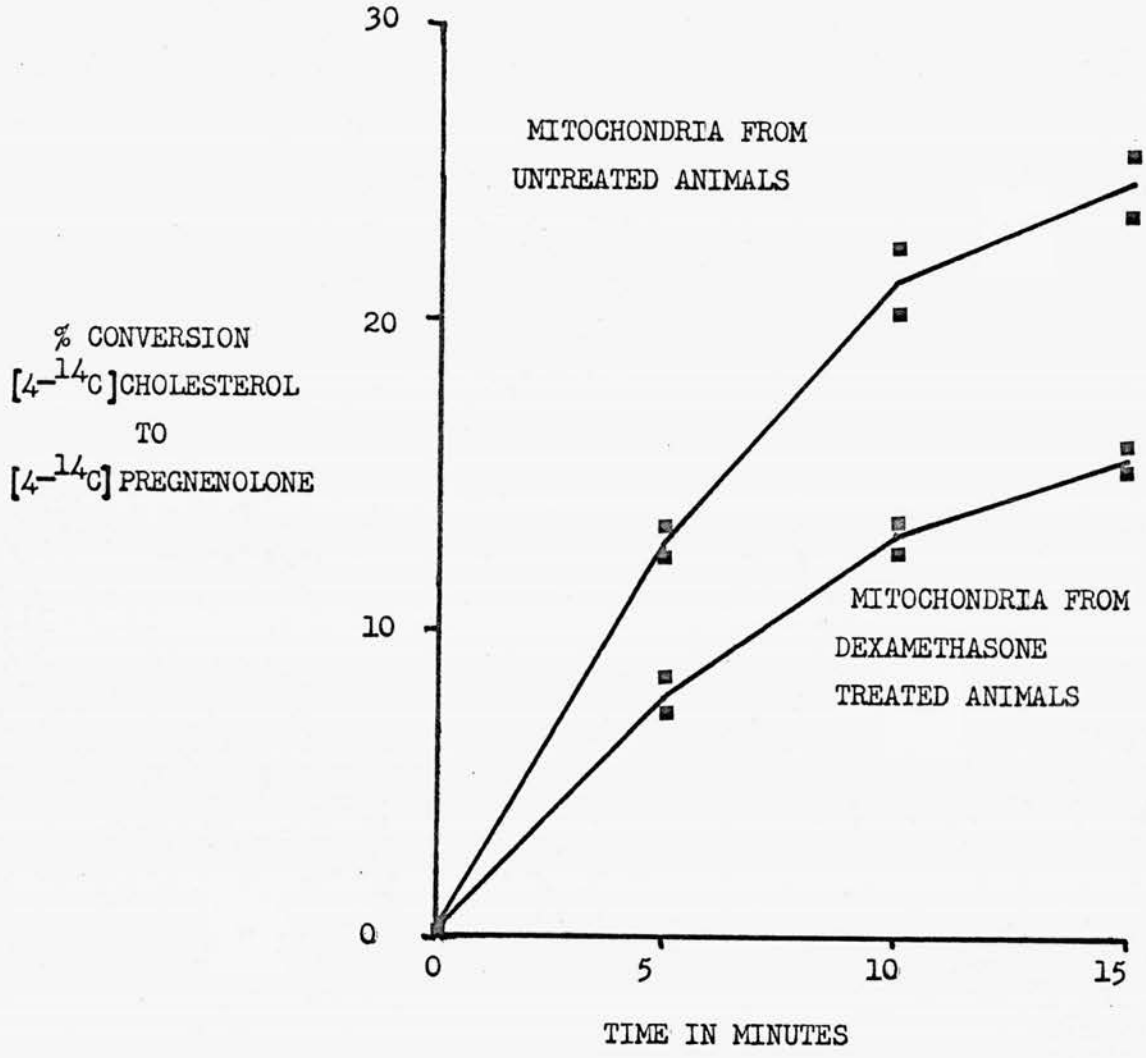


Figure 6.12. The effect of dexamethasone treatment of rats on the conversion of [4-^{14}C] cholesterol to [4-^{14}C] pregnenolone in luteal mitochondria. Side chain cleavage activity was measured as described for figure 6.10. Protein concentrations were 1.5 mg/ml for the mitochondrial incubations of the dexamethasone treated group of animals and 1.2 mg/ml for the control group. Shown on the graph are the results of duplicate assays.

Table 6.4

The effect of dexamethasone treatment of rats on adrenal and ovarian weight and cytochrome P450 content

	Dexamethasone treated	control
Adrenal weight mg/2 adrenals (average of 40 adrenals/group)	11	22
Adrenal cytochrome P450 content n moles/2 adrenals	0.38	1.12
n moles/mg wet weight	0.035	0.05
Ovarian weight mg/2ovaries (average 40 ovaries/group)	240	260
Ovarian cytochrome P450 content n moles/2 ovaries	2.88	2.72
Luteal mitochondrial cholesterol n moles/mg protein	25.32 $\pm$ 0.78	13.00 $\pm$ 0.17
	mean $\pm$ SEM four determinations	
Luteal mitochondrial cytochrome P450 n moles/mg protein	0.33	0.34

Rats were treated with dexamethasone as described in the text. Total adrenal and ovarian cytochrome P450 was measured in homogenates of the glands as described by Estabrook et al (1972). Luteal mitochondrial cholesterol and cytochrome P450 were measured as described in chapter 2.

The effects of dexamethasone treatment on cholesterol side chain cleavage in rat luteal mitochondria

To remove the effects of endogenous gonadotrophins in rats which were to be treated with LH, the animals could have been hypophysectomised. This would have been very difficult to do as up to fifty rats are needed to prepare mitochondria for one experiment. It was therefore decided to try to "chemically hypophysectomise" the rats for use in experiments. Dexamethasone, a synthetic corticosteroid, had been shown to suppress *in vivo* the secretion of progesterone by the human ovary and bovine corpus luteum (Fylling (1973); Edquist *et al* (1972)). This effect could have been due to a direct interaction of dexamethasone with steroidogenic enzymes or the steroid causing suppression of release of LH. More recently Baldwin and Sawyer (1974), using a radioimmunoassay, have shown dexamethasone can lower circulating levels of LH in the rat. Figure 6.12 shows that dexamethasone administered to rats for two days in their drinking water caused a 50% inhibition of production of $[4\text{-}^{14}C]$ pregnenolone in isolated luteal mitochondria in comparison to the production in untreated animals. The total cholesterol content of the luteal mitochondria was doubled by dexamethasone treatment of the rats (table 6.4). It was therefore unclear whether the decrease in $[4\text{-}^{14}C]$pregnenolone production in luteal mitochondria, caused by dexamethasone treatment, represented an actual decrease in pregnenolone production.

Ovarian cytochrome P450 and weight did not appear to be altered by dexamethasone treatment of the rats (table 6.4). Total cytochrome P450 in the ovarian homogenate was measured as the CO reduced minus CO difference spectrum (Estabrook *et al* (1972)). Also shown in table 6.4 are the adrenal weights and cytochrome P450 contents from the dexamethasone treated rats. The adrenal weights were cut by 50%

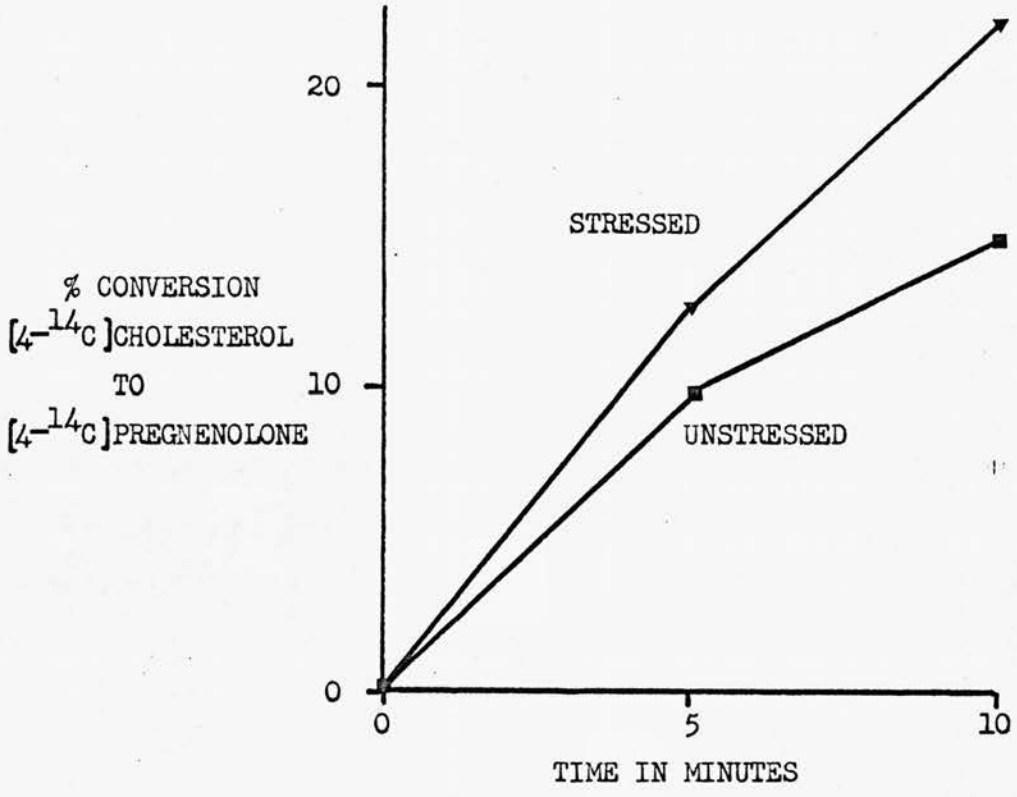


Figure 6.13. The effect of ether anaesthesia stress on cholesterol side chain cleavage in luteal mitochondria of dexamethasone treated rats. Side chain cleavage activity was measured as described for figure 6.10. Protein concentrations were 1.7 mg/ml in each group of incubations.

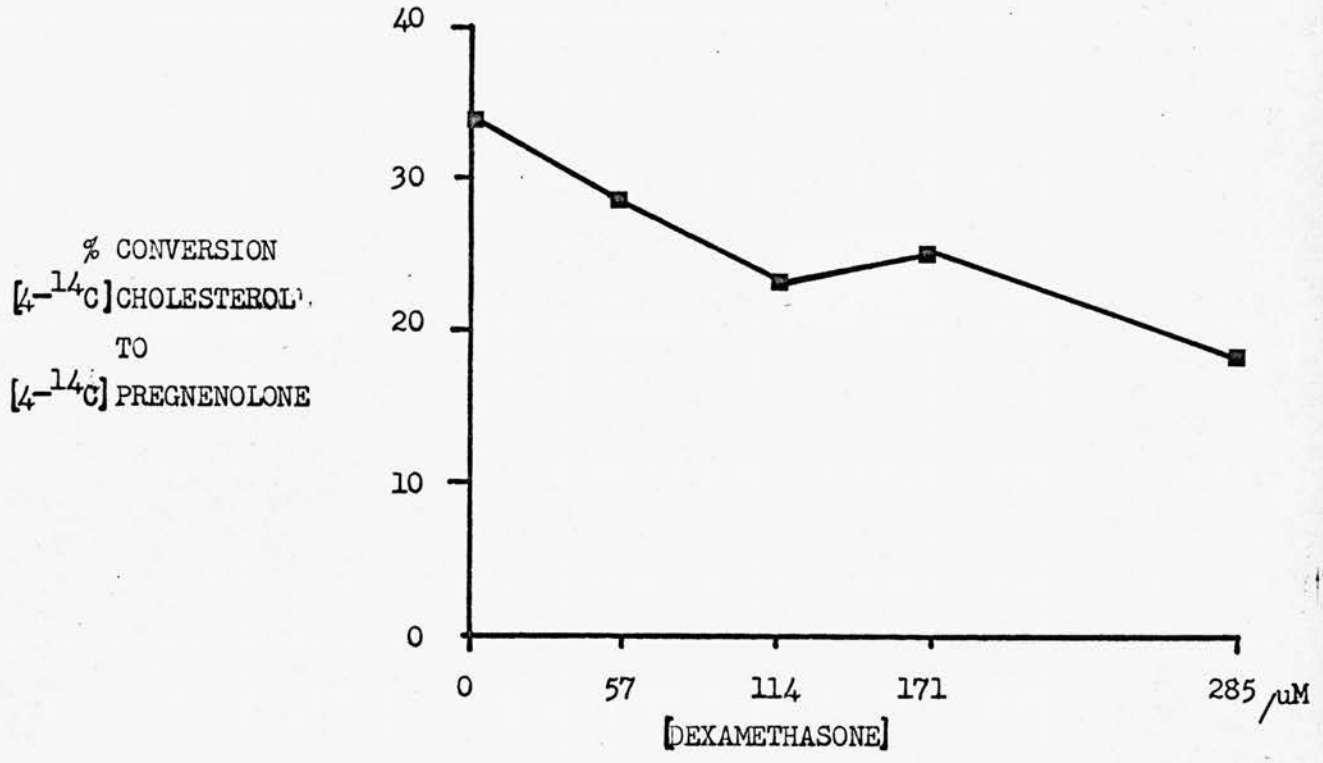


Figure 6.14. The inhibition of cholesterol side chain cleavage, *in vitro*, produced by the addition of dexamethasone to incubations of luteal mitochondria. The percentage conversion of [4-^{14}C] cholesterol to [4-^{14}C] pregnenolone was measured in 10 minute incubations.

Table 6.5

The effect of dihydrotestosterone treatment of rats on cholesterol side chain cleavage activity in luteal mitochondria

	% conversion			
	dihydrotestosterone		control	
	mitochondria		mitochondria	
time after addition of malate to incubations in mins	0; 0	0	0	0
	5; 29.5	26.6	28.3	25.6
	10; 42.6	36.9	40.1	36.3
	15; 49.1	44.2	48.6	44.5
mitochondrial cholesterol n moles/mg protein	12.6 ± 0.6		11.8 ± 0.3	
	mean ± SEM (4 determinations)			

The percentage conversion of [4-^{14}C] cholesterol to [4-^{14}C] pregnenolone supported by malate was measured in luteal mitochondria as described in chapter 2. Mitochondrial protein concentrations were 1.2 mg/ml and 1.5 mg/ml respectively for incubations of control and dihydrotestosterone mitochondria.

by this treatment as was the adrenal cytochrome P450 content and this suggests that the dexamethasone had suppressed pituitary ACTH release. The removal of ACTH by hypophysectomy causes the decay of adrenal weight and cytochrome P450 content in the rat (Purvis et al (1973)).

Dexamethasone treatment of rats did not prevent the effect of ether anaesthesia stress on luteal mitochondrial cholesterol side chain cleavage activity (figure 6.13). Therefore it would have been preferable to use hypophysectomy rather than treatment with a drug to prevent release of endogenous gonadotrophin in the rats used for this study. The use of dexamethasone had a further drawback as it was shown to have a direct inhibitory effect on the cholesterol side chain cleavage enzymes (figure 6.14).

The effects of dihydrotestosterone treatment on cholesterol side chain cleavage in rat luteal mitochondria

In the male rat and ovariectomised female rat treatment with dihydrotestosterone has been shown to cause decreases in circulating levels of LH (Beyer et al (1972); Naftolin and Feder (1973)). Treatment of superovulated rats with dihydrotestosterone 24 hours and 9 hours before killing (see methods) had no effect on cholesterol content or Δ^{4}-^{14}C pregnenolone production in isolated luteal mitochondria (table 6.5). Dihydrotestosterone therefore appeared to be ineffective for "chemical hypophysectomy". Suppression of LH release by dihydrotestosterone was observed in ovariectomised animals which have unusually high levels of circulating gonadotrophins (Beyer et al (1972)). Hence the drug may not have worked on animals with normal levels of circulating gonadotrophin.

Summary

(a) LH or HCG treatment of superovulated rats one hour before killing has been shown to cause increased conversion of [4 -^{14}C] cholesterol to [4 -^{14}C]pregnenolone in isolated luteal mitochondria, in comparison to mitochondria from saline injected controls.

(b) This increased conversion of [4 -^{14}C]cholesterol to [4 -^{14}C] pregnenolone represents a fall in total pregnenolone production/mg protein because LH/HCG treatment causes a decrease in the total luteal mitochondrial cholesterol content.

(c) In the presence of added cholesterol, luteal mitochondria from LH/HCG treated animals can produce more pregnenolone than those from saline injected controls.

(d) Total luteal mitochondrial cytochrome P450 and ovarian ferredoxin levels are unaltered by prior treatment of the rats with LH or HCG. Steady state levels of reduced ovarian ferredoxin during mitochondrial incubations are unchanged by prior LH treatment of rats.

(e) When cholesterol is first preincubated with luteal mitochondria cytochrome P450-pregnenolone induced difference spectra are increased by prior treatment of the rats with HCG.

(f) LH or HCG treatment of rats causes an increase in the cholesterol side chain cleavage activity of subsequently isolated luteal mitochondria. This activation can only be seen when sufficient cholesterol is added to the *in vitro* mitochondrial incubations.

(g) The pregnenolone production in luteal mitochondria is sensitive to prior stress to the rat either by tail vein injection or ether anaesthesia.

(h) Use of dexamethasone or dihydrotestosterone to "chemically hypophysectomise" rats is discussed.

CHAPTER 7

THE EFFECTS OF INHIBITORS OF PROTEIN SYNTHESIS ON CHOLESTEROL SIDE CHAIN CLEAVAGE IN RAT LUTEAL MITOCHONDRIA

Introduction

Stimulation of steroidogenesis, in the corpus luteum, by LH has been thought to involve protein synthesis. Marsh and Savard (1966b) and Marsh et al (1966) have shown that puromycin can prevent LH induced stimulation of steroidogenesis without preventing rises in cellular cyclic AMP concentrations. However results obtained with puromycin should be treated with some caution in view of the findings described later in this chapter. Cycloheximide, another inhibitor of protein synthesis, also prevents LH and cAMP stimulated rises in steroidogenesis in ovarian and luteal tissue (Gorski and Padnos (1966); Hermier et al (1971); (1972a, b); Flint et al (1973)). Gorski and Padnos (1966) found that actinomycin D did not prevent LH induced stimulation of steroidogenesis. As actinomycin D is an inhibitor of RNA synthesis, they suggested that LH stimulated the formation of a protein from a stable mRNA molecule. A protein has been postulated by Hermier et al (1971) to affect the availability of cholesterol to the mitochondrial cholesterol metabolising enzymes of the rat corpus luteum. This hypothesis was based on experiments with luteal slices and no actual measurements were made of the activities of the mitochondrial cholesterol side chain cleavage enzymes. Cholesterol side chain cleavage is thought to the rate limiting step of steroidogenesis in the corpus luteum (Chapter 1) as it is in the adrenal cortex (Stone

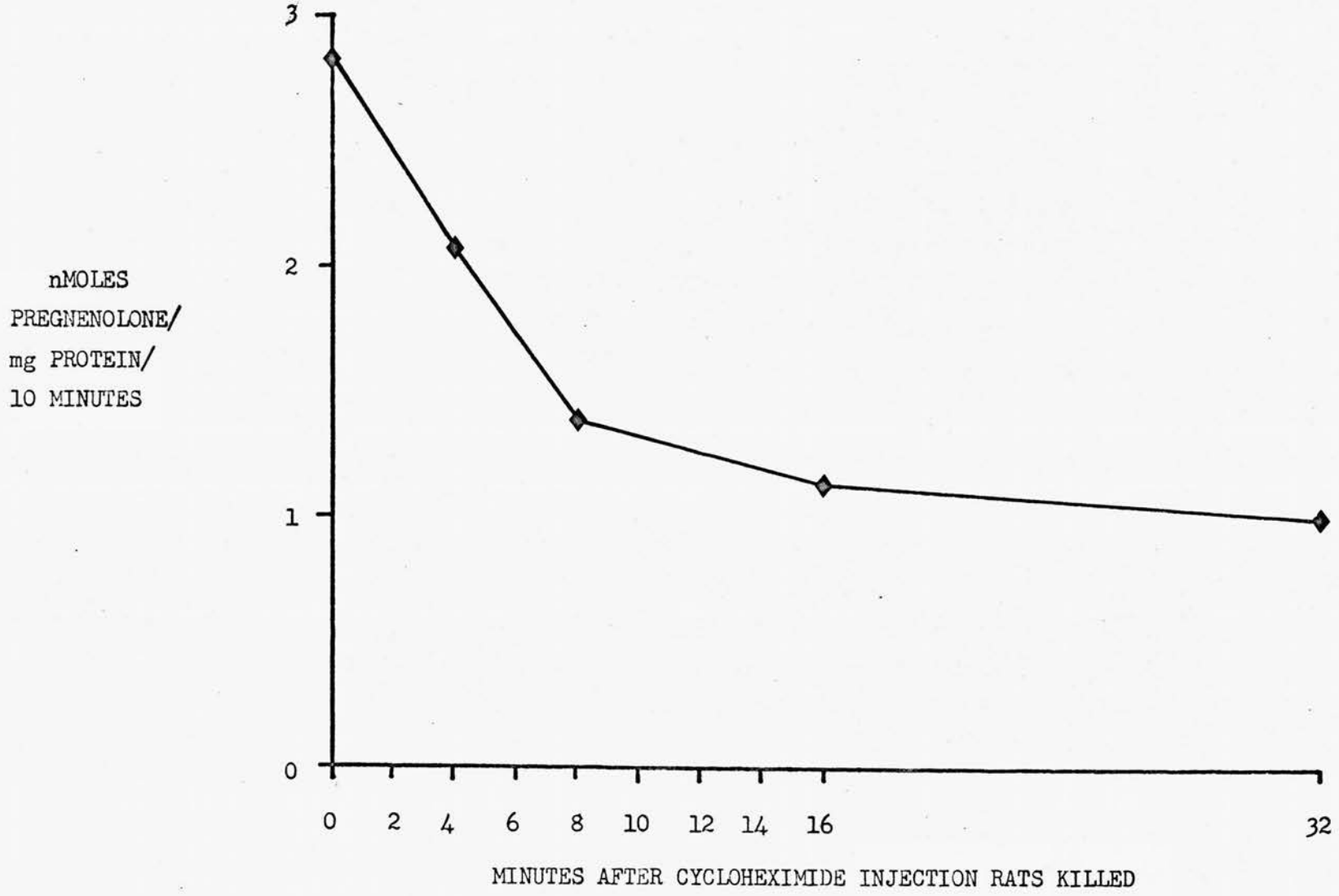


Figure 7.1. The effect of prior injection of rats with cycloheximide on the cholesterol side chain cleavage activity in isolated luteal mitochondria.

and Hechter (1954)). Simpson et al (1972); Brownie et al (1972) and Jefcoate et al (1974) have reported that in the adrenal cortex a rapidly turning over ACTH sensitive protein factor (Garren et al (1965)) influences the binding or transport of cholesterol to the mitochondrial cholesterol side chain cleavage cytochrome P450. Thus it was of interest to see if the cycloheximide sensitive factor in the rat corpus luteum (Hermier et al (1971)) affected the cholesterol metabolising enzymes and cytochrome P450 of the mitochondria. At the same time, effects of other inhibitors of protein synthesis on the cholesterol side chain cleavage enzymes were investigated.

The effects of cycloheximide treatment on the cholesterol side chain cleavage enzymes of rat luteal mitochondria

Superovulated rats were injected with cycloheximide (5 mgs./rat) as described in chapter 2. The rats (8/group) were killed 4,8,16 and 32 minutes after the cycloheximide injection. The ovaries were removed from the rats and homogenised immediately, mitochondria were then prepared from them as described in chapter 2. Cholesterol side chain cleavage activity was then determined in each group of mitochondria using malate as the electron donor. Pregnenolone production (n moles/mg protein/10 minutes) in these mitochondria is shown in figure 7.1. Six minutes after cycloheximide injection the luteal mitochondrial pregnenolone production had fallen to 50% of the control values. Sixteen minutes after the cycloheximide the mitochondrial pregnenolone production had fallen to 40% of the control values and did not decrease further. Thus cycloheximide administration to superovulated rats had a rapid inhibitory effect on luteal

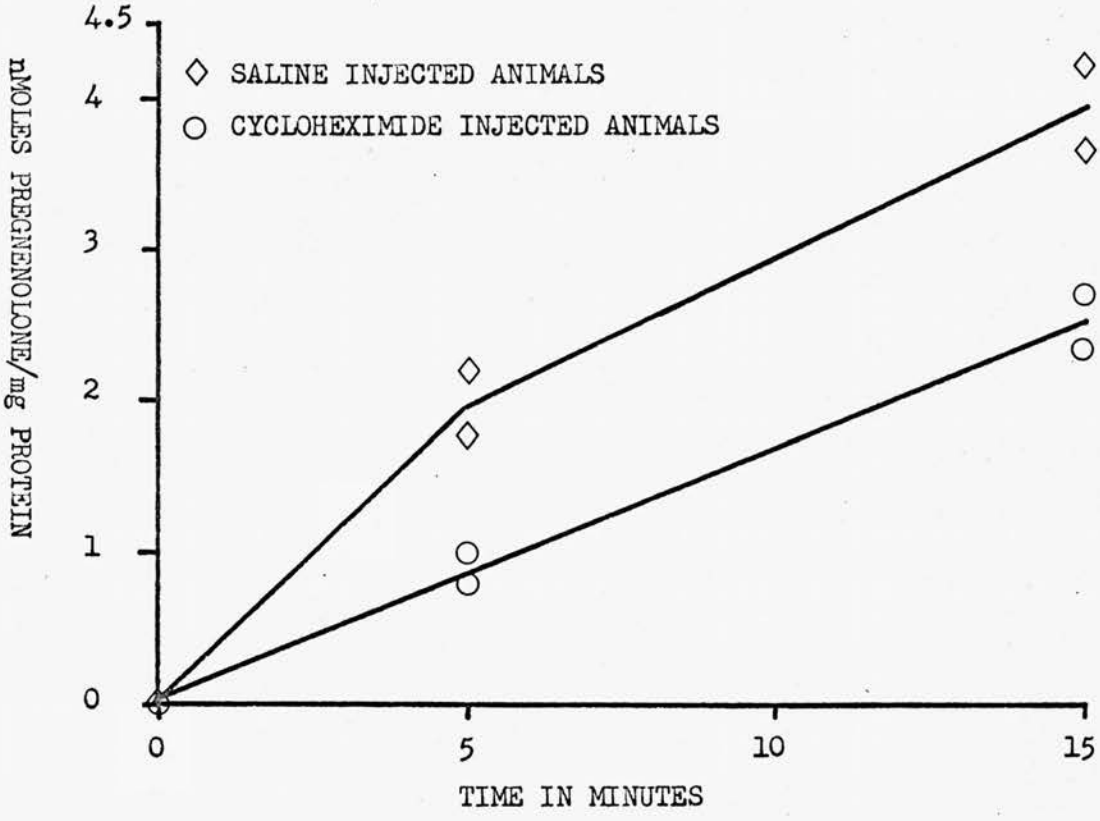


Figure 7.3. The effect of cycloheximide injection twenty minutes before killing on pregnenolone production in rat luteal mitochondria. Pregnenolone was measured as described in chapter 2 after side chain cleavage had been initiated in mitochondria with malate.

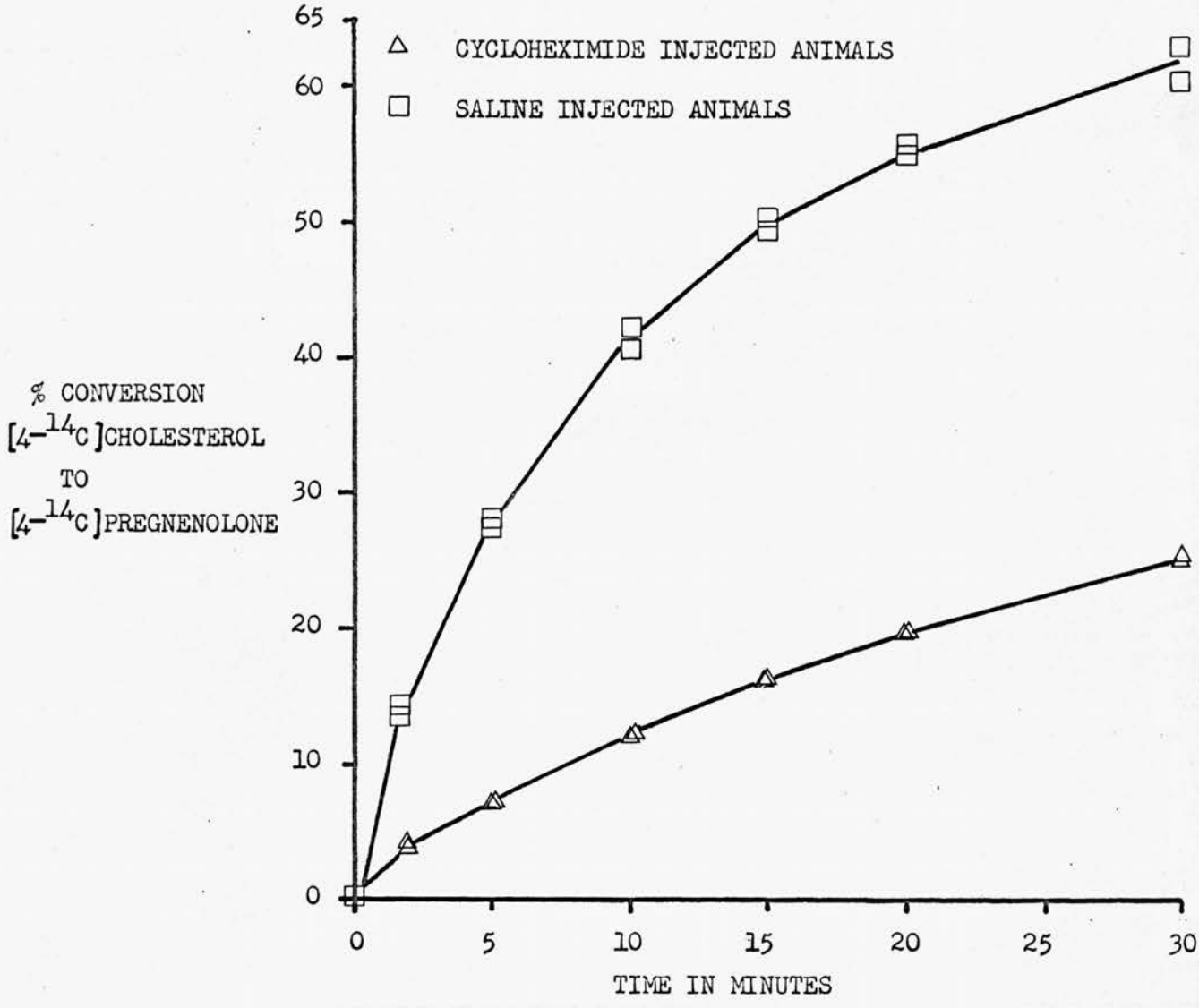


Figure 7.2. The effect of cycloheximide injection twenty minutes before killing on the conversion of [4-14 C] cholesterol to [4-14 C] pregnenolone in rat luteal mitochondria. The percentage conversion was measured using the methods described in chapter 2. Protein concentrations were 1.2 mg/ml in each incubation.

Table 7.1

Effect of cycloheximide 20 minutes before killing on cholesterol levels, cholesterol side chain cleavage activity, cytochrome P450 and pregnenolone induced difference spectra of isolated rat luteal mitochondria

	Saline Treated Animals	Cycloheximide Treated Animals
Mitochondrial cholesterol n moles/mg protein	17.55 ± 1.05(6)	22.0 ± 0.73(6)*
Pregnenolone formation n moles/mg protein/5 minute incubation	3.35 ± 0.53(6)	1.45± 0.21(6)*
Mitochondrial cytochrome P450 n moles/mg protein	0.48 ± 0.06(4)	0.47± 0.06(4)
Pregnenolone induced difference spectrum 10^3 x ΔAbsorbance (424-385nm)/ mg protein	15.1 ± 1.5 (7)	6.7 ± 0.3(7)+

Luteal mitochondria were isolated from rats which had previously been treated with cycloheximide or 0.9% saline for 20 minutes before killing. Initial cholesterol levels in the mitochondria and pregnenolone produced in 5 minute incubations were determined. Total mitochondrial cytochrome P450 was determined by the method of Omura and Sato (18). Pregnenolone induced difference spectra were determined as described in the methods section. All results are expressed as Mean ± S.E.M.; number of determinations in parenthesis
*$P < 0.01$, + $P < 0.001$.

mitochondrial cholesterol side chain cleavage. The effect of twenty minutes cycloheximide treatment on the rate of cholesterol side chain cleavage in luteal mitochondria was investigated using the radioactive assay (table 2.3). Figure 7.2 shows the time course of the appearance of $[4\text{-}^{14}C]$ pregnenolone from $[4\text{-}^{14}C]$ cholesterol in luteal mitochondria from rats treated with cycloheximide or 0.9% saline. The initial rate of formation of $[4\text{-}^{14}C]$ pregnenolone can be seen to have dropped to 25% of control values. This was greater than the inhibition of cholesterol side chain cleavage measured, when the actual conversion of cholesterol to pregnenolone was determined, in luteal mitochondria from cycloheximide treated and saline treated animals. In figure 7.1 the actual formation of pregnenolone only fell to 40% of control values after 16 or 32 minutes of cycloheximide treatment. This is reflected in the time course of pregnenolone production shown in figure 7.3. Here the initial rate of cholesterol side chain cleavage had fallen by 50% in the cycloheximide treated animals. Table 7.1 shows the pregnenolone production (n moles/mg protein/5 minutes) in luteal mitochondria from cycloheximide and saline treated animals from several experiments. The luteal mitochondria from cycloheximide treated animals showed 42% of the cholesterol side chain cleavage activity found in mitochondria from saline treated animals. Thus the measure of $[4\text{-}^{14}C]$ pregnenolone production, shown in figure 7.2, appeared to exaggerate the inhibitory effect of cycloheximide treatment on luteal mitochondrial cholesterol side chain cleavage. The reason for this became apparent when the levels of luteal mitochondrial cholesterol were measured in saline and cycloheximide treated rats (table 7.1). Cycloheximide treatment for twenty minutes induced a significant rise ($P < 0.01$) in the

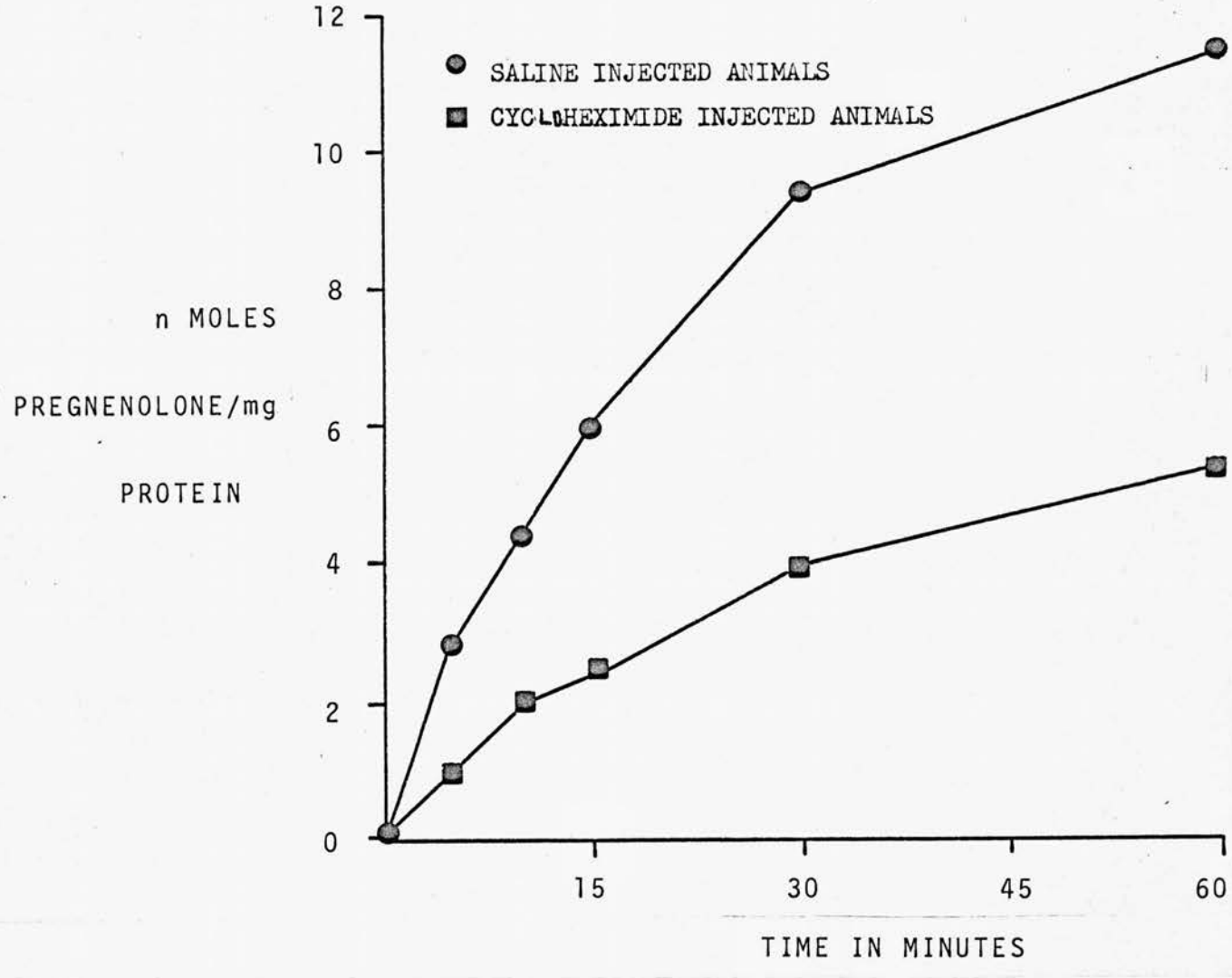


Figure 7.4. The effect of cycloheximide injection twenty minutes before killing on pregnenolone production in rat luteal mitochondria in the presence of added cholesterol. Conditions as described for figure 7.3 except both sets of incubations contained 260 μM cholesterol.

levels of luteal mitochondrial cholesterol when compared to the amounts in the mitochondria of saline treated animals. Hence it is better to compare the actual pregnenolone production/mg protein rather than the percentage conversion of cholesterol to pregnenolone, as the mitochondrial pool of cholesterol changed upon cycloheximide treatment.

In incubations, mitochondria were rapidly depleted of substrate cholesterol (figure 3.1 and 3.4). There was, however, more cholesterol in the luteal mitochondria of the cycloheximide treated animals. This suggested there was something preventing cholesterol being metabolised by the cholesterol side chain cleavage enzymes. Exogenous cholesterol was therefore added to incubations to see if pregnenolone production in the luteal mitochondria from cycloheximide treated or saline treated animals was affected. The results of this experiment are shown in figure 7.4. It can be seen from this that even after addition of an excess of cholesterol (10 times endogenous levels) there was still a 50% inhibition of pregnenolone production in the luteal mitochondria from cycloheximide treated animals.

Cycloheximide treatment had therefore inhibited luteal mitochondrial pregnenolone production despite the presence of more cholesterol in these mitochondria than there was in the mitochondria of saline treated animals. It seemed possible therefore that cycloheximide could have decreased the synthesis of the cholesterol side chain cleavage enzymes thus causing a reduction in pregnenolone production. Cytochrome P450 is considered to be a key enzyme in the cholesterol side chain cleavage system of rat corpora lutea (Sulimovici and Boyd (1968b)). The amount of cytochrome P450 in the luteal mitochondria

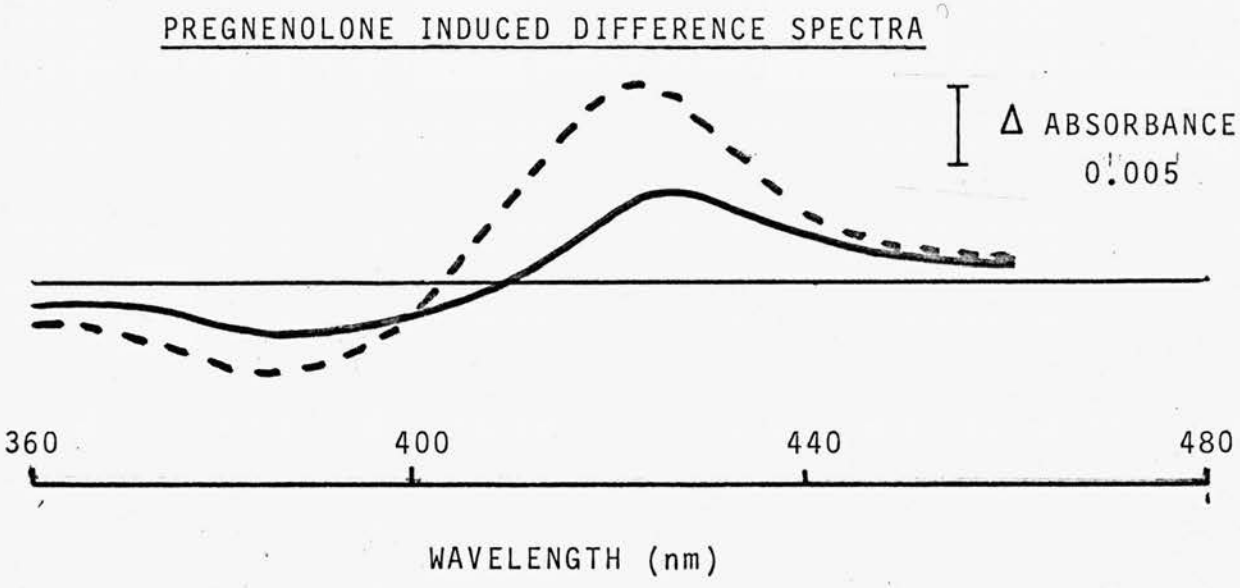


Figure 7.5. The effect of cycloheximide injection twenty minutes before killing on the pregnenolone induced difference spectrum of rat luteal mitochondria. Difference spectra were recorded as described in chapter 2; —— mitochondria from cycloheximide treated animals ..... mitochondria from saline treated animals.

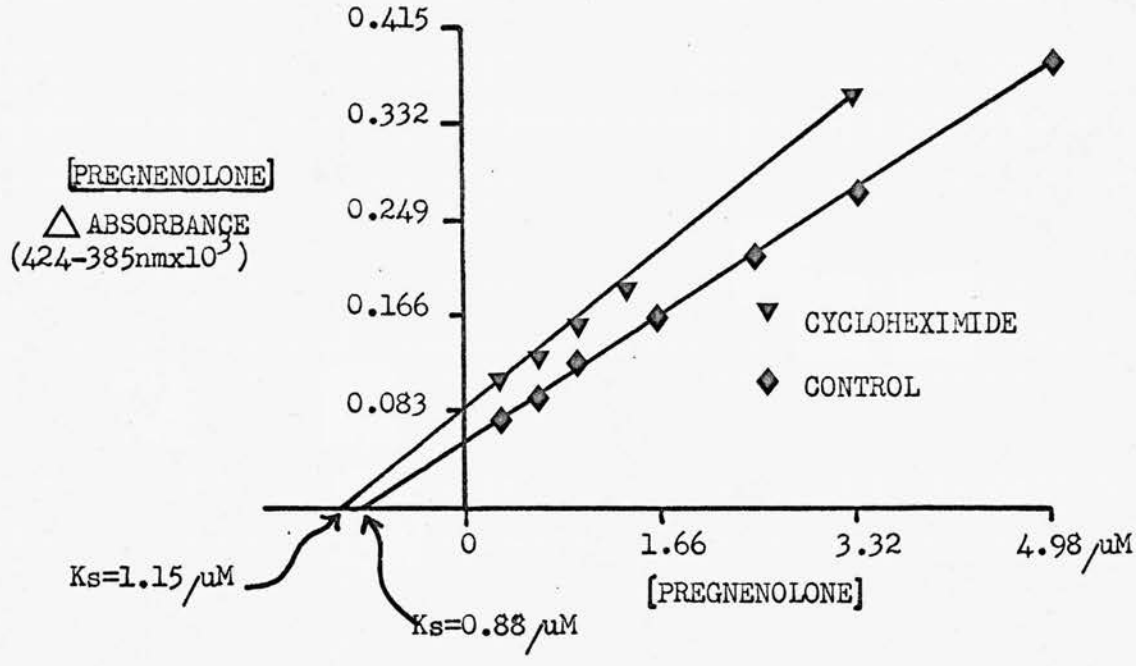


Figure 7.6. The determination of the binding constant (Ks) for the pregnenolone binding spectrum of rat luteal mitochondria. Shown are the results obtained with mitochondria from saline injected animals and animals which had been injected with cycloheximide twenty minutes before being killed. Protein concentrations were 0.9 mg/ml and 1.3 mg/ml respectively. The spectra were recorded and the Ks determined as described in chapter 2.

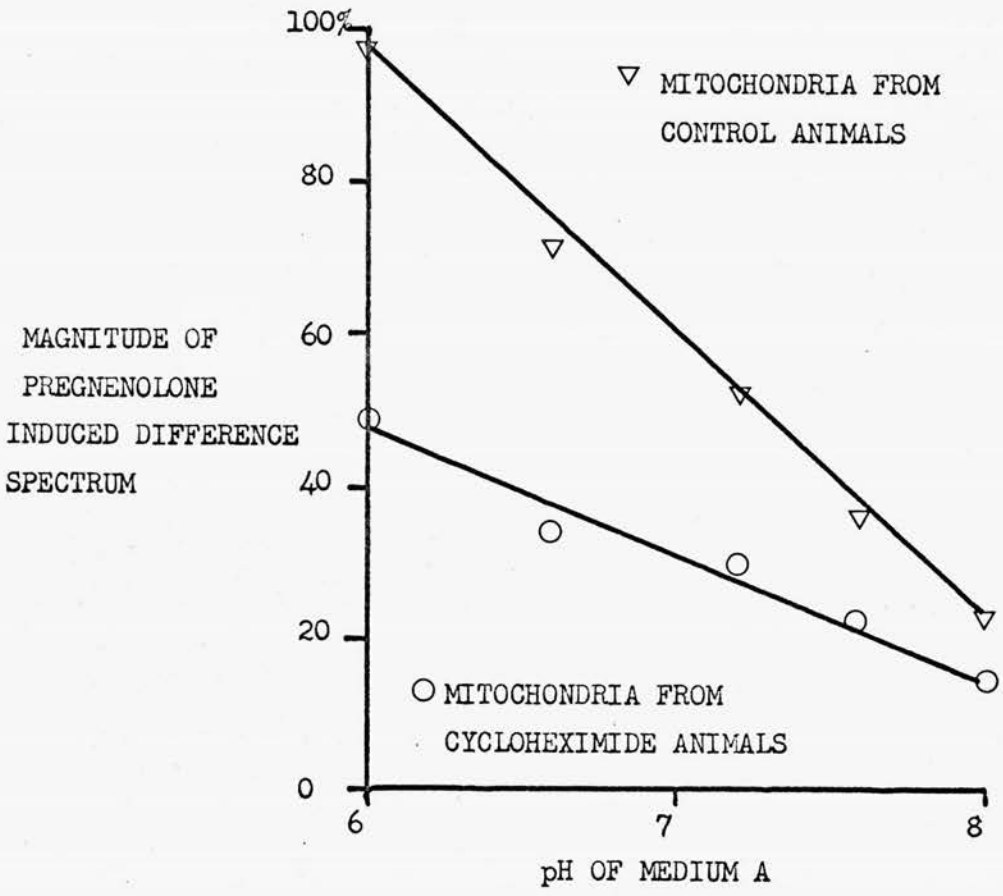


Figure 7.7. The effect of cycloheximide injection, twenty minutes before killing, on the pH dependency of the pregnenolone induced binding spectrum of rat luteal mitochondria. The spectra were recorded and plotted as described for figure 5.4.

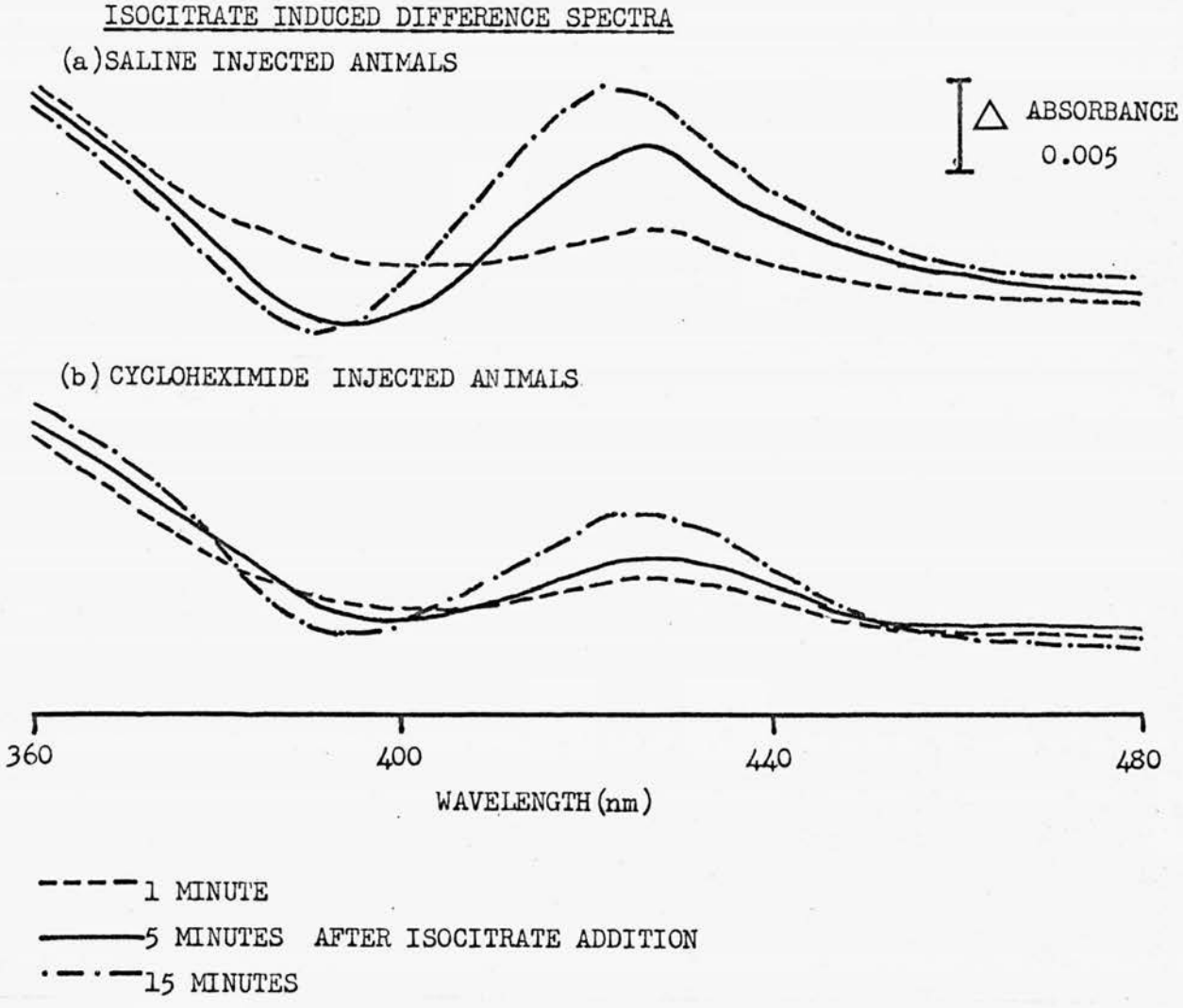


Figure 7.8. The effect of cycloheximide injection twenty minutes before killing on the isocitrate induced difference spectra of rat luteal mitochondria. The spectra were recorded as described for figure 5.5. Protein concentrations were 0.8 and 1.1 mg/ml for recording of spectra (a) and (b) respectively.

from cycloheximide treated and untreated rats was determined and no difference in the amount of cytochrome P450/mg protein in the two groups of mitochondria was found suggesting that cycloheximide could not effect a change in cytochrome P450 synthesis within twenty minutes (table 7.1).

As discussed in chapter 5 it was possible to obtain a measure of the cholesterol bound to the luteal mitochondrial cytochrome P450 by difference spectra induced by pregnenolone or Krebs cycle intermediates. Figure 7.5 shows the pregnenolone induced inverted type I difference spectra obtained with luteal mitochondria from rats treated with cycloheximide or 0.9% saline. The magnitude of the difference spectrum measured as $\triangle$ absorbance 424 - 385 nm was significantly decreased in the luteal mitochondria from cycloheximide treated animals (table 7.1). The binding constant for the pregnenolone induced spectral changes, in the two groups of mitochondria, was not however greatly altered (figure 7.6). The pregnenolone induced spectrum showed a pH dependency in the cycloheximide treated animals, like that previously found in the luteal mitochondria from control animals (figure 7.7). At different pH values the spectra, induced by pregnenolone, were 50% smaller with the mitochondria from the cycloheximide treated animals compared to the saline treated animals (figure 7.7). Thus although cycloheximide treatment had reduced the size of the pregnenolone induced spectrum from luteal mitochondria, it does not appear to be abnormal in any other way. The isocitrate induced difference spectra are also shown for the luteal mitochondria from animals treated with cycloheximide and saline for twenty minutes (figure 7.8). The isocitrate induced spectra after one minute are nearly the same in the mitochondria from both groups of animals. The difference

spectrum is like that of a reduced - oxidised b type cytochrome λ max 428 nm λ min 410 nm the Soret region of the spectrum. Five and fifteen minutes after isocitrate addition the spectra had developed to give a λ max at 424 nm and a λ min at 390 nm. The magnitude of the isocitrate induced spectral change was greater in the luteal mitochondria from saline treated animals (Δ absorbance 424-390 nm 0.0163/mg protein) compared to the mitochondria from cycloheximide treated animals (Δ absorbance 424-390 nm 0.0061/mg protein). Cycloheximide treatment therefore had a similar effect on the difference spectra induced by pregnenolone or isocitrate with rat luteal mitochondria. Spectra induced by malate isocitrate and pregnenolone with adrenal cortex mitochondrial cytochrome P450 have been interpreted to be due to displacement or metabolism of substrate cholesterol from a high spin complex with the cholesterol side chain cleavage enzyme (see chapter 5). The components and spectral properties of adrenal cortex and luteal mitochondrial cholesterol side chain cleavage systems have been shown to be very similar (McIntosh et al (1971), (1973); Jefcoate et al (1973); Simpson et al (1973); Sulimovici and Boyd (1968b); see also chapter 5). Thus it is suggested that the cycloheximide injection produced a fall in the pregnenolone induced inverted type I difference spectrum of rat luteal mitochondrial cholesterol side chain cleavage cytochrome P450; this could be due to a decrease in the amount of cholesterol bound to the enzyme. This change occurred even in the presence of increased amounts of cholesterol in the mitochondria of cycloheximide treated animals (table 7.1). Cycloheximide treatment had presumably blocked the synthesis of a rapidly turning over protein (Hermier et al (1971); (1972a,b)) which

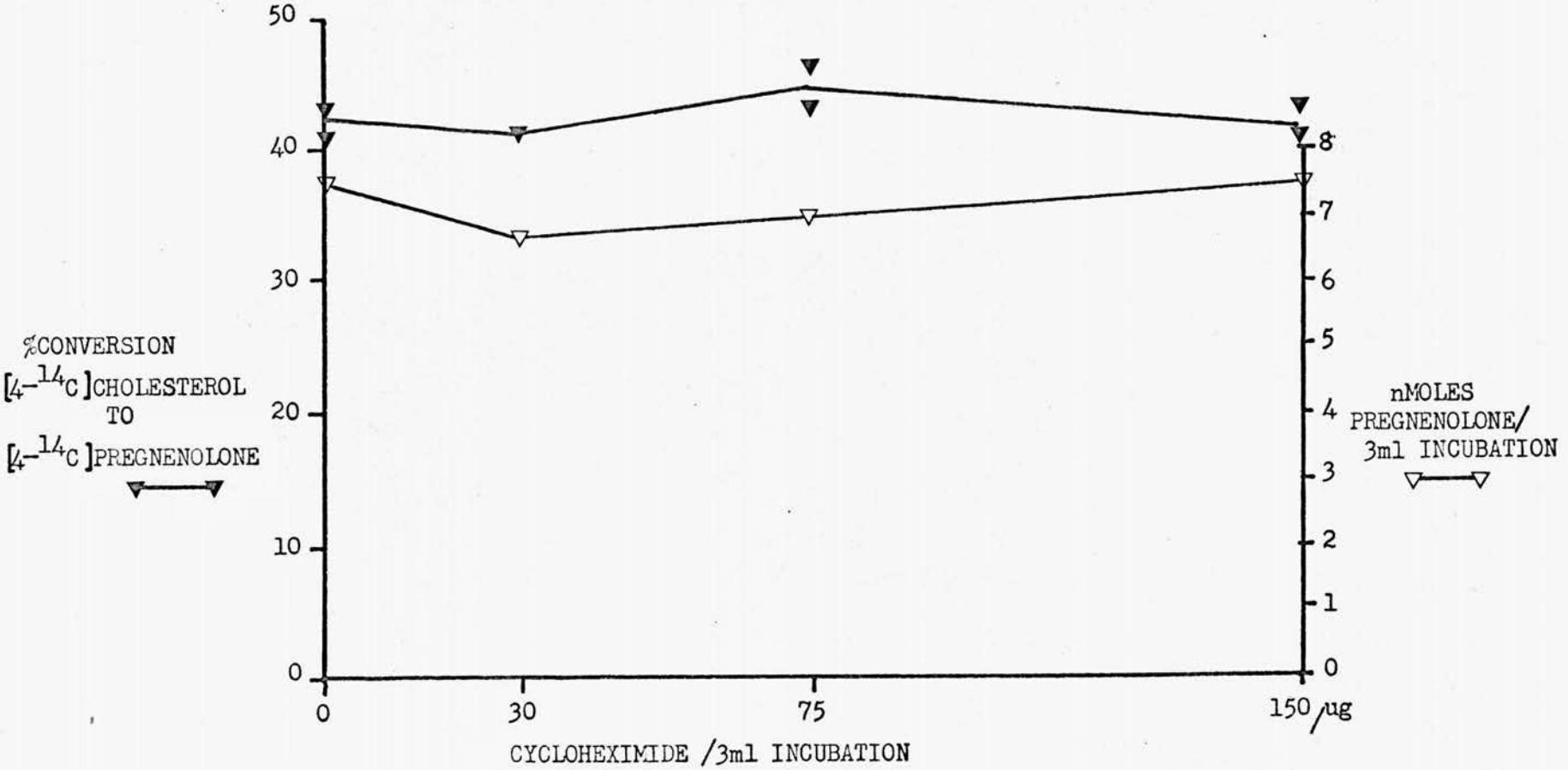


Figure 7.9. The effect of cycloheximide, added to incubations of luteal mitochondria, on cholesterol side chain cleavage activity. Side chain cleavage was measured in 10 minute incubations as described in chapter two. For the radioactive assay the protein concentration was 1.2 mg/ml. In the incubations to measure total pregnenolone the protein concentration was 0.8 mg/ml.

may influence the distribution of cholesterol within the luteal mitochondria. This labile factor could participate in the movement of cholesterol from the mitochondrial membrane to the cholesterol side chain cleavage cytochrome P450.

Cycloheximide could have exerted its effects on luteal steroidogenesis other than by inhibition of the synthesis of a rapidly turning over protein. It was possible that cycloheximide treatment had altered the supply of electrons to the cholesterol side chain cleavage cytochrome P450 or that cycloheximide had had a direct effect on cytochrome P450.

The effect of *in vitro* additions of cycloheximide on luteal mitochondrial cholesterol side chain cleavage

Figure 7.9 shows the effect of addition of cycloheximide in incubations on cholesterol side chain cleavage in rat luteal mitochondria. Cycloheximide in a concentration of up to 0.17 mM (150 µg/3mls) had no effect on the rate of cholesterol side chain cleavage measured as actual pregnenolone production or % conversion of [4-^{14}C] cholesterol to [4-^{14}C] pregnenolone. The amount of cycloheximide used for *in vitro* incubations was calculated on the assumption that all the chemical injected into the animal distributed evenly around the body of the rat. The ovaries constituted $< 0.2\%$ of the rat body weight therefore if they absorb 0.2% of the cycloheximide injected (5 mg), this would have been 10 µg/rat. The mitochondria used in an incubation came from 8 ovaries. Therefore assuming all the absorbed cycloheximide was carried through to the mitochondria 40 µg of the antibiotic would be found in an incubation. This calculation is of

Table 7.2

The effect, on luteal mitochondrial cholesterol side chain cleavage activity, of giving rats different doses of cycloheximide twenty minutes before killing

Dose cycloheximide/ rat	% conversion 10 mins	ratio % conversion untreated / % conversion treated
0	38	3.45
1 mg	11	
0	26	3.42
2.5 mg	7.4	
0	34.5	3.00
5 mg	11.4	

The percentage conversion of $[4\text{-}^{14}C]$ cholesterol to $[4\text{-}^{14}C]$ pregnenolone was measured in luteal mitochondria from rats which had been treated with cycloheximide or 0.9% saline 20 minutes before being killed.

course very speculative. To find the amount of cycloheximide absorbed by the ovary a ^{14}C label could be attached to the antibiotic. The amount of ^{14}C label in the ovary could then have been determined. In separate experiments lower amounts of cycloheximide were injected into the rats (table 7.2). It appeared that at all doses of cycloheximide tested (1-5 mg/rat) inhibition of cholesterol side chain cleavage activity measured by the radioactive assay was observed. If cycloheximide was exerting a direct effect on cholesterol side chain cleavage it would be expected that at higher doses it would cause a greater inhibition of the reaction. This argument would not apply if 1 mg injections of cycloheximide had already caused a maximal inhibition of steroidogenesis by a direct effect.

Cycloheximide ($<$ 0.2 mM) did not bind to luteal mitochondrial cytochrome P450, nor did it alter the magnitude of pregnenolone induced difference spectra when added to mitochondrial suspensions. While it was possible it did not seem likely that a metabolite of cycloheximide had been produced within six minutes which could have directly inhibited cholesterol side chain cleavage by 50% (figure 7.1). In the adrenal cortex, cycloheximide treatment inhibited the cholesterol side chain cleavage reaction but had no effect on another reaction dependent on cytochrome P450, namely the 11 β -hydroxylation of deoxycorticosterone (Garren et al (1971); Simpson et al (1972)). If a metabolite of cycloheximide was directly inhibiting one cytochrome P450 dependent reaction it was likely it should inhibit another reaction catalysed by a similar enzyme complex.

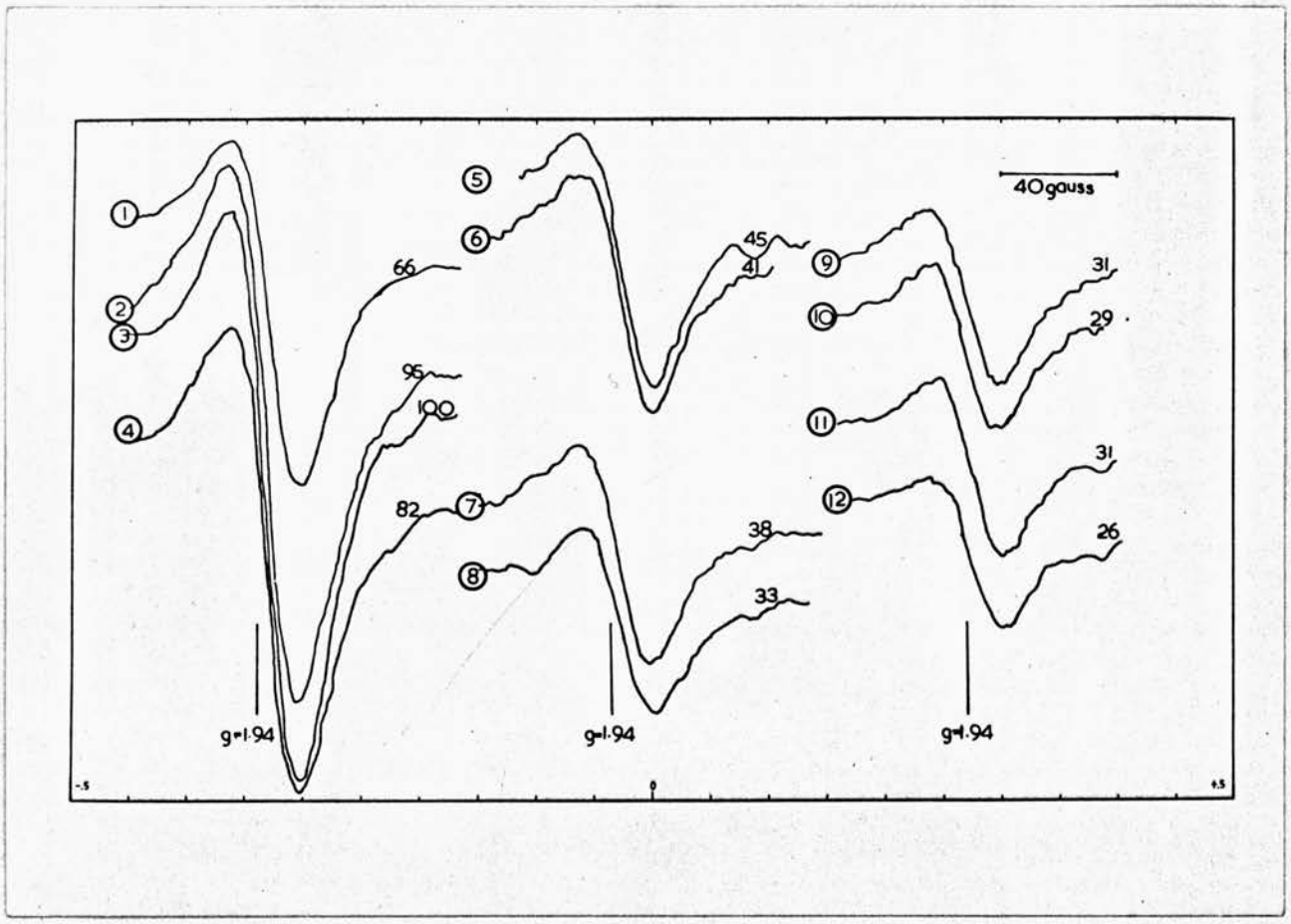


Figure 7.10. The E.P.R. spectra of luteal mitochondria from rats treated with cycloheximide or 0.9% saline twenty minutes before being killed. All spectra measured at gain of 4×10^3 and $-172^{\circ}C$ except (1) recorded at gain of 2×10^3 other conditions were similar to those used to record Figure 5.8. Small letters refer to the magnitude of the spectra (peak to trough), therefore (1) has a value of 132 for purposes of comparison with the other spectra. Experiments were carried out as described in the text. Protein concentrations were; 1 and 2, 3.8 mg/ml; 3 and 4, 3.7 mg/ml; 5 and 6, 1.5 mg/ml; 7 and 8, 1.4 mg/ml; 9, 10, 11 and 12, 1.1 mg/ml.

The effect of cycloheximide treatment on the luteal mitochondrial ovarian ferredoxin

It was also possible that cycloheximide may have affected the supply of NADPH and hence electrons to the cytochrome P450 involved in the cholesterol side chain cleavage reaction. This effect could have occurred via direct inhibition or decreased synthesis of the enzymes involved in NADPH generation. The ovarian ferredoxin which participates in the supply of electrons to the cholesterol side chain cleavage enzyme gives EPR signals at $g = 1.94$ and $g = 2.02$ when the protein is reduced (see chapter 5). The effect of cycloheximide treatment on the EPR signals of ovarian ferredoxin was therefore investigated (figure 7.10). Only the $g = 1.94$ signals are shown as the amplitude of this signal measured the amount of reduced ovarian ferredoxin (Estabrook et al (1973b) and chapter 5). The total amount of luteal mitochondrial ovarian ferredoxin was not decreased by cycloheximide treatment of rats twenty minutes before removal of the corpora lutea. Figure 7.10 nos. 1,3,5,7,9 and 11 show the $g = 1.94$ EPR signal obtained with the fully reduced luteal mitochondria from saline and cycloheximide treated animals. There appeared to be a slightly enhanced signal in the luteal mitochondria from cycloheximide treated animals (figure 7.10 nos. 1 and 5) than was observed from saline treated animals (figure 7.10 nos. 3 and 7). In another experiment similar $g = 1.94$ signals were obtained with both groups of mitochondria (figure 7.10 nos. 9 and 11). It was also possible to investigate the extent of reduction of the ovarian ferredoxin during pregnenolone production in rat luteal mitochondria. Luteal mitochondria from the same preparations in which total iron sulphur protein

had been determined were preincubated in medium A for 10 minutes before addition of malate. One to one and a half minutes after the malate had been added, the rate of pregnenolone production in these mitochondria was maximal (see figures 7.2 and 7.3). Samples of the incubations were therefore taken after this time and rapidly frozen in EPR tubes. The $g = 1.94$ signals of these samples are shown in figure 7.10 nos. 2,4,6,8,10 and 12. The sizes of the signals obtained in luteal mitochondria from saline treated animals were 81%, 84% and 87% of the signal obtained on maximum reduction (figure 7.10 nos. 4,8 and 12). Those obtained from cycloheximide treated animals were 94%, 79% and 91% of the maximal signal (figure 7.10 nos. 2,6 and 10). Therefore it seemed there was no constraint in the supply of reducing equivalents from NADPH reaching the mitochondrial iron sulphur proteins in rats which had received prior treatment with cycloheximide. It is likely therefore that cycloheximide did not affect NADPH formation during the oxidation of malate. If cycloheximide or a metabolite had a direct effect on the supply of reducing equivalents for the luteal mitochondrial cytochrome P450, it would have had to be on the transfer of electrons from the ovarian ferredoxin to cytochrome P450. This probably did not occur as cycloheximide had no effect on cholesterol side chain cleavage activity in vitro (figure 7.9). Thus cycloheximide treatment of rats did not appear to prevent the supply of NADPH and reducing equivalents to the luteal mitochondrial cholesterol metabolising enzymes. The major effect of cycloheximide treatment was to inhibit the synthesis of a rapdily turning over factor necessary for the binding of cholesterol to the cholesterol side chain cleavage

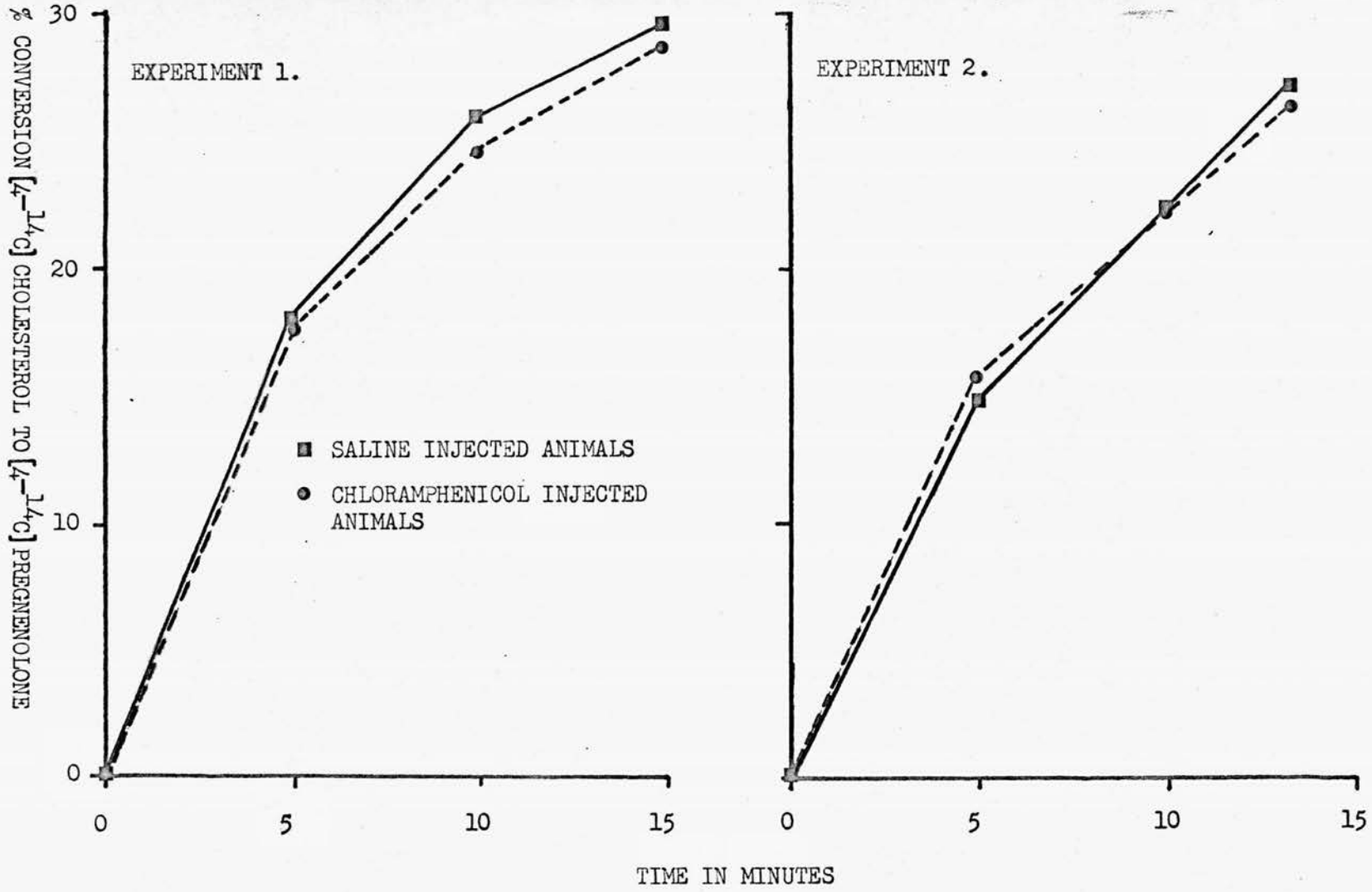


Figure 7.11. The effect of chloramphenicol injection on the conversion of [4-14C] cholesterol to [4-14C] pregnenolone in rat luteal mitochondria. Conversion of [4-14C] cholesterol to [4-14C] pregnenolone was determined as described in chapter 2. In experiment 1 protein concentrations were 1.2 mg/ml in each case. Rats were injected with chloramphenicol 3 hours before being killed. In experiment 2 protein concentrations were 1.1 mg/ml in each case. Rats were injected with chloramphenicol 3 and 10 hours before being killed.

Table 7.3

The effect of chloramphenicol injection 3 hrs or 10 hrs before killing on rat ovarian mitochondrial cholesterol and cytochrome P450

	Saline treated animals		Chloramphenicol treated animals	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
Mitochondrial cholesterol n Moles/mg protein	21.50±0.78	22.74±0.24	21.1±0.55	22.07±0.32
Mitochondrial cytochrome P450 n Moles/mg protein	0.46	0.46	0.43	0.47

Rats were injected once with chloramphenicol 3 hours (experiment 1) before killing or twice with chloramphenicol 10 and 3 hours (experiment 2) before killing. Mitochondria were prepared from the ovaries of these animals and free cholesterol and cytochrome P450 was determined in them. Results expressed as Mean ± SEM for six cholesterol determinations. Cytochrome P450's are expressed as mean of duplicate determinations.

cytochrome P450 of luteal mitochondria. This factor could be a protein (Hermier et al (1971); (1972a,b)) and it may participate in the redistribution of cholesterol within the mitochondria. The fact cycloheximide is able to cause such a large inhibition of luteal mitochondrial pregnenolone production suggested there was quite a considerable amount of endogenous gonadotrophin stimulating steroid production in the ovaries of the rats used in these investigations.

The effects of injections of chloramphenicol on the cholesterol side chain cleavage enzymes of rat corpus luteum

The rapidly turning over factor described above appeared to exert its effects on pregnenolone production intra-mitochondrially. An inhibitor of mitochondrial protein synthesis was therefore tested for its effects on luteal mitochondrial cholesterol side chain cleavage activity. Rats were injected intra-muscularly with chloramphenicol, an inhibitor of mitochondrial protein synthesis (Huang et al (1966); Ashwell and Work (1970)). The ovaries were removed from the rats three hours after the chloramphenicol injection, this time allowed the inhibitor to reach maximum concentrations in the blood (De Jonge and Hulsmann (1973)). No difference could be detected in the rate of luteal mitochondrial cholesterol side chain cleavage, using the radioactive assay, in the mitochondria from treated and untreated animals (experiment I, figure 7.11). The luteal cholesterol and cytochrome P450 levels were the same in both groups of mitochondria (Table 7.3). Similar results were obtained when rats were given two injections of chloramphenicol ten and three hours before killing (experiment 2, figure 7.11 and table 7.3). It has been reported that

chloramphenicol can inhibit the synthesis of rat adrenal cortex mitochondrial cytochrome P450 (Purvis et al (1973)) when injected for several days. However chloramphenicol injection appeared to have no short term effects on this superovulated system. It is concluded from these observations that it is possible that a rapidly turning over protein factor located in the mitochondrion but of cytoplasmic origin in the rat corpus luteum is involved in the cholesterol side chain cleavage reaction. Chloramphenicol has also been shown to be ineffective in inhibiting steroidogenesis in the adrenal cortex (Garren (1968); Rubin et al (1973)). This led these authors to believe that cytoplasmic rather than mitochondrial protein synthesis was important in the control of steroid production in the adrenal cortex.

The effect of puromycin injection and puromycin in vitro on the cholesterol side chain cleavage enzymes of rat luteal mitochondria

Puromycin, an inhibitor of protein synthesis, has been widely used in the study of steroidogenesis in the corpus luteum and adrenal gland (Marsh and Savard (1966b); Marsh et al (1966); Gorski and Padnos (1966); Ferguson (1963)). It was therefore of interest to see if treatments of rats with this antibiotic had any effect on the luteal mitochondrial cholesterol side chain cleavage enzymes. When rats were treated with puromycin (8 mg/rat) no inhibition of cholesterol side chain cleavage in isolated mitochondria could be detected. The total cytochrome P450 and cholesterol content of the luteal mitochondria were also unaffected by the puromycin treatment

Table 7.4

The effect of puromycin injection of rats on the cholesterol levels, cytochrome P450 levels and the cholesterol side chain cleavage activity of luteal mitochondria

	saline treated animals	puromycin treated animals
Mitochondrial cholesterol n moles/mg protein	13.8 ± 0.5	14.2 ± 0.4
	mean ± SEM 4 determinations	
% conversion of [4-^{14}C] cholesterol to [4-^{14}C] pregnenolone (10 minute incubations)	31.6%	33%
Mitochondrial cytochrome P450 n moles/mg protein	0.42	0.42

Rats were injected with puromycin (8mg) or 0.9% saline twenty minutes before being killed. Their luteal mitochondria were prepared and the above values measured as described in chapter 2.

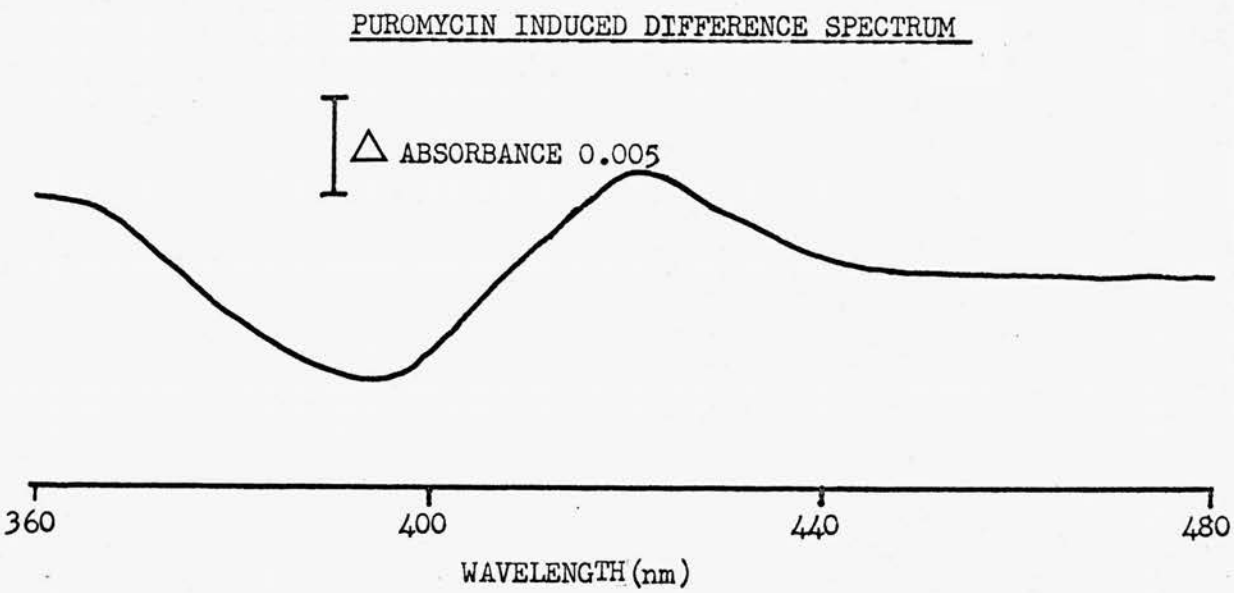


Figure 7.12. Puromycin induced difference spectrum of rat luteal mitochondria. Saturating amounts of puromycin (3.5 mM) were added to the sample cuvette dissolved in a small volume of incubation medium and an equal volume of medium was added to the reference cuvette (Protein concentration 2 mg/ml).

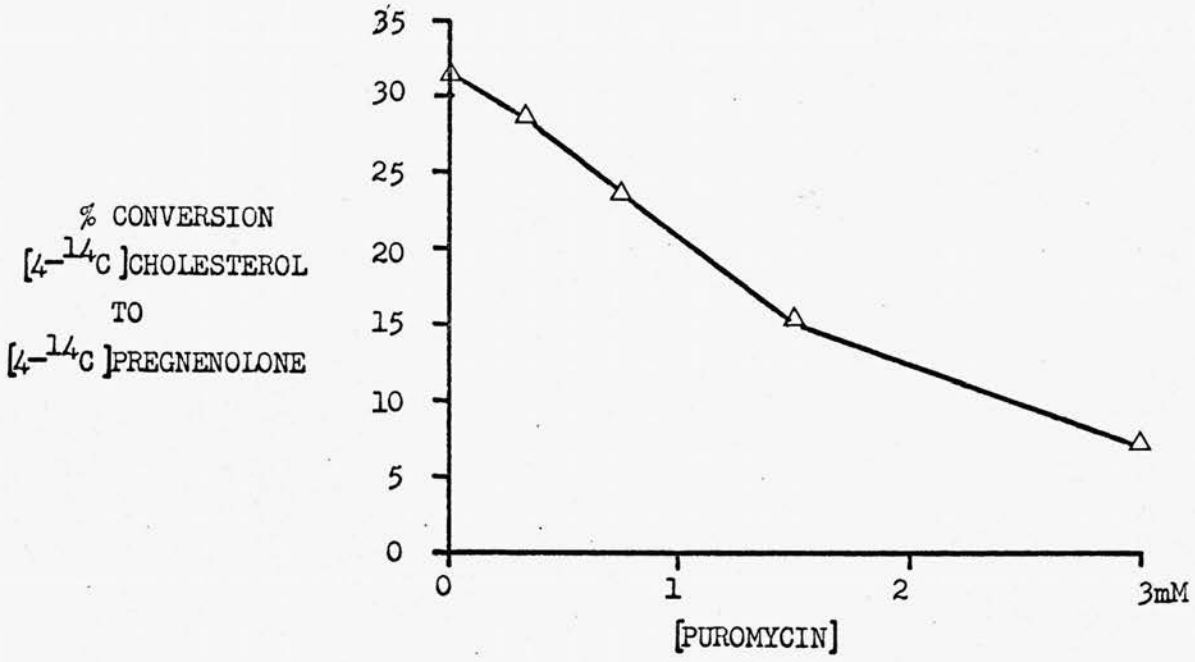


Figure 7.13. Inhibition of luteal mitochondrial cholesterol side chain cleavage in vitro by the addition of puromycin. The percentage conversion of [4-^{14}C] cholesterol to [4-^{14}C] pregnenolone was measured in 10 minute incubations. Results are the average of duplicate assays, protein concentrations were 1.1 mg/ml.

(table 7.4). Greater doses of puromycin were not used as this compound has been shown to bind to liver microsomal cytochrome P450 to give a type II difference spectrum (Soliman and Wade (1972)). Puromycin was found to bind to luteal mitochondrial cytochrome P450 to produce a type II difference spectrum (figure 7.12). The binding constant for this interaction was 0.7 mM using Δ absorbance 422 - 394 nm as a measure of binding. As shown in figure 7.13 puromycin also directly inhibited production of $[4\text{-}^{14}C]$ pregnenolone in mitochondrial incubations. 1.5 mM Puromycin in medium A caused a 50% inhibition of $[4\text{-}^{14}C]$ pregnenolone production; a similar concentration to that which produced the cytochrome P450 binding spectrum. It is therefore difficult to draw conclusions that puromycin can affect luteal steroid production through inhibition of protein synthesis rather than by a direct effect on the cholesterol side chain cleavage enzymes. Kowal (1970) has shown that puromycin is very toxic to adrenal cortex cells in culture, whereas cycloheximide is not. Rubin *et al* (1973) showed cycloheximide inhibition of adrenal steroidogenesis was reversible; inhibition by puromycin was not. Thus puromycin is probably unsuitable for use in the investigation of the role of protein synthesis in steroidogenesis as it can directly affect the steroid metabolising enzymes; an effect cycloheximide does not appear to have.

Table 7.5

and

The effects of cycloheximide, luteinizing hormone on cholesterol metabolism and cytochrome P450 in rat luteal mitochondria

	Pretreatment			
	0.9% Saline	Luteinizing Hormone	Cycloheximide	Luteinizing Hormone and Cycloheximide
Mitochondrial Cholesterol n Moles/mg protein Mean ± S.E.M.(3 determinations)	17.23 ± 0.79	16.9 ± 0.2	25.6 ± 1.73	29.06 ± 1.37*
Mitochondrial cytochrome P450 n Moles/mg protein	0.38	0.36	0.32	0.36
Pregnenolone binding spectrum	16.5	12.7	7.0	5.6 +
△Absorbance (424-385nm)x 10^3/ mg protein	18.0	22.0	6.0	6.0
Mitochondrial pregnenolone production	2.30	3.82	1.20	1.06*
nMoles/mg protein/10 minute	2.40	3.80	1.10	1.41
Mitochondrial pregnenolone production in the presence of 100 µM cholesterol	3.30	6.12	1.54	1.20*
n Moles/mg protein/10 minute	sample lost	5.0	1.40	1.20

* determinations from the same experiment. + determinations from different experiments.

The effects of luteinizing hormone and cycloheximide on cholesterol levels, cholesterol metabolism and cytochrome P450 in rat luteal mitochondria.

The effect of treating rats with cycloheximide and luteinizing hormone at the same time was investigated. Rats received injections of cycloheximide (5 mg/rat in 0.2 mls of 0.9% saline) or luteinizing hormone (40 µg/rat in 0.2 mls 0.9% saline) or both forty five minutes before being killed. Control animals received injections of 0.9% saline. The luteinized ovaries were removed from the rats and the mitochondria prepared from them. Cholesterol side chain cleavage activity, cholesterol content, cytochrome P450 and pregnenolone binding spectra were determined in the mitochondria of all groups (table 7.5). As found previously cycloheximide or luteinizing hormone treatment had no effect on total mitochondrial cytochrome P450. No change in total cytochrome P450 was observed when both treatments were administered. Luteinizing hormone produced a small rise in the magnitude of the luteal mitochondrial pregnenolone inverted type I difference spectrum in one experiment while in another experiment it gave a small decrease. Cycloheximide treatment produced a 55% to 65% decrease in the magnitude of the difference spectrum. This decrease also occurred when cycloheximide and luteinizing hormone were given in combination. These changes in the difference spectra were reflected in the cholesterol side chain cleavage activity of the four treatment groups of mitochondria. Pregnenolone production was measured in the mitochondria in 10 minute incubations in the presence of malate. Luteinizing hormone produced a 50% increase in pregnenolone production compared to the saline injected controls

whereas cycloheximide treatment produced a 50% inhibition in the activity. In the presence of extra cholesterol (100 μ M) in the incubations cycloheximide still produced an inhibition of pregnenolone production compared to saline injected controls. The luteal mitochondria from rats treated with luteinizing hormone showed a greater ability to metabolise the exogenous cholesterol compared to the mitochondria of saline injected controls. This phenomenon has already been discussed in chapter 6. The administration of cycloheximide, at the same time as the luteinizing hormone, prevented any effect of the hormone on the cholesterol side chain cleavage activity. The pregnenolone production in the mitochondria of rats receiving luteinizing hormone and cycloheximide was very similar to that found in the rats treated with cycloheximide alone. The accumulation of cholesterol in the isolated luteal mitochondria observed after cycloheximide treatment (table 7.5) was enhanced by a combination cycloheximide and luteinizing hormone treatment (table 7.5). This could have been due to the luteinizing hormone treatment which stimulates the luteal cytoplasmic cholesterol esterase (chapter 8). This cholesterol esterase liberates cholesterol from cholesterol esters in the lipid droplets in which it is stored in the luteal cell cytoplasm (Armstrong and Flint (1973)). The cholesterol thus liberated could not be rapidly metabolised in the mitochondria because of the cycloheximide treatment hence it accumulated. The cytoplasmic cholesterol esterase was insensitive to cycloheximide treatment (chapter 8) unlike the cholesterol side chain cleavage reaction. Thus cycloheximide treatment could overcome the effects of luteinizing hormone on luteal mitochondrial cholesterol side chain

cleavage. The effect of cycloheximide is probably to prevent the synthesis of a rapidly turning over factor which controls the rate of cholesterol side chain cleavage by altering the binding of cholesterol to cytochrome P450. This factor could be protein but the results did not rule out the possibility it could be some other molecule.

Summary

(a) Cycloheximide treatment of rats has been shown to inhibit the luteal mitochondrial cholesterol side chain cleavage system. This effect is very rapid, 50% inhibition of the enzymes occurred within six minutes of cycloheximide treatment.

(b) This inhibition of cholesterol side chain cleavage has been shown to be due to the inability of the substrate cholesterol to bind to the cytochrome P450 which converts it to pregnenolone. The reduced binding of cholesterol to cytochrome P450 in cycloheximide treated animals was demonstrated using isocitrate and pregnenolone induced difference spectra.

(c) Cycloheximide had no direct effect on the cholesterol side chain cleavage enzymes of isolated rat luteal mitochondria. Supply of NADPH to the luteal mitochondria appeared to be unchanged by cycloheximide treatment.

(d) Chloramphenicol, an inhibitor of mitochondrial protein synthesis, did not appear to affect luteal steroidogenesis.

(e) Puromycin has been shown to bind to luteal mitochondrial cytochrome P450 and directly inhibit the cholesterol side chain cleavage reaction.

(f) Cycloheximide treatment has been shown to prevent luteinizing hormone induced changes in the activity of luteal mitochondrial cholesterol side chain cleavage enzymes.

(g) These results (a) - (f) have been discussed in terms of a rapidly turning over protein factor which influences the binding of cholesterol to the luteal mitochondrial cytochrome P450.

CHAPTER 8

SOME PROPERTIES OF RAT LUTEAL CYTOPLASMIC CHOLESTEROL ESTERASE

Introduction

The work presented in chapters three to seven has been concerned with the luteal mitochondrial cholesterol side chain cleavage enzymes and how they are controlled. It has been shown that the supply of cholesterol to the enzymes may be an important step in the regulation of steroid hormone production. Cholesterol for steroidogenesis probably comes from the cholesterol esters stored in the lipid droplets of the luteal cell cytoplasm (see chapter 1). The cholesterol is liberated from the cholesterol esters by the enzyme cholesterol esterase. This enzyme has been demonstrated to be present in corpora lutea. Coutts and Stansfield (1968) found the activity resided in a 5000 x g pellet of a luteal homogenate. However, Behrman and Armstrong (1969) found the activity mainly in a 100,000 x g supernatant fraction of rat luteal tissue. This is similar to the distribution of the enzyme in rat adrenal tissue (Shima et al (1972); Trzeciak and Boyd (1973)). The luteal supernatant cholesterol esterase is stimulated by LH treatment of the rats, one hour before they are killed (Behrman and Armstrong (1969); Armstrong and Flint (1973)). The enzyme, however, appears to be insensitive to a direct effect of cyclic AMP (Behrman and Armstrong (1969); Flint et al (1973)). It has been shown recently that cyclic AMP per se will not appreciably stimulate cholesterol esterase activity in rat adrenal cortex 100,000 x g supernatant (Trzeciak and Boyd (1973)). However, it was demonstrated

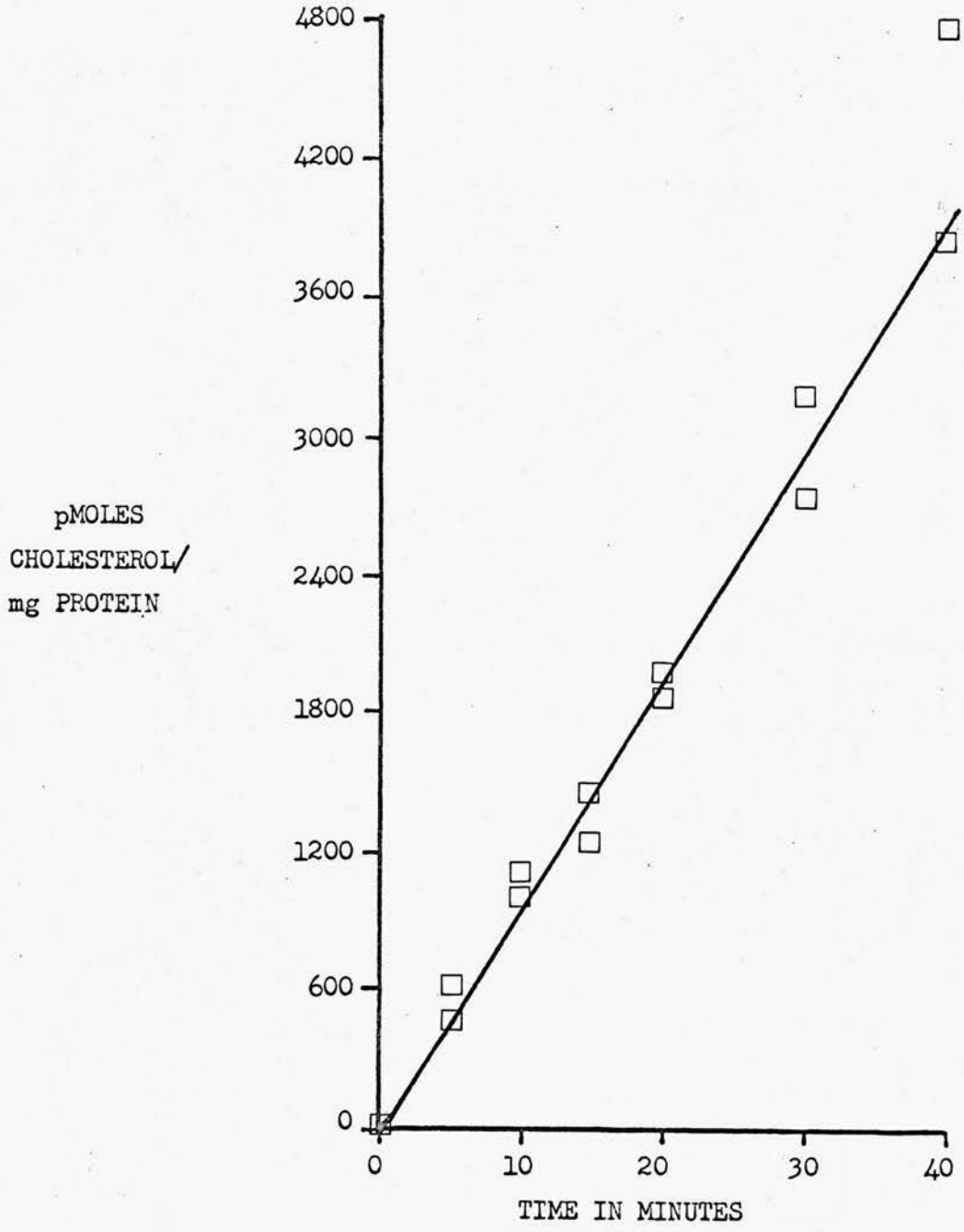


Figure 8.1. The time course of cholesterol appearance from cholesterol oleate catalysed by luteal cholesterol esterase. The assay was carried out as described in chapter 2 except that the time of incubation was varied.

that in the presence of ATP, cyclic AMP could stimulate cholesterol esterase activity (Trzeciak and Boyd (1973)). This effect was thought to be dependent on a cyclic AMP stimulated protein kinase, which activated the cholesterol esterase by phosphorylation (Trzeciak and Boyd (1974)). This kinase is activated by the removal of a regulatory protein which binds cyclic AMP (Gill and Garren (1971)). Cyclic AMP binding proteins have been isolated from rat corpus luteum homogenate 100,000 x g supernatant (Scott and Stansfield (1974a,b)). Also protein kinases have been purified from rat and bovine corpora lutea (Menon (1973); Webb and Stansfield (1974)). The kinases were found to be activated by 10^{-6}M cyclic AMP. The cytoplasmic cholesterol esterase in rat corpus luteum was investigated to establish how its activity could be altered in vivo by hormonal treatment of the animals or modified in vitro by addition of metal ions and adenine nucleotides.

General properties of the luteal cholesterol esterase

Cholesterol esterase activity was determined as described by Trzeciak and Boyd (1973) (see chapter 2). Figure 8.1 shows the time course of the appearance of cholesterol from cholesterol oleate incubated with a 100,000 x g supernatant fraction of luteal homogenate. The supernatant was first passed through a Sephadex G-25 column to remove low molecular weight compounds such as ATP and cyclic AMP. This procedure was carried out on the supernatant fractions used in all the experiments reported in this chapter. As can be seen from the graph (figure 8.1) cholesterol was released at a linear rate

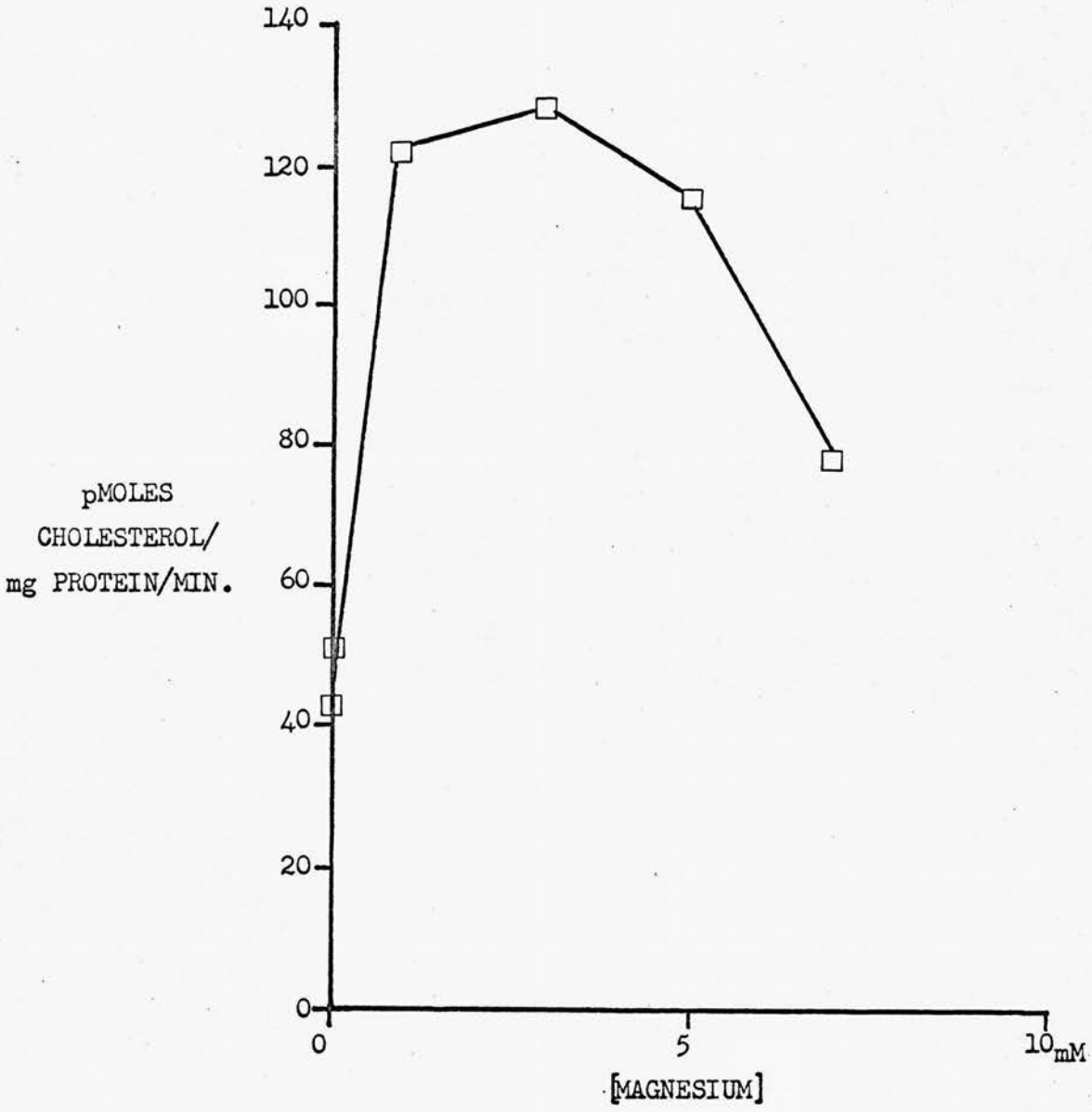


Figure 8.2. The effect of different concentrations of magnesium ions on the activity of rat luteal cholesterol esterase. The assay was carried out as described in chapter 2 except that the magnesium concentration was varied.

during forty minutes incubation. Figure 8.2 shows the effect of different concentrations of magnesium ions on cholesterol esterase activity in desalted 100,000 x g supernatant from luteal homogenates. Magnesium ions in the incubation medium (1-5 mM) produced a large stimulation of cholesterol esterase activity. 5 mM Magnesium was therefore included in incubations for the determination of cholesterol esterase activity. Shown in table 8.1 are the cholesterol esterase activities obtained on the addition of different cofactors to a preparation of luteal 100,000 x g supernatant. 5 mM Magnesium ions caused a 100% stimulation of esterase activity. 5 mM ATP caused a 50% stimulation of esterase activity. These stimulations appeared to be additive when magnesium and ATP were incubated together. 1 μM Cyclic AMP did not produce a stimulation of esterase activity in the presence of ATP with and without magnesium.

The effect of cycloheximide injection twenty minutes before killing or rat luteal cholesterol esterase activity

Cycloheximide injection of rats, which inhibited luteal mitochondrial cholesterol side chain cleavage activity, appeared to have no effect on luteal cholesterol esterase activity (table 8.2). The stimulation of esterase activity by 5 mM ATP was also unaffected. Cyclic AMP did not appear to cause any stimulation of activity in the presence or absence of ATP.

Table 8.1

Activators of rat luteal cholesterol esterase

Additions	Activity
	p mol/min/mg protein
None	48
5 mMATP	76
1 μM cAMP, 5 mMATP	75
5 mM Mg^{++}	106
5 mMATP/Mg^{++}	136
1 μM cAMP, 5 mMATP/Mg^{++}	116

Cholesterol esterase was assayed as described in chapter 2 except magnesium ions were omitted from the incubation medium; other additions are shown on the table. Incubations were carried out in duplicate.

Table 8.2

The effect of cycloheximide and saline injection twenty minutes before killing on the activity of rat luteal cholesterol esterase

Additions	Activity	
	Cycloheximide	saline
	p mol/min/mg protein	
None	62	54
1 μM cAMP	63	49
5 mMATP	122	115
1 μM cAMP, 5mMATP	134	118

Cholesterol esterase was assayed as described in chapter 2. Incubations were carried out in duplicate. Additions to the incubations are shown on the table.

Table 8.3

The effect of LH injection one hour before killing on the activity of rat luteal cholesterol esterase

Additions	Activity	
	LH	Saline
	p mol/min/mg protein	
None	33	14
1 μM cAMP	32	16
5 mMATP	65	49
1 μM cAMP 5 mMATP	73	65

Cholesterol esterase was assayed as described in chapter 2. Incubations were carried out in duplicate. Additions to incubations are shown on the table.

Table 8.4

The effect of injections with LH and cycloheximide, LH, cycloheximide and saline, forty five minutes before killing, on the activity of rat luteal cholesterol esterase

Additions	Activity			
	Cycloheximide	Saline	LH	LH/Cycloheximide
	p mol/min/mg protein			
None	17	15	34	30
5 $mMMg^{++}$	86	53	53	88
1 μM cAMP	17	9	40	29
5 $mMATP/Mg^{++}$	95	57	100	95
1 μM cAMP 5 $mMATP/Mg^{++}$	107	63	95	112

Cholesterol esterase was assayed as described in chapter 2, except magnesium ions were omitted from the incubation medium, other additions are shown on the table. Incubations were carried out in duplicate.

The effect of LH injection one hour before killing on the activity of rat luteal cholesterol esterase

Cholesterol esterase activity in luteal homogenate 100,000 x *g* supernatant was stimulated two fold by treatment of rats with LH, one hour before killing (table 8.3). This confirms the observations of Behrman and Armstrong (1969) and Armstrong and Flint (1973). It is interesting to note that ATP can still stimulate esterase activity in supernatant from the LH and saline injected animals. 1 μM Cyclic AMP did not affect esterase activity when added alone but it did appear to cause a small stimulation when added with ATP to the 100,000 x *g* supernatant from saline injected animals. In the presence of cofactors, the esterase activity was almost as great in the 100,000 x *g* supernatant from the saline injected animals as it was in the preparation from LH injected animals.

The effect of injections with LH/cycloheximide, LH, cycloheximide and saline forty five minutes before killing on the activity of rat luteal cholesterol esterase and the ratio of cholesterol/ cholesterol esters in the luteal lipid droplets

Cycloheximide treatment did not affect cholesterol esterase activity in luteal homogenate 100,000 x *g* supernatant (table 8.2) and LH treatment caused a stimulation of activity (table 8.3). Cycloheximide treatment did not, however, prevent the stimulatory effect of LH on cholesterol esterase (table 8.4); unlike the prevention of the trophic hormone effect on cholesterol side chain cleavage activity in luteal mitochondria (table 7.5). The effects of cycloheximide and LH treatment of rats on cholesterol esterase activity and

Table 8.5

The effect of injections with LH and cycloheximide, LH, cycloheximide and saline, forty five minutes before killing, on the ratio of cholesterol/cholesterol esters in the lipid droplets of the luteal cell.

Pretreatment	ratio cholesterol/cholesterol esters
Saline	0.014
LH	0.027
Cycloheximide	0.030
LH/Cycloheximide	0.044

The cholesterol and cholesterol ester content of lipid droplets were determined as described in chapter 2.

cholesterol side chain cleavage activity were reflected in the ratios of cholesterol to cholesterol esters observed in the lipid droplets (table 8.5). Administration of LH to rats forty five minutes before killing caused a doubling of the ratio of cholesterol/cholesterol esters in the luteal lipid droplets. This was probably due to the stimulation of cholesterol esterase activity. The increased cholesterol side chain cleavage activity of luteal mitochondria caused by LH treatment (figure 6.3) must not have been great enough to deplete the cholesterol levels in the lipid droplets, to below the levels seen in saline injected animals. Cycloheximide treatment of animals also caused an increase in the ratio of cholesterol/ cholesterol ester in the luteal lipid droplets. As cycloheximide treatment did not affect cholesterol esterase activity the inhibition of the cholesterol side chain cleavage reaction produced by the antibiotic must have caused cholesterol to accumulate in the lipid droplets. The greatest increase in the ratio cholesterol/cholesterol esters was seen when rats were treated with LH and cycloheximide. In this case the LH treatment would cause increased hydrolysis of the cholesterol esters in the lipid droplets and cycloheximide treatment would prevent the metabolism of the free cholesterol released. The combination of these two factors thus resulted in the greatest increase in free cholesterol in the lipid droplets.

Conclusions

These investigations are in agreement with previously published results which show that LH treatment of rats causes a stimulation in the 100,000 x g supernatant luteal cholesterol esterase activity (Behrman and Armstrong (1969); Armstrong and Flint (1973)). This activation may involve an effect of ATP or magnesium ions on the esterase enzyme (see figure 8.2 and table 8.1). ATP could activate cholesterol esterase via an allosteric effect. A protein kinase, such as the one which has been postulated to be involved in the activation of adrenal cortex cholesterol esterase (Trzeciak and Boyd (1974)), would also require ATP to activate the luteal cholesterol esterase. In the adrenal cortex the kinase is thought to be activated by cyclic AMP. No conclusive effect of cyclic AMP on luteal cholesterol esterase activity could be demonstrated. This may mean that cyclic AMP is not involved in the activation of the luteal cholesterol esterase. On the other hand a 0.1% contamination of the 5mM ATP, used in the experiments, with cyclic AMP may maximally stimulate the enzyme. Any further addition of cyclic AMP would then have no effect. The stimulatory effect of the magnesium ions on luteal cholesterol esterase activity is difficult to explain, especially as magnesium has an inhibitory effect on bovine adrenal cortex cholesterol esterase (G. Beckett personal communication). Magnesium ions may have a direct action on the luteal esterase or stimulate a protein kinase. Cycloheximide treatment of rats did not affect the luteal 100,000 x g supernatant cholesterol esterase. The cycloheximide sensitive factor involved in LH activation of cholesterol side chain cleavage is probably not involved in the

activation of cholesterol esterase. Thus the LH induced activity changes in the two enzymes are controlled by differing mechanisms.

Summary

(a) Cholesterol esterase activity in the 100,000 x g supernatant of rat luteal homogenates is stimulated by ATP and magnesium ions but apparently not by cyclic AMP.

(b) LH treatment stimulates and cycloheximide treatment has no effect on the luteal cholesterol esterase activity. Cycloheximide treatment of rats does not prevent LH induced changes in luteal cholesterol esterase activity.

(c) LH treatment of rats results in an increase in the release of free cholesterol from cholesterol esters in the luteal cytoplasmic lipid droplets. Thus cholesterol can then be used for steroid hormone production.

CHAPTER 9

GENERAL DISCUSSION

The object of the studies described in this thesis was to investigate certain aspects of the acute control of steroidogenesis in the corpus luteum by LH. The steroidogenic effect of LH is not its only effect on the corpus luteum. LH has been shown to increase the blood flow through the gland (Ellis (1961); Wurtman (1964)). The development and the long term maintainence of the corpus luteum may also depend on the effects of LH (see Savard (1973)). LH has been shown to stimulate the synthesis of RNA and proteins in the ovary (Reel and Gorski (1968); McKerns (1974); Jarlstedt *et al* (1973)). This effect may be mediated through stimulation of the enzyme ornithine decarboxylase by the trophic hormone (Kobayashi *et al* (1971); Kaye *et al* (1973); Maudsley and Kobayashi (1974)). Ornithine decarboxylase catalyses the first step in the formation of the polyamines spermine and spermidine which stabilise RNA and proteins in the cell. The LH induced stimulation of RNA and protein synthesis in the ovary is probably important in the development of the corpus luteum and the maintainence of its structure.

For the investigation of luteal steroidogenesis the rat was chosen as the experimental animal. This choice allowed *in vivo* treatment of the animal with hormones before the investigation of the steroidogenic enzymes of isolated luteal mitochondria. This procedure would have been inconvenient and impractical when dealing with the corpora lutea of larger animals such as sheep, pigs and cows. In

these species investigations of the effects of trophic hormones on luteal steroidogenesis have usually been conducted under *in vitro* conditions. Addition of the trophic hormones have been made to homogenates or tissue slices of the corpus luteum. In the case of tissue slices the circulation of blood to the gland is stopped with the result that the cells may tend to be anaerobic. There is also a problem of the penetration, by diffusion, of the hormones to all the cells of the tissue slice. Enzymes released from damaged cells are another problem of tissue slice experiments. These problems can be eliminated by making free cell preparations of luteal tissue. This method has been used by Sayers *et al* (1971) in studies of the effects of LH on the bovine corpus luteum.

Although the use of the rat as experimental animal makes it possible to administer trophic hormones *in vivo* there are also disadvantages. The ovary of the rat is very small ($<$ 20mg) and it may contain corpora lutea of up to three different ages (Hilliard (1973)). Thus it is difficult to obtain enough rat luteal tissue on which to perform experiments. However treatment of immature rats with pregnant mare-serum gonadotrophin and human chorionic gonadotrophin results in a massive formation of corpora lutea in the ovary (Parlow (1958)). The ovaries in one rat now weigh up to 300 mg and are over 95% corpora lutea (Channing and Villee (1966)). The formation of the corpora lutea is induced artificially before the rat has started a normal oestrus cycle. Hence the Parlow or superovulated rat is a convenient source of large numbers of similar corpora lutea. These corpora lutea actively produce progesterone and appear to be normal in all respects (Armstrong (1968)). The luteinized ovaries of

the Parlow rat respond to treatment of the animals with LH. The trophic hormone can be shown to cause the depletion of the levels of ascorbic acid and cholesterol in the luteinized ovary (Parlow (1958); Bell et al (1964, 1965); Clark and Zarrow (1968)). In vivo HCG or LH treatment also causes an increase in the blood concentration of progesterone in the superovulated rat (Behrman et al (1972); Major et al (1967); Rao and Saxena (1973); Rao and Carman (1973)). It was hoped in this study to investigate the mechanism by which LH achieved this effect.

The importance of the cholesterol side chain cleavage reaction in the control of steroidogenesis is now well established (Chapter 1 pages 15 and 23). It was therefore useful to determine the activity of this enzyme and to investigate the mechanism of its control by trophic hormones. The cholesterol side chain cleavage enzyme is assumed to be in the mitochondria of steroidogenic cells (review Sulimovici and Boyd (1969b)). Therefore luteal mitochondria were prepared and investigated before any attempt was made to establish how steroidogenic activity was affected by trophic hormones. It was essential to have a preparation of mitochondria which was well characterised and appeared to be intact. The reason for this was that many factors affect the activity of the cholesterol side chain cleavage complex. Leakage of NADP(H) from the mitochondria during preparation could decrease the supply of reducing equivalents for the cholesterol side chain cleavage reaction. Ions such as calcium and magnesium can have a large effect on adrenal cortex cholesterol side chain cleavage activity (Simpson et al (1974)). Mechanical disturbances such as ultrasonication can alter adrenal mitochondrial pregnenolone production and the spin state of cytochrome P450 (Jefcoate et al (1974)).

The mitochondrial preparation used in these studies appeared to be intact using criteria defined by Chance and Williams (1956). They exhibited good respiratory control ratios with several Krebs cycle intermediates and in the absence of any electron donor endogenous oxygen uptake was very low. The ADP/O ratios calculated for the various Krebs cycle intermediates (table 4.1) were close to theoretical values which is a further indication of the mitochondrial intactness. The inability of the mitochondria to measurably oxidise added NADPH or NADH in incubations suggests that the inner mitochondrial membrane had not been disrupted during their preparation. Normally the inner mitochondrial membrane is almost impermeable to pyridine nucleotides (Vingradov et al (1972)). The buffer medium, protein concentrations and Krebs cycle intermediate concentrations in the oxygen electrode studies were the same as those which were used in the studies of cholesterol metabolism. Hence the luteal mitochondria used in this study were tightly coupled under the conditions of incubation.

The assay of cholesterol side chain cleavage played an important part in this study. Previous studies of mitochondrial cholesterol side chain cleavage have relied on the measurement of the percentage conversion of ^{14}C or ^{3}H cholesterol to radioactive products (Simpson and Boyd (1967); Sulimovici and Boyd (1968a); Robinson and Stevenson (1971a); McIntosh et al (1971)). This has not allowed the absolute rate of the reaction to be measured. Another difficulty in the use of this method is that in comparing steroidogenesis in different preparations of mitochondria it is essential that the cholesterol pool size in each be identical. The assumption also has to be made that the radioactive cholesterol is able to completely equilibrate with

each pool of mitochondrial cholesterol. In the measurement of the rate of cholesterol side chain cleavage another difficulty is encountered in that two or sometimes three steroid products are formed in incubations (Simpson and Boyd (1967b); Sulmovici and Boyd (1968a)). This difficulty can be overcome by the use of cyanoketone; so that pregnenolone is the only steroid product of side chain cleavage. Cyanoketone prevents further metabolism of pregnenolone by inhibition of the 3 β-hydroxysteroid dehydrogenase (Goldmann et al (1967)) while pregnenolone can be conveniently assayed in mitochondrial incubations (Simpson et al (1971), (1972)).

Inclusion of cyanoketone in incubations of luteal mitochondria, with [4 -^{14}C] cholesterol and malate, resulted in [4 -^{14}C] pregnenolone being the only steroid product of side chain cleavage (figure 3.5, table 3.1). In the absence of cyanoketone [4 -^{14}C] progesterone was the major product, only small amounts of [4 -^{14}C] pregnenolone could be found. These large changes in the amount of [4 -^{14}C] pregnenolone did not seem to affect the rate of side chain cleavage (figure 3.5). Cyanoketone was therefore included in all incubations in which cholesterol side chain cleavage activity was being measured. This greatly simplified the assay as only one product had to be measured.

When malate was added to incubations of luteal mitochondria at the same time as [4 -^{14}C] cholesterol, [4 -^{14}C] pregnenolone appeared at a linear rate for up to half an hour. When the [4 -^{14}C] cholesterol was preincubated for 10 minutes with the mitochondria before the addition of malate [4 -^{14}C] pregnenolone was formed rapidly for 5 - 10 minutes and then the rate declined (figure 3.1). This preincubation effect took about seven minutes to become maximal (figure 3.2). Although the [4 -^{14}C] cholesterol had to be equilibrated with the

mitochondria for seven minutes it was found to be bound or adsorbed to them within 1-2 minutes of being added to incubations (figure 3.3). The fact that the [4 -^{14}C] cholesterol had equilibrated with the endogenous mitochondrial cholesterol was confirmed by comparing the percentage conversion of total steroid and radioactive steroid to products in incubations (table 3.2). After pre-incubation the time course of conversion of [4 -^{14}C] cholesterol to [4 -^{14}C] pregnenolone (figure 3.1) was very similar to the time course of the appearance of pregnenolone from endogenous cholesterol in mitochondrial incubations (figure 3.4). It was therefore possible to use a radioactive assay for cholesterol side chain cleavage as long as the tracer [4 -^{14}C] cholesterol was first preincubated with the mitochondria. It was convenient to use the radioactive assay as it was more sensitive than the absolute assay for pregnenolone. Radioactive assays could be performed in 0.5 ml incubations with 0.5 mg of protein. Measurement of total pregnenolone in incubations required at least 2 mg protein. The radioactive assay, however, could be used to compare cholesterol side chain cleavage activity within one group of mitochondria. Activity is then expressed as a percentage conversion; if two preparations of mitochondria had differing cholesterol pool sizes comparison of percentage conversions as an indication of a rate would be misleading (see figures 6.7 and 7.2).

During incubations luteal mitochondrial cholesterol esters appeared not to be depleted and therefore were probably not a source of cholesterol for pregnenolone production (table 3.2). Depletion of mitochondrial free cholesterol (n moles/mg protein) mirrored exactly the appearance of pregnenolone (n moles/mg protein) in incubations

(table 3.2, figure 3.4). Free cholesterol therefore is probably the immediate substrate for cholesterol side chain cleavage in rat luteal mitochondria.

Although the cholesterol side chain cleavage enzyme complex has generally been thought to be mitochondrial in the corpus luteum (review Sulimovici and Boyd (1969a)) recent reports have suggested that the enzyme may also be present in the microsomes (Flint and Armstrong (1971a, b), (1972)). No evidence could be obtained for microsomal cholesterol side chain cleavage activity in the preparations used in these studies.

The pH optimum of the mitochondrial cholesterol side chain cleavage enzyme was found to be fairly broad and centred on pH 7.4 (figure 3.6). The activity of the enzyme is independent of protein concentration above 0.75 mg/ml. Below this protein concentration $NADP^+$ may leak from the mitochondria and thus become rate limiting in steroidogenesis (figure 3.7).

The incubation medium for optimal cholesterol side chain cleavage activity was found to be 250 mM sucrose, 20 mM KCL, 15 mM triethanolamine hydrochloride, 10 mM potassium phosphate, 5 mM $MgCl_2$, 0.2 mM EDTA and 0.1% BSA pH 7.4. The effects of variation of each of the components of the incubation medium are discussed in chapter 3. Medium A was used in spectral, oxygen electrode and cholesterol metabolism studies with luteal mitochondria.

In incubations of luteal mitochondria the rate of production of pregnenolone began to fall off after about five minutes (figures 3.1 and 3.4). This could be corrected for by addition of cholesterol to the incubations in an acetone solution (figure 4.1). The cholesterol side chain cleavage in the presence of extra cholesterol, continued at a linear rate for at least thirty minutes (figure 4.1). Under

incubation conditions described in this thesis, supply of cholesterol to the cholesterol side chain cleavage enzymes would appear to be important. Supply of other substrates for the cholesterol side chain cleavage reaction could also have been rate limiting in steroidogenesis. Alterations in the supply of NADPH has often been proposed as a mechanism by which trophic hormones exert their effects on steroidogenesis (see Chapter 1 page 16 and Chapter 3 page 71). It was for this reason that the levels of reduced iron sulphur protein were investigated in luteal mitochondria during steroidogenesis. This iron sulphur protein is involved in the transfer of electrons from the NADPH flavoprotein dehydrogenase to the cytochrome P450 (Omura et al (1966); Sulimovici and Boyd (1968b); Baron (1973); see figure 1.3). In incubations of luteal mitochondria, 1-2 minutes after the addition of malate or isocitrate, the rate of pregnenolone production is operative at the maximum rate. The demand for NADPH should be maximal at this time. When the amount of reduced iron sulphur protein was measured under these conditions of steroidogenesis it was found to be 66% - 80% of the total amount seen on dithionite reduction of the mitochondria (figures 5.9, 5.10, 6.8 and 7.10). Thus, even when cholesterol side chain cleavage is occurring at maximal rates, there appears to be a large excess of reduced iron sulphur protein available for steroidogenesis. It appears therefore that NADPH supply does not limit the rate of steroidogenesis under these incubation conditions. Any limit in the supply of electrons to cytochrome P450 would have to occur during their transfer from the iron sulphur protein (see fig. 1.3).

The supply of oxygen for cholesterol side chain cleavage in incubations was not investigated. The Km for the 7 α -hydroxylation of cholesterol, a cytochrome P450 dependent reaction, is 20 μM

(Boyd et al (1973)) this is far below the oxygen concentration in medium A, which was found to be 250 μM (Chapter 4).

In incubations therefore availability of cholesterol to the enzyme appears to be important in the maintainence of cholesterol side chain cleavage activity. In some incubations up to 70% of the mitochondrial cholesterol appeared to be converted into pregnenolone (figures 3.1, 6.2 and 7.2). This conversion was not exceeded even on prolonged incubation. Thus, it may be that only $<70\%$ of the total mitochondrial cholesterol is available for steroidogenesis. The other 30% of the cholesterol may play a purely structural role in the mitochondrion. When labelled cholesterol was added to the mitochondria it appeared to equilibrate with all the cholesterol present (table 3.2). It is therefore possible that all the mitochondrial cholesterol may play a structural and a functional role. While the steroid is a substrate for steroidogenesis it may also act in maintaining the structure of the mitochondrial membrane. It is probably important in vivo that the cholesterol is replaced in the membrane as soon as it is metabolised. When the mitochondrial cholesterol has been depleted to the extent of 70% the membrane may be affected in such a way as to prevent the formation of any more pregnenolone. However when more cholesterol is added to mitochondria which have stopped producing pregnenolone from endogenous steroid, cholesterol side chain cleavage activity restarts (figure 4.4 and 4.7). In the experiment shown in figure 4.7 the depletion of endogenous cholesterol must have affected the mitochondrial membrane, because the cholesterol added after 30 minutes could not be metabolised until $NADP^+$ had been added to the incubations. The cholesterol in the

membranes of liver mitochondria appear to contribute towards their stability (Graham and Green (1970)). Cholesterol has been shown to have a similar stabilising effect on liposomes which have been made artificially with phospholipids (Demel et al (1968); De Gier et al (1968)). Cholesterol depletion of the luteal mitochondrial membrane may therefore have decreased its stability allowing $NADP^+$ to escape and fall below levels necessary to maintain a supply of electrons for cholesterol side chain cleavage. In vivo a large depletion of luteal mitochondrial cholesterol should not occur. The cytoplasmic LH sensitive cholesterol esterase liberates free cholesterol from the cholesterol esters stored in the cytoplasmic lipid droplets (Armstrong and Flint (1973)). This cholesterol may then be available for steroidogenesis. Free cholesterol liberated from lipid droplet stores of cholesterol ester can probably equilibrate with all the membranes of the cell. This could account for the observation that depletion of cholesterol from esters in ovarian cells on LH stimulation far exceeds subsequent progesterone production (Solod et al (1966)). The hormonal stimulation would have to liberate enough cholesterol to go into the mitochondrial and other cellular membranes. Only the cholesterol in the mitochondria would be available for steroidogenesis. Some of the cholesterol released from the lipid droplets may be lost from the cell into the plasma. Such a loss of cholesterol has been reported on LH stimulation of leydig tumour cells (Moyle et al (1973a)).

Cytochrome P450 an essential component of the cholesterol side chain cleavage enzyme system was found to be present in the luteal mitochondria at a concentration of 0.41 ± 0.02 n moles/mg protein. The cholesterol side chain cleavage cytochrome P450 of the rat luteal mitochondria was found to have similar properties to the cytochrome

from adrenal cortex and sheep luteal mitochondria (see Simpson et al (1973); Jefcoate et al (1973), (1974)). The rat luteal mitochondria gave an inverted type I difference spectrum with pregnenolone. This type of spectrum, also observed with preparations of adrenal cortex mitochondrial cholesterol side chain cleavage cytochrome P450 indicates a high to low spin transition of the cytochrome P450 (Jefcoate and Boyd (1971)). The magnitude of the spectrum depends on the amount of high spin cytochrome P450 initially present (Jefcoate et al (1973), (1974)). The high spin form of cytochrome P450 is thought to be due to it complexing with substrate, in luteal mitochondria this would be cholesterol (see chapter 5). The magnitude of the pregnenolone induced difference spectrum varied with the pH of the buffer the luteal mitochondria were suspended in. This pH dependent change in spin state is a property unique to the cholesterol side chain cleavage cytochrome P450. It is not displayed by forms of cytochrome P450 involved in other reactions (Jefcoate et al (1973), (1974)). Changes in pH have been shown to alter the configuration of liver mitochondrial subparticles (Wrigglesworth and Packer (1971)). Localised pH changes in luteal cells could bring about conformational changes in the mitochondria. This could then cause an alteration of the spin state of the cholesterol side chain cleavage cytochrome P450, which is in the mitochondrial membrane. The rate of cholesterol side chain cleavage appears to depend on the amount of high spin cytochrome P450 in mitochondria (Simpson et al (1972) and see Chapters 6 and 7). Thus LH stimulation of luteal cells would increase steroidogenesis via a pH change induced around the mitochondrial membrane, which would cause an increase in the amount of high spin cytochrome P450. When the properties of the luteal mitochondrial cholesterol side chain

cleavage system were established it was then possible to investigate how hormonal stimulation could alter the enzymic activity.

LH or HCG treatment of rats, 15 minutes or 1 hour before killing, was shown to cause a stimulation of the luteal mitochondrial cholesterol side chain cleavage activity (figures 6.3 and 6.5). In some cases this stimulation could not be observed, in incubations of the mitochondria, until extra cholesterol was added. However after LH treatment of the rats even if the luteal mitochondria produced less pregnenolone from endogenous cholesterol they have potentially an increased cholesterol side chain cleavage activity. The increased activity of the cholesterol side chain cleavage system does not appear to be due to an increased supply of electrons from NADPH (figure 6.8). The LH treatment one hour before killing did not alter the total levels of iron sulphur protein in the luteal mitochondria nor did it appear to increase the amount of reduced iron sulphur protein observed during steroidogenesis in incubations.

The supply of cholesterol to the side chain cleavage enzymes appeared to be the most important factor in LH induced changes of pregnenolone production. The isolated mitochondria from LH treated animals had less cholesterol in them but under certain conditions were capable of producing more pregnenolone. This lowered level may have represented a decrease in the steady state levels of mitochondrial cholesterol on LH stimulation. The cholesterol side chain cleavage enzymes becoming more active could metabolise the cholesterol more quickly thus lowering the quantity of steroid in the mitochondrion. The cholesterol would not disappear from the mitochondria due to increased supplies of cholesterol from the lipid droplets (Chapter 8).

The diminished cholesterol levels in luteal mitochondria, from animals treated with LH for one hour, could reflect a lowered progesterone output by the corpus luteum. Steroidogenesis in the sheep corpus luteum becomes insensitive to LH stimulation after the hormone has been infused into the gland for half an hour. This was suggested to be due to depletion of cholesterol necessary for steroidogenesis (Collet et al (1973)). Whether the reduced levels of luteal mitochondrial cholesterol were due to increased turnover of the cholesterol side chain cleavage enzymes or due to a depletion in the supply of substrate cholesterol could be resolved. Measurement of progesterone output by the rat corpus luteum in vivo, in response to prolonged LH stimulation, would show the length of the steroidogenic response.

The ability of the luteal mitochondria to metabolise cholesterol at an increased rate in response to LH stimulation could have been due to increased synthesis of the cholesterol side chain cleavage enzymes. However as shown in tables 6.2 and 6.3 and figure 6.8 this probably does not happen. LH had probably induced an activation of the cholesterol side chain cleavage enzymes. This was reflected in the magnitude of the inverted type I pregnenolone binding spectrum observed in the luteal mitochondria from LH treated animals in comparison to those from animals receiving saline injections (figures 6.6 and 6.7). This increased binding spectrum, due to LH treatment could only be observed when the mitochondria had been preincubated with cholesterol before the addition of pregnenolone. The pregnenolone inverted type I binding spectrum is thought to measure the high spin ferric cytochrome P450 cholesterol side chain cleavage complex (Simpson et al (1972); Jefcoate et al (1973), (1974)). It appears

that the luteal mitochondrial cholesterol side chain cleavage cytochrome P450 from LH treated animals has a greater ability to form an active enzyme substrate complex than does the cytochrome P450 from saline treated animals. Formation of this high spin cholesterol complex is a necessary step in the cholesterol side chain cleavage reaction prior to the reduction of cytochrome P450 (Simpson et al (1971)). Thus an increased ability to form the complex represents an increased ability to metabolise cholesterol.

The mechanism whereby LH enhances the binding of cholesterol to luteal mitochondrial was studied using inhibitors of protein synthesis. Inhibitors of protein synthesis have been shown to prevent LH stimulation of steroidogenesis in the corpus luteum (Marsh et al (1966); Hermier et al (1971), (1972a,b)). Hermier et al (1971) suggested that a rapidly turning over protein caused the redistribution of cholesterol in the luteal cell. This redistribution was thought to be the transfer of cholesterol into the mitochondrion. Administration of cycloheximide to the superovulated rats caused a rapid decrease in the cholesterol side chain cleavage activity of luteal mitochondria (figure 7.1). This decrease in activity was not associated with lower levels of ovarian ferredoxin or cytochrome P450 in the mitochondria (table 7.1; figure 7.10). The supply of electrons to the luteal mitochondrial cholesterol side chain cleavage system did not seem to be affected (figure 7.10). The cycloheximide treatment did however decrease the amount of cholesterol bound to the luteal mitochondrial cytochrome P450 even though there was an increase in the levels of cholesterol in the mitochondria (table 7.1). Presumably the cycloheximide treatment inhibited the cholesterol side chain cleavage activity, in luteal

mitochondria, which was maintained by endogenous gonadotrophins. The treatment of rats with cycloheximide did not affect luteal cytoplasmic cholesterol esterase activity (table 8.2 and 8.4). This coupled with the fact there was more cholesterol in the mitochondria of cycloheximide treated rats suggests that any factor involved in the control of steroidogenesis by LH works at the mitochondrial level rather than at the level of transfer of cholesterol into the mitochondria. The "rapidly turning over" cycloheximide sensitive factor influences the binding of cholesterol to cytochrome P450. It may cause a redistribution of mitochondrial cholesterol to allow the steroid to bind to cytochrome P450 or it could act directly on the cytochrome P450 to increase its capacity to bind steroid. It is very difficult to distinguish between these two possibilities. Treatment with cycloheximide inhibits endogenous cholesterol side chain cleavage activity in luteal mitochondria and also prevents LH induced changes in luteal mitochondrial pregnenolone production (table 7.5). As shown in table 7.5 the mitochondrial levels of cholesterol in rats treated with LH and cycloheximide are raised above the levels of those treated with cycloheximide alone. The cholesterol side chain cleavage enzymes in the mitochondria were inhibited by cycloheximide treatment of the rats and therefore could not metabolise the increased supply of cholesterol from the stimulated cholesterol esterase. This is also apparent when the ratio of cholesterol/cholesterol ester in the lipid droplets is examined (table 8.5). There is a greater increase in the ratio in animals which had been treated with LH and cycloheximide rather than LH alone. Thus LH can cause a release of free cholesterol in the luteal cell. This free cholesterol could not be metabolised when the rats had been pre-treated with cycloheximide,

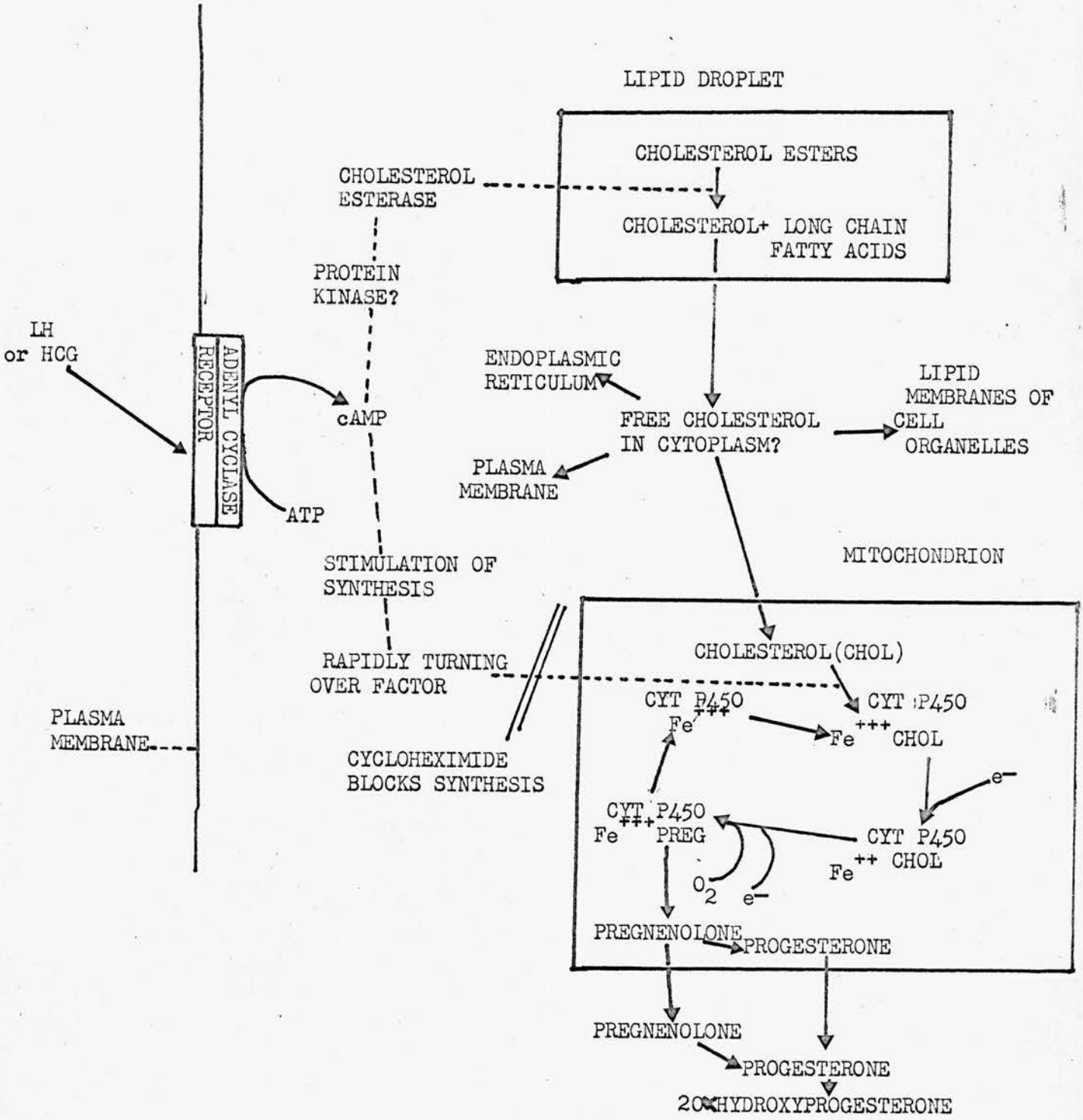


Figure 9.1. A proposed mechanism for the stimulation of steroidogenesis by LH and HCG in the rat corpus luteum.

and thus it accumulated in the lipid droplets and in the mitochondria.

The information presented in this thesis and previously published work suggest a mechanism for the acute effect of LH on steroid production in the luteal cell. This is summarised in figure 9.1. Other possible effects of LH on the cell are not shown.

LH may bind to the plasma membrane of the cell at specific receptor sites. It seems that HCG will also bind to the luteal cell at these sites (Channing and Kammerman (1974); Koch et al (1974)). This causes an activation of adenyl cyclase which in turn results in the conversion of ATP to cyclic AMP. The increased levels of cyclic AMP may then mediate the actions of LH on steroidogenesis. In testis and adrenal tissue it has been shown that stimulation of steroidogenesis with very low doses of trophic hormone can occur without any detectable change in the concentrations of cyclic AMP in the cell (Rommerts et al (1973); Beall and Sayers (1972); Moyle and Ramachandran (1973); Rao and Saxena (1973)). This may mean that cyclic AMP does not have a direct role in stimulation of steroidogenesis or alternatively that changes in cyclic AMP concentration necessary to stimulate steroidogenesis can not be detected using conventional techniques. It must be remembered however that administration of cyclic AMP or dibutyryl cyclic AMP to animals and tissue slices can mimic the effects of trophic hormone stimulation on steroidogenesis (e.g. Savard et al (1966); Hermier and Jutisz (1969); Mahaffee et al (1974)). The raised levels of cyclic AMP in the cell may cause the stimulation of luteal cytoplasmic cholesterol esterase observed on LH treatment of rats (Behrman and Armstrong (1969) and Chapter 8). The stimulated cholesterol esterase can then cause the release of more cholesterol

from the cholesterol stored in the lipid droplets of the cell. This cholesterol will then probably equilibrate with all the cholesterol in the lipid membranes in the cell and only a small portion of it becoming available to the mitochondrial side chain cleavage enzymes (See Chapter 1 pages 18-19). The activity of the luteal mitochondrial cholesterol side chain cleavage enzymes is also altered by LH treatment of the rats. This effect is probably mediated by a rapid turnover factor which could be a protein produced in the cell cytoplasm. (See figures 7.1 and 7.11; Garren et al (1965); Hermier et al (1971)). This factor affects the amount of cholesterol bound to cytochrome P450 in the luteal mitochondria (figures 6.6, 6.7, 7.5 and 7.8). This cholesterol is then available for metabolism to pregnenolone and progesterone. Thus the rapidly turning over protein allows cholesterol to bind to the side chain cleavage cytochrome P450 and in doing so causes an increased output of pregnenolone and progesterone. How LH and cyclic AMP induce or stimulate the synthesis of this factor is uncertain. Walton et al (1971) have shown that an ACTH stimulation of protein kinase in adrenal cortex cells can catalyse the phosphorylation of ribosomes. They postulate, this may be important in the stimulation of a rapidly turning over protein important in the steroidogenic response of adrenal cortex cells to ACTH. More recently in an abstract Caron et al (1974) have reported that phosphorylation of a cytochrome P450 fraction, of a reconstituted bovine corpus luteum mitochondrial cholesterol side chain cleavage system can cause stimulation of steroidogenesis. Cycloheximide does not prevent trophic hormone stimulation of cholesterol esterase which presumably occurs via a protein kinase. Thus phosphorylation of cytochrome

P450 may not be necessary for the mediation of the effects of trophic hormones reported in this thesis.

The acute stimulatory effect of LH on steroidogenesis in the rat luteal cell probably depends on two processes. Cholesterol esterase activity is increased in the cell cytoplasm and cholesterol side chain cleavage activity is stimulated in the mitochondria. The activation of the mitochondrial cholesterol side chain cleavage system depends on a rapidly turning over protein of extramitochondrial origin which enhances the binding of cholesterol to mitochondrial cytochrome P450.

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The Effect of Inhibitors of Protein Synthesis on Cholesterol Side-Chain Cleavage in the Mitochondria of Luteinized Rat Ovaries

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1. Conversion of cholesterol to pregnenolone, termed cholesterol side-chain cleavage, has been studied in isolated mitochondria from luteinized rat ovaries.

2. Cholesterol in these mitochondria in the presence of cyanoketone is converted to one product pregnenolone on addition of a suitable electron donor. The percentage conversion of total mitochondrial cholesterol to pregnenolone is comparable to that measured by the percentage conversion of [4-^{14}C]cholesterol to [4-^{14}C]pregnenolone when the label has been preincubated at 29 °C for 10 min with the mitochondria.

3. In mitochondrial incubations the depletion of endogenous cholesterol follows the production of pregnenolone. The initial rate of production of pregnenolone (0.8 nmol × mg protein^{-1} × min^{-1}) was maintained in incubations for 5–6 min. Addition of free cholesterol to incubations allowed the production of pregnenolone to continue at the initial rate for at least 30 min.

4. Treatment of rats with cycloheximide 20 min prior to killing, caused a 58% inhibition of luteal mitochondrial cholesterol side-chain cleavage and a small but significant rise in the cholesterol content of isolated mitochondria. The cycloheximide treatment did not affect the total luteal mitochondrial cytochrome *P*-450 but caused a 56% decrease in the inverted type I pregnenolone-binding spectrum, suggesting cholesterol binding to cytochrome *P*-450 had been reduced.

5. Rats treated with chloramphenicol 3 and 10 h before killing exhibited no differences in total luteal mitochondrial cytochrome *P*-450 or mitochondrial cholesterol metabolism when contrasted with saline-injected animals.

6. Puromycin was shown to bind to the rat luteal mitochondrial cytochrome *P*-450 to produce a type II difference spectrum and to inhibit luteal mitochondrial cholesterol side-chain cleavage *in vitro*.

7. The results are discussed in terms of a protein which is rapidly turning over and which may be synthesised extra-mitochondrially and in some way affect the binding of cholesterol to luteal mitochondrial cytochrome *P*-450.

The formation of pregnenolone as a result of the cholesterol side-chain cleavage reaction is thought to be the rate-limiting step in steroidogenesis in the corpus luteum [1–3]. This cleavage is carried out by a mitochondrial enzyme complex containing an NADPH-specific flavoprotein dehydrogenase, an iron-sulphur protein (ovarian ferredoxin) and cytochrome *P* 450 [4].

Abbreviations. Cholesterol, 5 cholesten-3β-ol; pregnenolone, 3β-hydroxypregn-5-en-20-one; progesterone, 4-pregnene-3, 20-dione.

Enzymes. Steroid 3β-ol dehydrogenase or 3β-hydroxy steroid NAD(P) oxidoreductase (EC 1.1.1.51); steroid Δ^4-Δ^5 isomerase or 3-ketosteroid Δ^4-Δ^5 isomerase (EC 5.3.3.1); cholesterol esterase or sterol-ester hydrolase (EC 3.1.1.13).

The source of reducing equivalents for these reactions is thought to be the oxidation of Krebs cycle intermediates such as malate and isocitrate [5,6] or the oxidation of long-chain fatty acids [7]. The role of protein synthesis in luteotropin-induced changes in steroidogenesis has been widely reported [8–10]. Hermier *et al.* [10] have suggested that a protein factor which is rapidly turning over is involved in the supply of cholesterol to the cholesterol metabolism enzymes of mitochondria from the corpus luteum. Recently Simpson *et al.* [11] have reported that in the adrenal gland an adrenocorticotropin-sensitive protein factor which is rapidly turning over [12] influences the binding or transport of cholesterol to the cytochrome *P*-450

of mitochondrial side-chain cleavage. This paper reports the influence of some inhibitors of protein synthesis on cytochrome *P*-450 and on the metabolism of cholesterol in isolated mitochondria of luteinized ovaries from the rat. Luteinized ovaries in immature rats pretreated by the method of Parlow [13] were used in this study. These animals are a convenient source of large quantities of metabolically active corpora lutea [14]. The rats being immature are small and easy to inject prior to the corpora lutea being removed for experiments. Study of cytochrome *P*-450 of cholesterol side-chain cleavage in corpus luteum mitochondria is very desirable as it is not complicated by the presence of other forms of cytochrome *P*-450 as it is in the adrenal gland [15].

MATERIALS AND METHODS

Animals and Pretreatment

Female rats of the Wistar strain obtained from The Small Animal Breeding Station (University of Edinburgh) were used in all experiments. Luteinized ovaries were obtained from these animals using the pretreatment procedure devised by Parlow [13]. Rats 21–24 days old were injected subcutaneously with pregnant-mare serum gonadotrophin (Gestyl-Organon) (50 IU) followed three days later by an injection of human chorionic gonadotrophin (Pregnyl-Organon) (25 IU) or a second injection of pregnant-mare serum gonadotrophin (50 IU) Sulimovici and Boyd [14]. Hormones were dissolved in 0.5 ml 0.9% saline for injection. The rats were used 5–7 days after the second injection of hormone. Ovarian weight varied from 120–150 mg.

Cycloheximide (5 mg/rat) and puromycin dissolved in 0.2 ml 0.9% saline were administered to the rats by intra-peritoneal injection. Control animals received similar injections of 0.9% saline. The animals were usually killed 20 min after the injection or as otherwise indicated in the results section.

Chloramphenicol (10 mg/rat) was administered by intra-muscular injection suspended in 0.2 ml 0.9% saline. The animals were killed 3 h later by which time the inhibitor had reached the maximum blood levels [16]. In experiments which involved the use of inhibitors of protein synthesis 25 rats were injected with the inhibitor and 25 rats were given injections of a 0.9% solution of saline as controls. Ovaries from each treated group were pooled and mitochondria prepared as described below.

Preparation of Luteal Mitochondria

Animals were killed by cervical dislocation, the ovaries removed by a dorsal approach, trimmed free of fat and immediately placed in ice-cold 0.25 M sucrose. The trimmed ovaries were homogenised at 4 °C in 5 vol. 0.25 M sucrose/g tissue using a Potter Elvehjem homogeniser with a teflon pestle. The homogenate was centrifuged at $650 \times g$ for 10 min at 4 °C in an M.S.E. 18 highspeed centrifuge to remove nuclei, erythrocytes and unbroken cells. The supernatant was centrifuged at $8500 \times g$ for 10 min to give the mitochondrial pellet. This pellet was resuspended in one half of the original homogenate volume of 0.25 M sucrose and centrifuged again at $8500 \times g$ for 10 min. This "washing" was repeated and the final mitochondrial pellet suspended in 0.25 M sucrose to give a protein concentration of 6–8 mg/ml.

Determination of Cholesterol Side-Chain Cleavage Activity

The buffer which was used for cholesterol metabolism studies of ovarian mitochondria was 250 mM sucrose, 20 mM KCl, 15 mM triethanolamine hydrochloride, 10 mM potassium phosphate, 5 mM $MgCl_2$, 0.1 mM EDTA and 0.1% bovine serum albumin (type F-Sigma) pH 7.4. Mitochondrial protein concentrations were 1–2 mg/ml. Incubations were performed at 29 °C and were 2 or 3 ml in volume.

Cholesterol metabolism was initiated using 5 mM L-malate; [4-^{14}C]cholesterol (60000 counts/min, 50 μCi/μmol) in 5 μl acetone was added either 10 min before the malate or at the same time. Cyanoketone (2α-Cyano-4, 4-17α-trimethyl-17β-hydroxy-5-androsten-3-one) which is an inhibitor of steroid 3β-ol dehydrogenase [17], was added to incubations to prevent the conversion of pregnenolone to progesterone. Incubations were stopped by the addition of 10 ml methanol. The percentage conversion of [4-^{14}C]cholesterol to [4-^{14}C]pregnenolone was measured as described by Simpson *et al.* [11]. Total cholesterol and pregnenolone were also measured in incubations using the methods described by Simpson *et al.* [11] for adrenal mitochondria.

Mitochondrial Cholesterol Esters

Mitochondrial cholesterol ester was measured after tracer amounts of [4-^{14}C]cholesterol oleate were added to incubations after all enzymic activity had been stopped with methanol. This method corrected for procedural losses during the isolation of the cholesterol esters. The solvent system used to separate steroids in the cholesterol side-chain cleavage assay (70 ml di-isopropyl ether : 30 ml light petroleum 60 °C/80 °C boiling fraction: 2 ml glacial acetic acid) [11] was used to isolate cholesterol esters which migrated with the solvent front. Cholesterol esters were eluted with acetone from the silica gel, dried under a stream

of nitrogen then hydrolysed at 37 °C for 3 h in 30% methanolic KOH. The reaction mixture was neutralized with conc. HCl and extracted with chloroform. The extract was subjected to thin-layer chromatography using the solvent system described above for the cholesterol side-chain cleavage assay. The cholesterol liberated from the ester was measured using gas-liquid chromatography [11].

Spectral Studies

Spectral measurements were made using an Aminco Chance split beam dual wavelength spectrophotometer. Mitochondrial cytochrome *P*-450 was measured using the method of Omura and Sato [18]. Pregnenolone-induced spectra were measured using methods described by Simpson *et al.* [11].

Puromycin hydrochloride difference spectra were obtained by adding the chemical dissolved in a small amount of the incubation medium to the sample cuvette. A similar volume of medium was added to the reference cuvette. The pH of the mitochondrial suspension was not altered by the addition of puromycin. All spectral measurements were made on mitochondria in the same buffer medium as used for incubations. Protein concentrations in these studies were 1 – 2 mg/ml.

Proteins

Protein determinations were carried out using the biuret method [19].

Chemicals, Radiochemicals and Hormones

All chemicals used were of the highest grades obtainable; solvents were redistilled before use. Puromycin dihydrochloride was obtained from Calbiochem (San Diego, California, U.S.A.), cycloheximide from Koch-Light (Colnbrook) and chloramphenicol from Parke Davis (London). Cyanoketone was a gift from Dr J. L. McCarthy (Southern Methodist University, Dallas, Texas). Radioactive steroids were purchased from the Radiochemical Centre (Amersham) and purified by thin-layer chromatography on silica gel before use.

RESULTS

The Cholesterol Side-Chain Cleavage System

When the mitochondrial preparation was incubated with 5 mM L-malate in the presence of cyanoketone, [4-^{14}C]cholesterol was added to the mitochondria in some experiments at the same time as the malate and in other experiments 10 min before the malate. The formation of [4-^{14}C]pregnenolone from the [4-^{14}C]cholesterol is shown in Fig. 1. When the labelled cholesterol was preincubated with the mitochondria for 10 min [4-^{14}C]pregnenolone was produced at a more rapid rate than that observed without preincubation. After 5 – 10 min the rate of production of [4-^{14}C]pregnenolone decreased.

The actual time course of pregnenolone formation in a typical mitochondrial incubation is shown in Fig. 2.

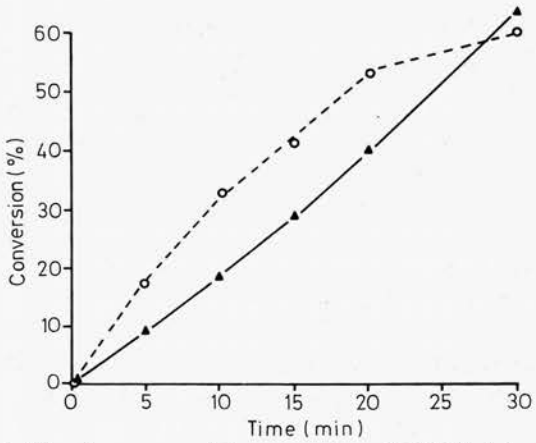

Fig. 1. *The time course of the conversion of [4-^{14}C]cholesterol to [4-^{14}C]pregnenolone in luteal mitochondria.* The reaction was initiated by adding 5 mM malate at time zero. Mitochondrial protein concentration was 1.3 mg/ml, cyanoketone 6.4 μM (▲) [4-^{14}C]cholesterol added at the same time as the malate; (○) [4-^{14}C]cholesterol preincubated with mitochondria 10 min before the addition of malate

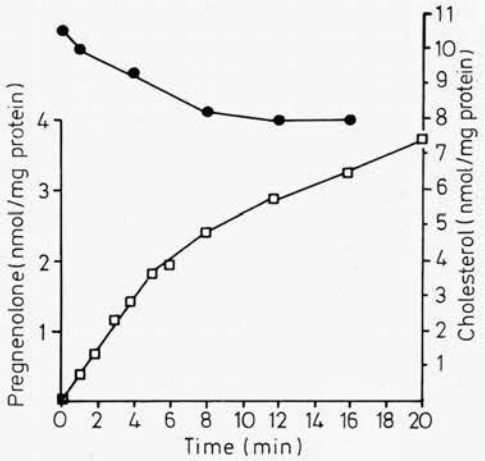

Fig. 2. *The time course of pregnenolone appearance from endogenous cholesterol and the depletion of endogenous cholesterol in luteal mitochondria.* Mitochondria were preincubated for 10 min in buffer before addition of malate to initiate side-chain cleavage. Protein concentrations were 1.4 mg/ml, cyanoketone 6.4 μM. (□) Pregnenolone; (●) cholesterol

Table 1. *Measurement of cholesterol side-chain cleavage in luteal mitochondria*
Results expressed as mean ± S.E.M for 6 determinations. Cholesterol esters, cholesterol, pregnenolone and conversion of [4-^{14}C]cholesterol to [4-^{14}C]pregnenolone were assayed as described in the text. Incubation volume 3 ml, protein concentrations 1 mg/ml and all contained 6.4 μM cyanoketone. Mitochondria were preincubated for 10 min with [4-^{14}C]cholesterol before addition of 4 mM malate. 10 ml methanol were added to the incubations at the same time as or 5 min after the malate to stop enzymatic activity. n.d. = not detectable

Sample taken after	Cholesterol esters	Free cholesterol	Pregnenolone	Conversion of [4-^{14}C]cholesterol into pregnenolone	
				total	radioactive
	nmol steroid/mg protein			%	
Time zero	7.0 ± 0.6	12.9 ± 0.3	n.d.	–	–
5-min incubation	6.8 ± 1.3	9.6 ± 0.4	3.6 ± 0.1	28.92 ± 1.4	27.30 ± 0.8

It will be observed that the rate of formation of pregnenolone begins to fall off after 5–10 min; this follows very closely the time course of appearance of [4-^{14}C]pregnenolone observed in the radioactive assay when the [4-^{14}C]cholesterol was preincubated with the luteal mitochondria (Fig. 1). Table 1 shows a comparison of the two methods of measuring cholesterol side-chain cleavage when applied to the same luteal mitochondrial preparation. It will be noted that in a 5-min incubation, the percentage conversion of cholesterol to pregnenolone is the same whether measured as actual conversion of endogenous cholesterol to pregnenolone or conversion of [4-^{14}C]-cholesterol to [4-^{14}C]pregnenolone. The depletion of cholesterol during the 5-min incubation was 3.3 nmol/mg protein and corresponds very closely to the formation of pregnenolone which was 3.6 nmol/mg protein. The cholesterol ester content of the ovarian mitochondrion, which was small, did not change significantly during the 5-min incubation. Fig. 2 shows the depletion of luteal mitochondrial cholesterol during a typical incubation. The depletion of free cholesterol reflects the appearance of pregnenolone suggesting that free cholesterol, rather than cholesterol ester, is the substrate for cholesterol side-chain cleavage in these mitochondria.

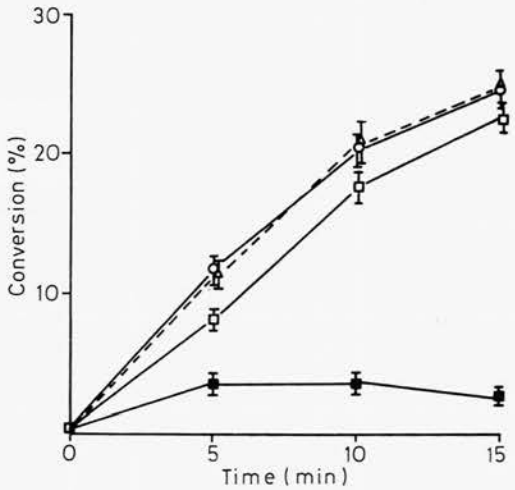

Fig. 3. *The effect of cyanoketone on the time course of cholesterol side-chain cleavage of [4-^{14}C]cholesterol.* Side-chain cleavage was initated in mitochondria by malate at time zero after they had been preincubated for 10 min with [4-^{14}C]-cholesterol, protein concentrations 1.4 mg/ml. (△) Total products; (■) [4-^{14}C]pregnenolone; (□) [4-^{14}C]progesterone formed in absence of cyanoketone; (○) pregnenolone formed in presence of cyanoketone. Results mean ± S.E.M. for 4 determinations

Cyanoketone, an inhibitor of 3β-hydroxysteroid dehydrogenase was included in incubations to prevent the conversion of pregnenolone to progesterone. Fig. 3 shows the conversion of [4-^{14}C]cholesterol to products in the presence and absence of cyanoketone. The experiments show that the total conversion of [4-^{14}C]-cholesterol to products in the presence and absence of cyanoketone is the same. In the presence of cyanoketone one product, [4-^{14}C]pregnenolone is formed; when it is not present two products are formed [4-^{14}C]-pregnenolone and [4-^{14}C]progesterone. The identity of these two products has been confirmed by recrystallisation to constant specific activity with authentic steroids (Table 2). In the absence of cyanoketone [4-^{14}C]progesterone is the major product, little [4-^{14}C]pregnenolone remaining. Thus the addition of cyanoketone has no effect on the measured rate of cholesterol side-chain cleavage despite the fact that it blocks further metabolism of the product of the reaction resulting in a large rise in the mitochondrial pregnenolone concentration. Cholesterol side-chain cleavage in rat luteal mitochondria can therefore be measured by following the appearance of pregnenolone in the presence of cyanoketone, a method which has been described for adrenal cortex mitochondria [11].

Table 2. *Recrystallisation of [4-^{14}C]pregnenolone and [4-^{14}C]-progesterone to constant specific activity*
Areas of radioactivity corresponding to standard pregnenolone and progesterone were scraped from thin-layer plates of incubation extracts. The radioactive steroids were extracted and mixed with authentic steroid (Organon). Recrystallisations were then carried out from redistilled methanol. Duplicate determinations of specific activity of each recrystallisation are shown in the table

A. Incubations without cyanoketone

Stage of purification	[4-^{14}C]-Pregnenolone	[4-^{14}C]-Progesterone
	dis. $\times$ min^{-1} $\times$ mg^{-1}	
After addition of standard pregnenolone or progesterone	143 141	2237 2061
1st recrystallisation	139 176	2015 2070
2nd recrystallisation	141 140	2189 2120
3rd recrystallisation		2201 2190

B. Incubations with cyanoketone

Stage of purification	[4-^{14}C]Pregnenolone
	dis. $\times$ min^{-1} $\times$ mg^{-1}
After addition of standard pregnenolone	3681 3637
1st recrystallisation	3687 3769
2nd recrystallisation	3761 3766

Table 3. *Inhibition of cholesterol side-chain cleavage in luteal mitochondria* in vitro *by addition of pregnenolone*
Cholesterol side-chain cleavage was measured as percentage conversion of [4-^{14}C]cholesterol to [4-^{14}C]pregnenolone after 5 min. Pregnenolone and malate were added after mitochondria were preincubated for 10 min with [4-^{14}C]cholesterol. Results are the average of duplicate assays, protein concentration 1.2 mg/ml, all incubations contained 6.4 μM cyanoketone

Pregnenolone	Conversion	Inhibition
μM	%	
0	11.0	0
2.5	11.0	0
5	9.5	14
10	7.8	23
20	6.8	37
40	5.1	53

Addition of Pregnenolone to Luteal Mitochondrial Incubations

Addition of pregnenolone to mitochondrial incubations was investigated to see if this had any effect on the rate of cholesterol side-chain cleavage.

The accumulation of endogenously produced pregnenolone in mitochondrial incubations as a result of the addition of cyanoketone was shown above to have no effect on the measured rate of cholesterol side-chain cleavage. Table 3 shows the effect of added pregnenolone on the cholesterol side-chain cleavage reaction. Concentrations of pregnenolone in the range 0 to 10 μM caused little inhibition of the reaction while 40 μM pregnenolone was required to reduce the rate of cholesterol side-chain cleavage by 50%. When the rate of cholesterol side-chain cleavage from endogenous substrate in luteal mitochondria decreases during incubations after 5–10 min (see Fig. 2 and 4) the pregnenolone concentration is only 2–3 μM. Hence this reduction in the rate of this reaction is unlikely to be due to product inhibition.

Addition of Cholesterol to Luteal Mitochondrial Incubations

As product inhibition does not appear to be important in the conditions of mitochondrial incubations, the availability of substrate for cholesterol side-chain cleavage was investigated.

Fig. 4 shows the effect of the addition of cholesterol to mitochondrial incubations. There is no obvious dramatic effect on the initial rate of pregnenolone production. When the production of pregnenolone from endogenous cholesterol shows a decrease after 5–10 min, the added cholesterol corrects this and results in the pregnenolone production rate continuing at the initial rate of 0.8 nmol $\times$ mg protein^{-1} $\times$ min^{-1} for at least 30 min. Addition of a further quantity of cholesterol did not increase this rate of pregnenolone production above the initial rate observed with endogenous substrate. This suggests that under experimental conditions apparent saturation kinetics for cholesterol had been established.

Effect of Cycloheximide Treatment on Cholesterol Side-Chain Cleavage in Luteal Mitochondria

The effect on cholesterol metabolism in luteal mitochondria of injecting rats with cycloheximide was investigated. Superovulated rats were injected with cycloheximide as described in Methods. The rats (8/group) were killed 4, 8, 16 and 32 min after the cycloheximide injection and mitochondria were pre-

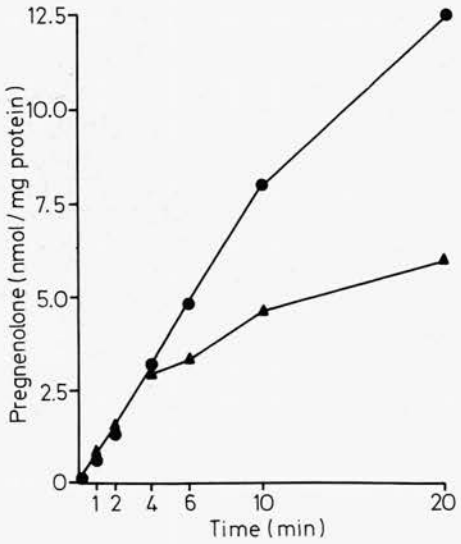

Fig. 4. *Formation of pregnenolone from endogenous and added cholesterol by luteal mitochondria.* Conditions as in legend for Fig. 2. Protein concentrations were 0.9 mg/ml. (▲) Pregnenolone formed from endogenous substrate; (●) pregnenolone formation in the presence of added 215 μM cholesterol

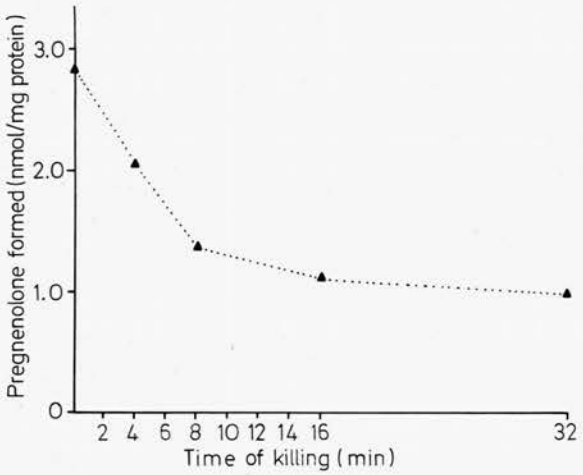

Fig. 5. *Effect of cycloheximide injection on the rate of cholesterol side-chain cleavage in rat luteal mitochondria.* Rats were injected with cycloheximide as indicated in Methods and killed 4, 8, 16 and 32 min afterwards. Ovaries were removed from those rats and mitochondria prepared from them. (▲) Pregnenolone formed per 10 min in these mitochondria

pared from the ovaries in the usual way. Pregnenolone production (nmol/mg protein per 10 min) in these mitochondria is shown in Fig. 5. Within 6 min after the cycloheximide injection pregnenolone production in rat luteal mitochondria had dropped by 50%. 16 min after cycloheximide injection, the pregnenolone production in ovarian mitochondria had dropped to

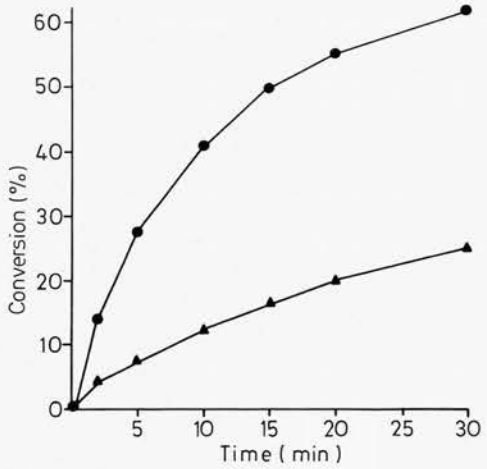

Fig. 6. *Effect of cycloheximide injection on the conversion of [4-^{14}C]cholesterol to [4-^{14}C]pregnenolone in rat luteal mitochondria.* Mitochondria were preincubated with [4-^{14}C]-cholesterol for 10 min before the addition of malate to initiate cholesterol side-chain cleavage. Protein concentrations were 1.2 mg/ml in each case and cyanoketone 6.4 μM. (●) Saline-injected animals; (▲) cycloheximide-treated animals

40% of the control values. Fig. 6 shows the time course for the appearance of [4-^{14}C]pregnenolone from [4-^{14}C]cholesterol in luteal mitochondria from rats treated with cycloheximide for 20 min compared to control animals which were saline-injected. The initial rate of cholesterol side-chain cleavage can be seen to have dropped to 25% of control values in the cycloheximide-treated animals. This is greater than the inhibition observed of total pregnenolone production in rat luteal mitochondria from cycloheximide-treated rats (Fig. 5, Table 4). This difference can be explained by a more detailed analysis of the effects of cycloheximide injection on rat luteal mitochondrial cholesterol and cholesterol metabolism (Table 4). The cholesterol pool in luteal mitochondria from ovaries of the cycloheximide-treated rats was found to be significantly greater than was found in the mitochondria from saline-injected rats ($P < 0.01$). Hence it is essential to compare the actual pregnenolone production/mg protein rather than the percentage conversion of cholesterol to pregnenolone as the "mitochondrial pool of cholesterol" changes upon cycloheximide treatment. The rate of pregnenolone production (nmol/mg protein per 5 min) is significantly different ($P < 0.01$) in luteal mitochondria from cycloheximide-treated and saline-treated rats.

The difference in the rates of pregnenolone production between luteal mitochondria from rats treated with saline and cycloheximide is preserved even when an additional 215 nmol cholesterol/mg protein is added to the incubations (Fig. 7).

Table 4. *Effect of giving cycloheximide 20 min before killing on cholesterol levels, cholesterol side-chain cleavage activity, cytochrome P-450 and pregnenolone-induced difference spectra of isolated rat luteal mitochondria*
Luteal mitochondria were isolated from rats which had previously been treated with cycloheximide or 0.9% saline for 20 min before killing. Initial cholesterol levels in the mitochondria and pregnenolone produced in 5 min incubations were determined. Total mitochondrial cytochrome *P*-450 was determined by the method of Omura and Sato [18]. Pregnenolone-induced difference spectra were determined as described in Methods. All results are expressed as mean ± S.E.M.; number of determinations in parentheses

Treatment	Mitochondrial cholesterol	Pregnenolone formation	Mitochondrial cytochrome *P*-450	$10^3 \times$ Pregnenolone-induced difference spectrum
	nmol/mg protein			$\Delta A_{424-385}$/mg protein
Saline	17.55 ± 1.05 (6)	3.35 ± 0.53 (6)	0.48 ± 0.06 (4)	15.1 ± 1.5 (7)
Cycloheximide	22.0 ± 0.73[a] (6)	1.45 ± 0.21[a] (6)	0.47 ± 0.06 (4)	6.7 ± 0.3[b] (7)

[a] $P < 0.01$.
[b] $P < 0.001$.

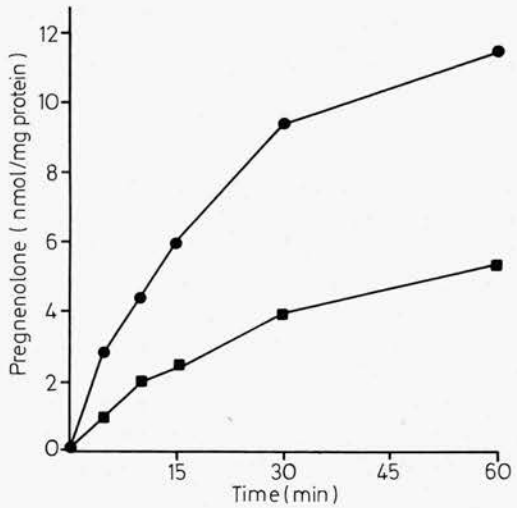

Fig. 7. *Pregnenolone formation from exogenous cholesterol in ovarian mitochondria from saline and cycloheximide-treated rats.* Conditions as in legend for Fig. 2. Protein concentrations 0.8 mg/ml in each case, cyanoketone 6.4 μM. (●) Saline-treated plus 215 μM cholesterol; (■) cycloheximide-treated plus 215 μM cholesterol

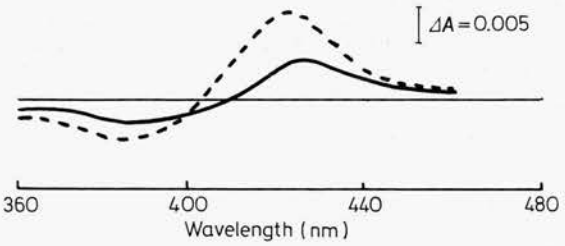

Fig. 8. *Pregnenolone-induced difference spectrum of rat luteal mitochondria.* 12 μM pregnenolone was added to sample cuvette in ethanol and an equal volume of ethanol added to the reference cuvette. Protein concentrations were 1 mg/ml in each case, cyanoketone 6.4 μM. (-----) Mitochondria from saline-treated animals; (——) mitochondria from cycloheximide-treated animals

Effect of Cycloheximide Treatment on Cytochrome P-*450 in Luteal Mitochondria*

Total mitochondrial cytochrome *P*-450 was measured by the method of Omura and Sato [18]. There was found to be no significant difference in values between animals injected with cycloheximide and saline (Table 4).

Cycloheximide treatment has, however, an effect on the inverted type I pregnenolone-binding spectrum of rat luteal mitochondrial cytochrome *P*-450. The magnitude of the spectral change (change in absorbance between 424–385 nm) on the addition of saturating amounts of pregnenolone to the luteal mitochondria was reduced by 56% in the cycloheximide-treated rats (Fig. 8 and Table 4). Cycloheximide did not produce a difference spectrum with rat luteal mitochondria nor did it affect cholesterol side-chain cleavage when added directly to mitochondrial incubations.

Effect of Chloramphenicol Treatment on Cholesterol Metabolism and Cytochrome P-*450 in Luteal Mitochondria*

Chloramphenicol was administered to rats 3 h before they were killed; this allows the drug to attain maximum blood concentrations [16]. No difference could be detected in the rate of cholesterol side-chain cleavage, using the radioactive assay, in luteal mitochondria from treated and untreated animals (Expt 1, Fig. 9). There was no difference in the cytochrome *P*-450 or cholesterol levels in luteal mitochondria from

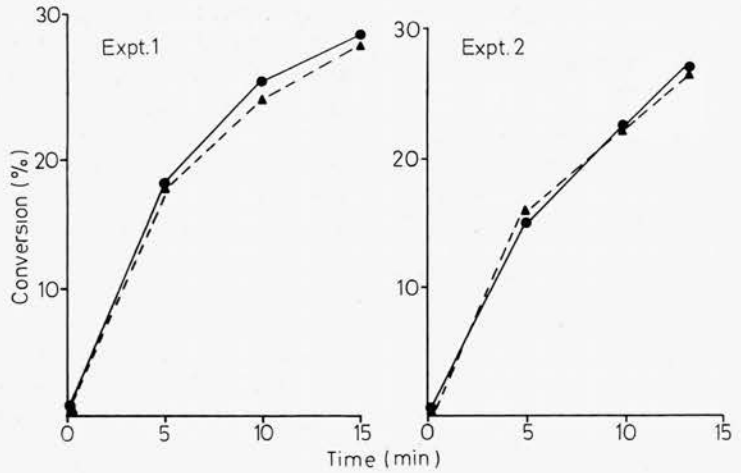

Fig. 9. *Effect of chloramphenicol injection on the conversion of [4-^{14}C]cholesterol to [4-^{14}C]pregnenolone in rat mitochondria.* Mitochondria were preincubated for 10 min with [4-^{14}C]-cholesterol before addition of malate to initiate side-chain cleavage. Experiment 1, protein concentrations 1.2 mg/ml in each case cyanoketone 6.4 μM. (●) Saline; (▲) chloramphenicol. Rats injected with chloramphenicol 3 h before killing. Experiment 2, protein concentrations 1.1 mg/ml in each case cyanoketone 6.4 μM. (●) Saline; (▲) chloramphenicol. Rats injected with chloramphenicol 3 and 10 h before killing

Table 5. *The effect of chloramphenicol injection 3 h or 10 h before killing on rat luteal mitochondrial cholesterol and cytochrome* P-*450*
Rats were injected once with chloramphenicol 3 h (experiment 1) before killing or twice with chloramphenicol 10 and 3 h (experiment 2) before killing. Mitochondria were prepared from the ovaries of these animals and free cholesterol and cytochrome *P*-450 was determined in them. Results expressed as mean ± S.E.M. for six cholesterol determinations. Cytochrome *P*-450 is expressed as mean of duplicate determinations

Treatment	Expt	Cholesterol	Cytochrome *P*-450
		nmol/mg protein	
Saline	1	21.50 ± 0.78	0.46
	2	22.74 ± 0.54	0.46
Chloramphenicol	1	21.1 ± 0.55	0.43
	2	22.07 ± 0.32	0.47

two groups of animals (Table 5). Similar results were obtained when the rats were given two injections of chloramphenicol 10 h and 3 h before killing (Expt 2, Fig. 9 and Table 5).

Effect of Puromycin Treatment on Cholesterol Metabolism and Cytochrome P-*450 in Luteal Mitochondria*

Doses of puromycin 2 mg or 8 mg were administered to rats 20 min before killing. This treatment had no effect on cholesterol side-chain cleavage or total cytochrome *P*-450. It was not considered practical to

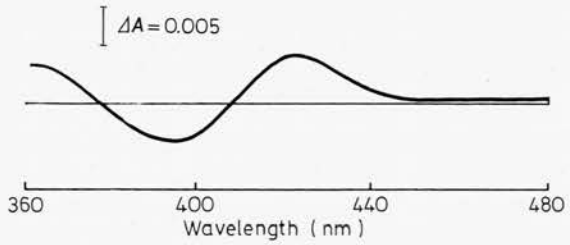

Fig. 10. *Puromycin-induced difference spectrum of rat luteal mitochondria.* Saturating amounts of puromycin (3.5 mM) were added to the sample cuvette dissolved in a small volume of incubation medium and an equal amount of medium was added to the reference cuvette. Protein concentration 2 mg/ml

use greater doses of puromycin as it would have become difficult to distinguish its effects on protein synthesis from its direct effects on the enzymes of mitochondrial cholesterol side-chain cleavage. Puromycin has been shown to bind to liver microsomal cytochrome *P*-450 [20] to give a type II difference spectrum. It was found to bind to luteal mitochondrial cytochrome *P*-450 to produce a type II difference spectrum (Fig. 10). The binding constant of this interaction is 0.7 mM using the change in absorbance between 422 – 394 nm as a measure of binding. Puromycin added *in vitro* to luteal mitochondrial incubations inhibited cholesterol side-chain cleavage in similar concentrations to those which produced a difference spectrum. 1.5 mM puromycin was needed *in vitro* to inhibit cholesterol side-chain cleavage in luteal mitochondria by 50% (Table 6).

Table 6. *Inhibition of cholesterol side-chain cleavage in luteal mitochondria* in vitro *by addition of puromycin*
Cholesterol side-chain cleavage was measured as percentage conversion of [4-^{14}C]cholesterol to [4-^{14}C]pregnenolone in 10 min after addition of malate. The [4-^{14}C]cholesterol and puromycin were preincubated with the luteal mitochondria for 10 min before addition of malate to initiate cholesterol side-chain cleavage. Results are average of duplicate assays, protein concentrations were 1.1 mg/ml, all incubations contained 6.4 μM cyanoketone

Puromycin	Conversion
mM	%
0	31.6
0.37	28.9
0.75	23.1
1.5	15.3
3.0	7.3

DISCUSSION

This paper describes the cholesterol-metabolising properties of a rat luteal mitochondrial preparation. In these mitochondria the rate of pregnenolone formation from endogenous substrate declines from the initial rate after 4–5 min. This appears to be due to depletion of the substrate cholesterol. As can be seen from Fig. 4 addition of cholesterol to a final concentration of 215 μM allows pregnenolone production to continue at the initial rate for at least 30 min. The results suggest that the supply of cholesterol to the enzyme system of cholesterol side-chain cleavage in luteal mitochondria is critical and mitochondrial stores of cholesterol can be depleted rapidly under these situations *in vitro*.

Inhibition of cholesterol side-chain cleavage by the product, pregnenolone, has been suggested as being important in the control of steroidogenesis in the adrenal cortex. Koritz and Kumar [21] suggested that adrenocorticotropin stimulated steroidogenesis in the adrenal cortex by releasing the product inhibition on the rate-limiting step, cholesterol side-chain cleavage [22] by a mechanism involving the efflux of pregnenolone from the mitochondrion. Such a control of luteal steroidogenesis under the experimental conditions outlined in this paper would appear not to exist. Pregnenolone concentrations will probably not build up in luteal mitochondria *in vivo* due to the presence of an active mitochondrial 3β-hydroxysteroid dehydrogenase [23]. Alteration of mitochondrial pregnenolone concentrations by omitting cyanoketone did not affect the overall rate of cholesterol side-chain cleavage (Fig. 3) suggesting product inhibition was not important in controlling the rate of the reaction. Pregnenolone inhibited cholesterol side-chain cleavage when concentrations were added which exceeded by 4 or 5-fold the concentration (2–3 μM) encountered in incubations when the rate of pregnenolone production started to decline after 5–10 min (Table 3, Fig. 2).

Protein synthesis has been implicated as being involved in the control of steroidogenesis in the ovary [8–10]. Hermier *et al.* [10] have concluded that a protein factor may be involved in the control of mitochondrial cholesterol metabolism in the rat corpus luteum. This is a similar situation to that which has been reported for the adrenal gland by Davis and Garren [12]. The activity of the cholesterol side-chain cleavage system in rat luteal mitochondria can be inhibited 50% by prior treatment of the rats with cycloheximide a potent inhibitor of protein synthesis for 6 min (Fig. 6). Although there is a fall in the rate of mitochondrial cholesterol metabolism there is no fall in the amount of what is considered to be a key enzyme in this connection [4] namely cytochrome *P*-450 in these mitochondria (Table 4). This suggests that cycloheximide administration under these conditions did not decrease synthesis of the cholesterol-metabolizing cytochrome-*P*-450-dependent enzymes in this short time. Also cycloheximide had no effect on these enzymes *in vitro* in cholesterol side-chain cleavage studies in rat luteal mitochondria. The fall by 56% of the inverted type I difference spectrum produced by pregnenolone in the luteal mitochondria of the cycloheximide-treated rats (Fig. 8) is similar to the 58% fall in the mitochondrial cholesterol side-chain cleavage activity (Table 4). These spectral changes can be interpreted to indicate the change in the amount of cholesterol bound to the cytochrome *P*-450 enzyme of cholesterol side-chain cleavage as a consequence of the cycloheximide treatment. The inverted type I difference spectrum produced by pregnenolone on cytochrome *P*-450 of adrenal cortex mitochondria can be interpreted to be due to the displacement of substrate cholesterol from the high-spin cholesterol complex of the cytochrome *P*-450 of cholesterol side-chain cleavage [24]. The initial rate of cholesterol side-chain cleavage in rat adrenal cortex mitochondria has been correlated to the amount of the high-spin cholesterol complex of the cytochrome *P*-450 of mitochondrial cholesterol side-chain cleavage [11]. The components of the cholesterol side-chain cleavage system in the adrenal gland and the corpus luteum are very similar [4]. The spectral properties of the side-chain cleavage cytochrome *P*-450 in luteal and adrenal mitochondria have also been shown to be similar [15,25,26]. Hence it is suggested that the cycloheximide injection induced a fall in the pregnenolone (inverted type I) difference spectrum of cytochrome *P*-450 of cholesterol side-chain cleavage in rat luteal mitochondria which could be due to a decrease in the amount of

the cholesterol bound to the enzyme. The total amount of luteal mitochondrial free cholesterol is increased by 25% in cycloheximide-treated rats (Table 4), whereas the amount of cholesterol bound to the specific cholesterol side-chain cleavage cytochrome *P*-450 is decreased. This suggests that the the cycloheximide injection which has presumably blocked the synthesis of a protein [10] has influenced the distribution of cholesterol within the mitochondrion. This "cycloheximide-sensitive factor" could be important in effecting the redistribution or transport of cholesterol within the luteal mitochondrion, for instance by participating in the movement of cholesterol to the cholesterol side-chain cleavage cytochrome *P*-450.

The fact that there is an increase of free cholesterol in the rat luteal mitochondria as a whole from cycloheximide-treated rats supports the evidence that cycloheximide administration had no inhibitory effect on the luteal cytoplasmic hormone-sensitive cholesterol esterase (Arthur, J. R., Beckett, G. J., and Boyd, G. S., unpublished observations). This esterase effects the hydrolysis of cholesterol esters stored in the lipid droplets of the luteal cell cytoplasm [27]. Presumably therefore cholesterol was released from the lipid droplets, by the action of the esterase and was accumulated in the mitochondria. Although cycloheximide treatment has been shown to be without effect on the rat adrenal cortex adrenocorticotropin-sensitive cholesterol esterase [28] it does prevent the adrenocorticotropin induced rise in the cholesterol side-chain cleavage activity in adrenal mitochondria. In the rat corpus luteum, cycloheximide treatment probably does not affect the hydrolysis of cholesterol esters but blocks the subsequent metabolism of cholesterol in the mitochondrion. This blockage could be due to a lack of a factor which is rapidly turning over, which could be protein, produced extra mitochondrially in the cell.

Additional of a large excess of cholesterol to incubations of luteal mitochondria from cycloheximide-treated rats did not prevent the decrease in cholesterol side-chain cleavage activity in luteal mitochondria observed on cycloheximide treatment (Fig. 7). Thus even when there is an excess of cholesterol available to the luteal mitochondria some factor is needed to facilitate cholesterol metabolism by the mitochondrial cholesterol side-chain cleavage enzyme system.

Chloramphenicol treatment of the rats was investigated to establish whether this inhibitor of mitochondrial protein synthesis [29, 30] had any short term effects on luteal mitochondrial cholesterol metabolism. It has been reported that chloramphenicol inhibits the synthesis of adrenal cortex mitochondrial cytochrome *P*-450 [31]. However chloramphenicol injection to rats appeared to have no short-term effect in this superovulated system. Metabolism of cholesterol in luteal mitochondria and the luteal mitochondrial cytochrome *P*-450 system were unaffected (Fig. 9, Table 5). It is concluded from these observations that it is possible that a protein factor which is rapidly turning over and is located within the mitochondria but of cytoplasmic rather than mitochondrial origin in the rat corpus luteum is involved in the cholesterol side-chain cleavage reaction.

Puromycin was shown to interact directly with luteal mitochondrial cytochrome *P*-450 (Fig. 10) and to inhibit cholesterol side-chain cleavage *in vitro* (Table 6). It is therefore difficult to draw conclusions that puromycin can effect luteal steroidogenesis through inhibition of protein synthesis rather than by a direct effect on the cholesterol side-chain cleavage enzymes.

In conclusion, the rate of cholesterol side-chain cleavage in the superovulated rat ovarian mitochondrion is affected by a factor which is rapidly turning over, which could be protein. This is a similar situation to the proposed control of cholesterol side-chain cleavage in rat adrenal cortex mitochondria [11]. Further work should elucidate the nature and function of this factor in the superovulated rat ovary.

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