STUDIES ON STEROL OXYGENASES IN MAMMALIAN TISSUES

by

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SUMMARY

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

The sterol mono-oxygenase studied in this investigation was the cholesterol side-chain cleavage enzyme system of human term placenta.

(a) A rapid and sensitive assay of cholesterol side-chain cleavage activity is described.

(b) The enzyme system was found to be located in the mitochondrial fraction and to have the characteristics of a mono-oxygenase or "mixed function oxidase", i.e. required NADPH and molecular oxygen.

(c) Using native placental mitochondrial preparations, the effect of various intramitochondrial sources of reducing equivalents and also other reducing agents on the enzymic activity was examined.

(d) The enzyme system was successfully extracted in a soluble fraction using three different techniques, viz , ultrasonication, a combination of ultrasonication and lyophilization and acetone powder preparation.

(e) Disruption of the mitochondria led to the activation of an endogenous inhibitor of the enzyme system. The inhibitor was firmly associated with the particulate material and appeared to be mitochondrial phospholipid. Lecithin was found to be the most potent phospholipid inhibitor. A mechanism for the inhibition is discussed.

(f) The characteristics of the 'solubilised' enzyme system have been investigated with special consideration being given to the effect of NADPH and ionic strength on the enzymic activity as well as the activation energy of the reaction.

(g) The enzyme system was found to be sensitive to carbon monoxide. A study of data on the release of this inhibition by light of various wavelengths implied the involvement of cytochrome P-450 in the enzyme system.

(h) The soluble enzyme system was resolved into three protein fractions - a haem-containing fraction (presumably cytochrome P-450), a non-haem iron protein and an NADPH diaphorase fraction. The enzymic activity was restored after recombination of these three protein fractions.

The human placental mitochondrial cholesterol side-chain cleavage enzyme system, therefore, appeared similar to the enzyme system present in bovine adrenal cortex and rat ovary.

ABBREVIATIONS USED IN THE TEXT

The following standard abbreviations will be used throughout the text:-

$NADP^+$	-	nicotinamide-adenine dinucleotide phosphate
NADPH	-	reduced nicotinamide-adenine dinucleotide phosphate
NAD^+	-	nicotinamide-adenine dinucleotide
NADH	-	reduced nicotinamide-adenine dinucleotide
G6P	-	D-glucose-6-phosphate disodium salt
G6PD	-	D-glucose-6-phosphate dehydrogenase
ACTH	-	adrenocorticotrophic hormone
GSH	-	reduced glutathione
ATP	-	5'-adenosine triphosphate
PPO	-	2,5-diphenyloxazole
POPOP	-	1,4-bis(2-(5-phenyloxazolyl))-benzene
TLC	-	thin-layer chromatography
GLC	-	gas-liquid chromatography
EPR	-	electron paramagnetic resonance

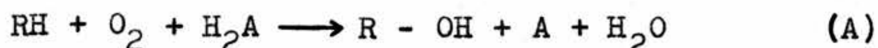
Pregnenolone	-	3β-Hydroxypregn-5-en-20-one
Progesterone	-	Pregn-4-ene-3,20-dione

CHAPTER 1

INTRODUCTION

INTRODUCTION

Enzymes that incorporate one atom of molecular oxygen into substrates while concomitantly reducing the other atom to water are termed mixed-function oxidases (Mason, 1957, 1965) or mono-oxygenases. The stoichiometry of this type of reaction may be represented as follows:



$$RH + O_2 + H_2A \longrightarrow R - OH + A + H_2O \qquad (A)$$

RH is the substrate and, in most instances, the reductant; H_2A is either reduced nicotinamide-adenine dinucleotide phosphate (NADPH) or reduced nicotinamide-adenine dinucleotide (NADH). The importance of mono-oxygenases in metabolism is evident from the wide range of substances with which they react - such as, carbohydrates, lipids, amino acids, steroids, drugs and hormones. The nature of mixed function oxidase-catalyzed reactions has been reviewed by Mason (1965), and is the topic of several reviews (King et al., 1965; Bloch and Hayaishi, 1966; Konstanz Symposium on the "Mechanisms of Mixed Function Oxygenation", 1968). Although considerable effort in the past decade has been devoted to the enzyme components, coenzymes and cofactors required for oxygenation, the reaction mechanism of mono-oxygenases at the molecular level remains poorly understood. Studies have been complicated by both the particulate nature and the lability of the enzyme components. Although in a few cases the systems within which mixed-function reducing equivalents are brought into reaction with oxygen and substrate are soluble, such as phenylalanine hydroxylase (Kaufman, 1962) and camphor oxidase

(Katagiri et al., 1968), they are usually associated with organised subcellular structures. In higher organisms, this is either the mitochondrion or the endoplasmic reticulum.

For certain mixed-function oxidases it has been possible to determine the precise stoichiometry of the reaction sequence (A). The electron donor in many mammalian mono-oxygenase systems is NADPH, but NADH can serve as a source of reducing equivalents in certain cases.

Hydroxylases

Hydroxylases belong to the class of mixed-function oxidase enzymes. Enzymic hydroxylation refers to the introduction of a hydroxyl group into various substrates and is catalyzed by different types of enzymes. The source of oxygen in the hydroxyl group may be derived from (a) molecular oxygen, (b) an oxygen atom of a water molecule, or (c), possibly, from some other compound. Certain aspects of the metabolism of lipids and aromatic compounds are often initiated by hydroxylation. These compounds apparently need to be oxygenated in order to become biologically active or more soluble in an aqueous environment. But, possibly because of their hydrophobic nature, molecular oxygen seems to be the preferred hydroxylating agent rather than water. When the oxygen atom of the hydroxyl group is derived from molecular oxygen, the enzyme is, by definition, an oxygenase. Mono-oxygenases are concerned with hydroxylation reactions of aromatic as well as aliphatic compounds. They also catalyze an apparently diverse group of reactions including epoxide

formation, N- or S-oxide formation, dealkylation, decarboxylation, deamination, desaturation and so forth. Though the overall reactions catalyzed by mono-oxygenases appear grossly different, the primary chemical events involved are basically identical since all the processes are initiated by the incorporation of one atom of molecular oxygen into the substrate (Guroff et al., 1967).

In mammals, mono-oxygenases are located in certain specific organs and subcellular particles. Many of the enzymes involved in the hydroxylation of physiological substrates and the enzymes that take part in the hydroxylation (detoxification) of foreign compounds are located in the liver endoplasmic reticulum (Gillette et al., 1969). Certain selective hydroxylations are also carried out by liver mitochondria (Saito et al., 1957).

Mono-oxygenases are also found in the steroid-producing tissues (adrenals, gonads and placenta) and play an important role in steroid metabolism. In these tissues, they are associated with both the mitochondria and the endoplasmic reticulum (see Dorfman and Ungar, 1965).

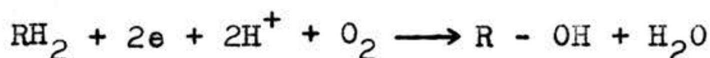
Enzymic hydroxylation has recently been reviewed by Hayaishi (1969).

Sterol and Steroid mono-oxygenases

Hydroxylation reactions pertaining to mammalian steroid metabolism have been noted at carbons 7α, 20α, 22R and 26 in cholesterol and at carbons 2α, 2 (aromatic), 6α, 6β, 7α, 11β, 15α, 16α, 16β (possible, but not certain), 17α, 18, 19 and 21 in other steroids. With the suitable deoxy-steroid substrate it is likely that

hydroxylation could be shown to occur at any position on the steroid molecule (Dorfman and Ungar, 1965).

There is considerable evidence that the source of reducing equivalents for mammalian steroid mixed-function oxidases is NADPH (Ryan and Engel, 1957; Sweat and Lipscomb, 1955). The requirement for molecular oxygen was demonstrated by Hayano et al. (1955) who showed the incorporation of molecular $^{18}O_2$ during the 11β-hydroxylation of deoxycorticosterone. Cooper et al. (1962) established the validity of the stoichiometry of the 'mixed-function oxidase' equation proposed by Mason (1957), i.e.



$$RH_2 + 2e + 2H^+ + O_2 \longrightarrow R - OH + H_2O$$

These workers showed that for the steroid 21-hydroxylase the ratio of NADPH oxidised to 17α,21-dihydroxyprogesterone produced to the O_2 reduced is 1:1:1, as predicted by the above equation.

The most thoroughly studied mammalian steroid hydroxylase is the 11β-hydroxylase which is found in the mitochondria of the adrenal cortex. However, the first indication as to the nature of steroid hydroxylases came from an observation of Ryan and Engel (1957) that the 21-hydroxylation of 17α-hydroxyprogesterone occurring in adrenocortical microsomes was inhibited by carbon monoxide, and that this inhibition was reversed by light. In 1958, Garfinkel and Klingenberg independently observed an intense light absorption band at 450 nm when liver microsomes reduced with dithionite were gassed with carbon monoxide. Estabrook et al. (1963) observed a similar type of 450 nm-absorbing pigment when adrenal

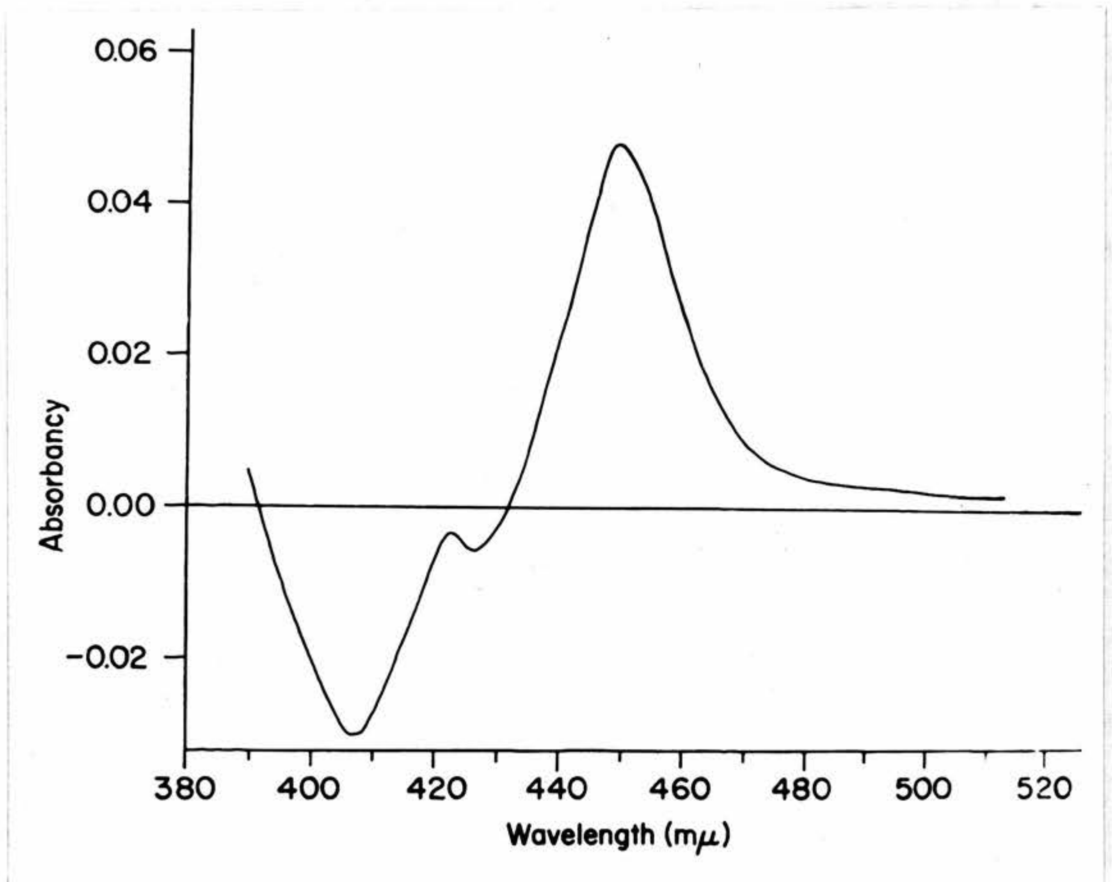


Fig. 1.1. The difference spectrum of the CO-complex of cytochrome P-450 of bovine adrenocortical microsomes. The difference in light absorbancy was determined as a function of wavelength; a reaction cuvette containing NADPH was gassed with CO; a reference cuvette contained NADPH. After Estabrook <u>et al.</u> (1963).

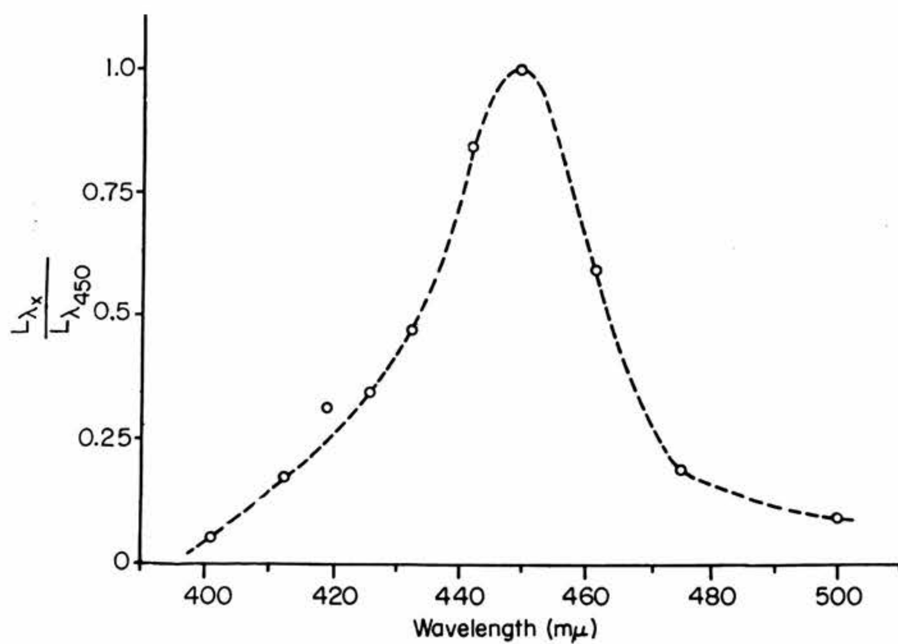
microsomes were treated with NADPH and carbon monoxide. This pigment was shown to be a protoporphyrin haemoprotein of the cytochrome b type and named cytochrome P-450 by Omura and Sato (1964a,b). Figure 1.1 shows a typical difference spectrum of the reduced, carbon monoxide complex of this haemoprotein. Estabrook et al. (1963) further demonstrated that cytochrome P-450 was the terminal oxidase involved in the steroid 21-hydroxylation reaction.

The Function of Cytochrome P-450 in Mono-oxygenase-catalysed Reactions

Estabrook et al. (1963) studied the carbon monoxide inhibition of the steroid 21-hydroxylation reaction in adrenal cortex microsomes by observing the rate of hydroxylation of 17α-hydroxyprogesterone under an atmosphere composed of different carbon monoxide to oxygen ratios. The result was expressed as a partition coefficient:

$$K = \frac{n}{1 - n} \cdot \frac{CO}{O_2}$$

for each ratio of the gases, where n is the ratio of hydroxylation rate with carbon monoxide present to the uninhibited rate, as defined by Warburg (1949). The partition constant, K, was found to vary between 0·6 and 1·8 for different preparations of adrenal microsomes. These values were found to be similar to the partition constant for cytochrome P-450 as determined by spectrophotometric titration. The value of the partition constant for the carbon monoxide inhibition of steroid 21-hydroxylation is quite different from the partition constants of haemoglobin and cytochrome oxidase



Photochemical action spectrum for the light-reversal of CO inhibition of 21-hydroxylation of 17α-hydroxyprogesterone. Incubations were gassed with a mixture of 4.4% O_2 and 9.5% CO in nitrogen. The results are plotted in terms of relative light sensitivity L_{λ}/L_{452}, versus wavelength of the light applied.

Fig. 1.2. Photochemical action spectrum for the light reversal of the CO inhibition of the steroid 21-hydroxylase of bovine adrenocortical microsomes. After Omura _et al._ (1965).

which are $1 \cdot 8 - 8 \cdot 0 \times 10^{-3}$ and about 10 - 20, respectively. (Keilin and Wang, 1946).

These results suggested a role for cytochrome P-450 in steroid 21-hydroxylation. Use of the photochemical action technique of Warburg (1949) provided more conclusive evidence that the carbon monoxide complex of cytochrome P-450 is the carbon monoxide derivative of the terminal oxidase of the hydroxylase system. The technique utilizes the fact that the degree of dissociation of the CO complex of a haemoprotein by monochromatic light of different wavelengths, but equal intensities, corresponds to the light absorption spectrum of the CO derivative of the reduced haemoprotein. In the case of an hydroxylase system, the light-induced dissociation of the CO complex can be measured by the release of the CO inhibition of the hydroxylation reaction. The photochemical action spectrum obtained for the steroid 21-hydroxylase by Omura _et al_. (1965) is shown in Fig. 1.2. The similarity to the Soret band of the CO complex of cytochrome P-450 is further evidence for the identity of adrenal microsomal cytochrome P-450 with the terminal oxidase of the steroid 21-hydroxylase enzyme system.

The photochemical action spectrum technique has subsequently been employed to show a similar role for the cytochrome P-450 of adrenal cortex mitochondria in the steroid 11β-hydroxylase (Wilson _et al_. (1965); Rosenthal and Cooper, 1967), cholesterol side-chain cleavage enzyme (Simpson and Boyd, 1967a) and the 18-hydroxylase systems (Greengard _et al_., 1967), as well for several other steroid

hydroxylases and drug metabilizing systems of liver endoplasmic reticulum (Voigt, 1968; Omura et al., 1965).

Thus, there is considerable evidence, that in the adrenal cortex, cytochrome P-450 is responsible for oxygen activation in steroid mono-oxygenases, both mitochondrial and microsomal.

Substrate binding by cytochrome P-450: Studies of the adrenal cortex microsomal 21-hydroxylase system (Narasimhulu, 1965) revealed that cytochrome P-450 is also the site of substrate binding. Addition of 17α-hydroxyprogesterone to adrenal cortex microsomes in the absence of NADPH, produced a change in the spectral properties of the microsomal haemoprotein. The resultant difference spectrum is characterised by the formation of an absorption minimum at 420 nm and a maximum at 390 nm. Addition of NADPH to the system resulted in the gradual disappearance of the spectral characteristics. The cyclic appearance and disappearance of this absorbance change occurred on readdition of the steroid substrate. Other mono-oxygenase systems such as the 11β-hydroxylation of DOC by adrenocortical mitochondria and the induced drug hydroxylation system of liver microsomes have been shown to produce similar substrate binding spectra (Cooper et al., 1965; Schenkman et al., 1967).

Titration of adrenocortical microsomes with 17α-hydroxyprogesterone produced saturation of the binding sites at high substrate concentrations (Narasimhulu et al., 1965). From such data, an apparent substrate dissociation constant (K_s) was calculated and was found to be close to the K_m value

(4μM) for the overall 21-hydroxylation reaction. However, Estabrook _et al_. (1968) suggested that, since the substrate-induced spectral change was not independent of the protein concentration, the value of K_s obtained was not equivalent to a true K_m but rather was representative merely of stoichiometric binding of the steroid to a reaction site associated with the hydroxylation reaction.

Induction of spectral alterations in adrenal microsomes or mitochondria have now been shown with the corticosteroid intermediates, 20α-hydroxycholesterol, pregnenolone, 17α-hydroxyprogesterone, deoxycorticosterone and 11-deoxycortisol (see Harding _et al_., 1969). The data of Cammer and Estabrook (1967) suggest that it is the oxidised Fe^{3+} form of cytochrome P-450 that binds substrates, since cytochrome P-450 in adrenocortical mitochondria is mainly in the oxidised form under aerobic steady state conditions.

Forms of cytochrome P-450. The specificity of the reactions concerned in steroid biosynthesis and the existence of genetic defects in humans involving the absence of one or other hydroxylation activities (Eberlein and Bongiovanni, 1956) predicts the existence of additional enzymes in the multi-enzyme hydroxylating pathway or the existence of substrate specific cytochrome P-450s.

The studies of Hildebrant _et al_. (1968) have indicated the presence in liver microsomes of two interconvertible forms of cytochrome P-450 (named P-450 and P-446) which have different absorption maxima in the oxidised state. One

form (P-446) is characteristic of a high spin type haemoprotein, whilst the other (P-450) resembles a low-spin type haemoprotein. Each of these forms is induced by one of two classes of compound, typified by phenobarbital in the case of P-450 and 3-methylcholanthrene in the case of P-446. Imai and Sato (1968) also observed the presence of two spectrally different states in the ethyl isocyanide complex of reduced cytochrome P-450. Mitani and Horie (1969a) from studies on adrenocortical mitochondria found that the absolute absorption spectra of cytochrome P-450 in the presence and absence of substrates suggested that type I and type II - difference spectra resulted from increases in the amount of high-spin and low-spin components, respectively. The spectrum in the absence of substrate was intermediary between the high and low-spin types of spectrum.

Harding et al. (1969) have studied the effect of 20α-hydroxycholesterol on the binding of deoxycorticosterone by adrenocortical mitochondria. Their hypothesis which explains the data requires that there be two specific P-450s in adrenocortical mitochondria. Type I P-450 binds 11β-hydroxylation substrates (420 nm, trough; 385 nm peak). The type II P-450 binds the 22R-hydroxylation substrate but can bind other steroids if they are present in high concentration.

Cytochrome P-450 - EPR Spectrum. In addition to the optical spectrum, cytochrome P-450 is also characterised by its electron paramagnetic resonance (EPR) spectrum. Addition of steroid substrate produces a change in the EPR spectrum as well as the optical spectrum (see Harding et al., 1969;

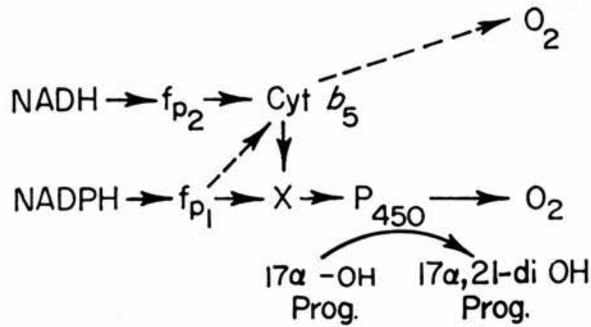
Simpson et al., 1969; Mitani and Horie, 1969b). The EPR signals obtained for adrenocortical mitochondrial cytochrome P-450 (Mitani and Horie, 1969b) correspond to those reported by Hashimoto et al. (1962) for liver microsomal Fe_x.

Electron Transport System of Mono-oxygenases

In the classical mitochondrial respiratory chain, the reaction of pyridine nucleotides with haemoproteins is mediated via electron carriers such as flavoproteins, quinones and non-haem iron proteins. Similarly, electron carriers are involved in the supply of reducing equivalents from NADPH to the cytochrome P-450. Omura et al. (1966) succeeded in resolving the steroid 11β-hydroxylase of adrenocortical mitochondria into three fractions - a particulate fraction containing cytochrome P-450, a FAD-containing flavoprotein and a non-haem iron protein which has been called 'adrenodoxin' (Suzuki and Kimura, 1965).

The flavoprotein associated with the 11β-hydroxylase system has a molecular weight of about 60,000 and contains FAD as its prosthetic group. This flavoprotein functions as a diaphorase only when NADPH is an electron donor. (Omura et al., 1966). The combination of both flavoprotein and non-haem iron protein is required for electron transfer from NADPH to cytochrome C.

The non-haem iron protein, adrenodoxin, has a molecular weight of about 12,000 and contains 2 atoms of iron and 2 atoms of acid-labile sulphur per molecule (Kimura and Suzuki, 1967). The absorption spectrum of the oxidised form of adrenodoxin has absorption bands at 415 nm and 455 nm



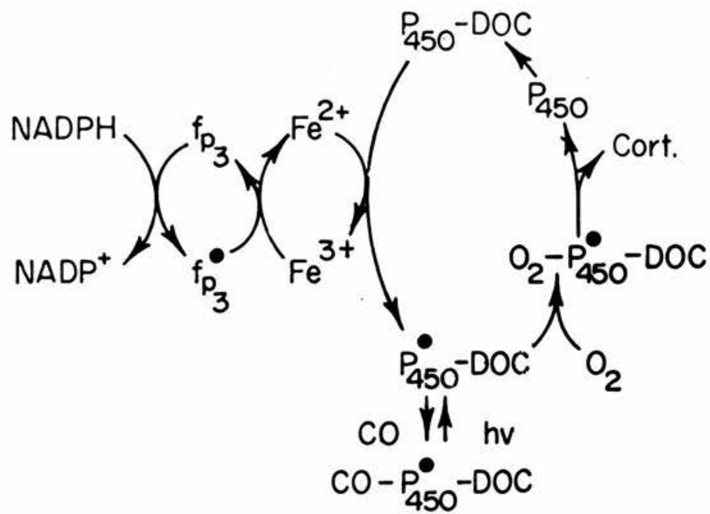


Fig. 1.3. Scheme of electron transport in microsomal and mitochondrial mixed-function oxidases of bovine adrenal cortex. After Simpson _et al._ (1969).

in the visible spectrum. The EPR spectrum of the reduced form has a prominent signal at $g = 1 \cdot 94$, typical of many non-haem iron proteins from both plant and animal ferredoxins (Beinert and Sands, 1960). A similar non-haem protein has been purified from testis mitochondria (Kimura and Ohno, 1968) which could substitute for adrenodoxin in adrenal steroid 11β-hydroxylation. Rat ovarian mitochondria also contain a protein with very similar properties (Kimura and Ohno, 1968; Sulimovici and Boyd, 1968b). The biochemical aspects of non-haem proteins such as adrenodoxin have been reviewed by Kimura (1968).

The flavoprotein and adrenodoxin constitute the NADPH-cytochrome P-450 reductase system. Combination of these components with cytochrome P-450 is required to restore 11β-hydroxylase activity.

The cholesterol side-chain cleavage system of adrenal mitochondria has been resolved into the same three components (Simpson and Boyd, 1967b; Bryson and Sweat, 1968). There is the possibility of the presence of a fourth component (Kimura, 1968) to determine the specificity of the particular hydroxylation reaction since 18-hydroxylation also occurs in adrenal cortex mitochondria (Greengard et al., 1967).

In microsomes, no EPR signal corresponding to the mitochondrial non-haem iron protein signal has been detected. Microsomal electron transport systems are less well understood, though certainly a flavoprotein is involved (Sweat et al. 1969). Williams and Kamin (1962) isolated a flavoprotein from liver microsomes which functions in drug hydroxylating systems.

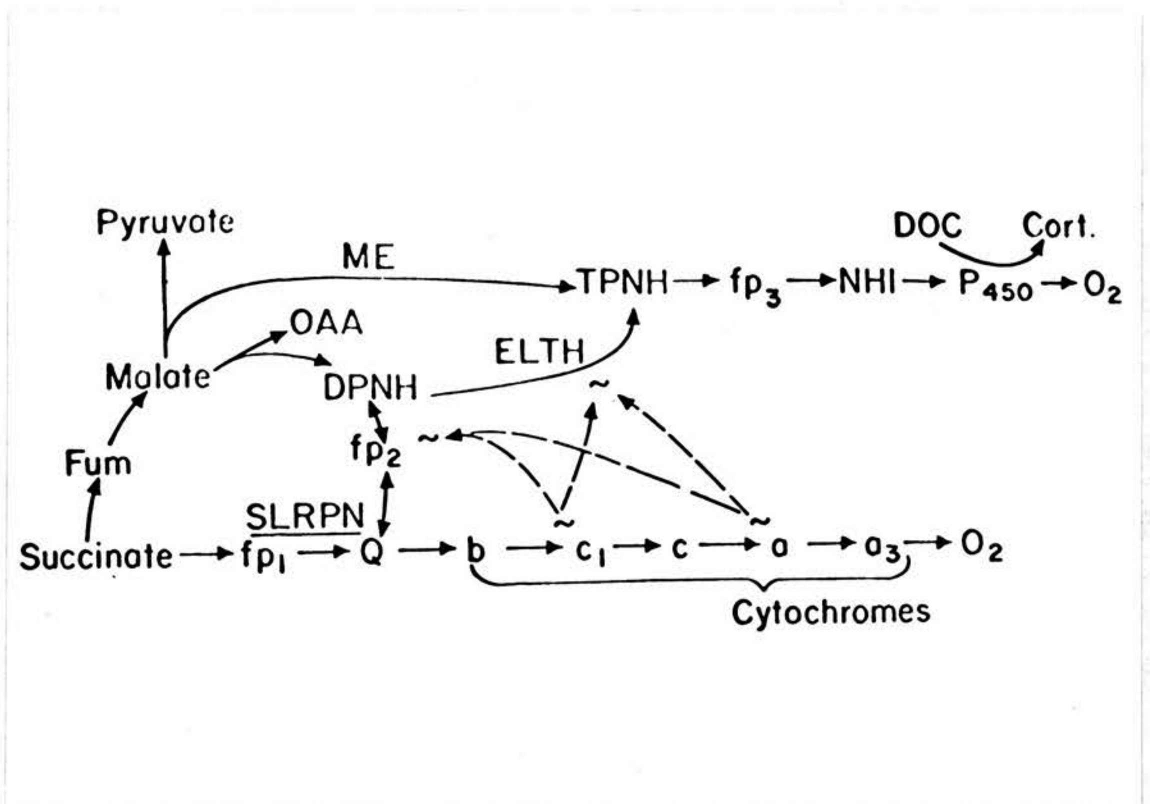


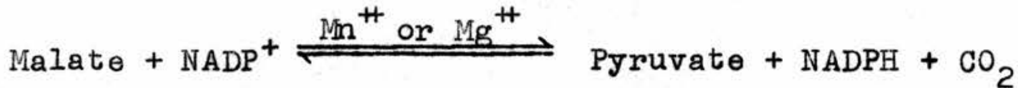
Fig. 1.4. Schematic representation of electron transport in bovine adrenal cortex mitochondria, showing the postulated role of energy-linked transhydrogenase (ELTH) and succinate-linked reduction of pyridine nucleotide (SLRPN) via reversible electron transport. OAA, oxaloacetate; ME, malic enzyme. After Cammer _et al_. (1968).

Fig. 1.3 shows schemes for the microsomal and mitochondrial mono-oxygenase electron transport pathways, as far as they are understood at the moment.

Supply of Reducing Equivalents during Steroid Hydroxylation

The immediate source of reducing equivalents for steroid mono-oxygenase reactions is NADPH. Presumably in the microsomal systems, cytochrome P-450 and the respiratory electron transport pathway are in functional contact with the cytoplasmic NADPH-generating systems such as glucose-6-phosphate dehydrogenase. Mitochondrial hydroxylation activity is, however, dependent upon the integrated action of the mitochondrial dehydrogenases and also the respiratory chain as shown in Fig. 1.4. The ability of Krebs tricarboxylic acid cycle intermediates to support the 11β-hydroxylation of deoxycorticosterone in adrenal cortex mitochondria has been known for a considerable time (Kahnt and Wettstein, 1951; Hayano and Dorfman, 1953; Brownie and Grant, 1954). Sweat and Lipscombe (1955) suggested that the tricarboxylic cycle intermediates supported 11β-hydroxylation by reducing the $NADP^+$ to NADPH, possibly via a transhydrogenase. Grant (1956) demonstrated the reduction of $NADP^+$ in adrenal mitochondrial acetone powders using fumarate as substrate. Grant and Brownie (1955) suggested the involvement of 'malic enzyme' in the reduction of $NADP^+$, since pyruvate was formed during the oxidation of fumarate in the presence of $NADP^+$. The difficulty was that 'malic enzyme' was generally thought to be exclusively a cytosol enzyme (Hsu and Lardy, 1967).

However, Simpson and Estabrook (1968) demonstrated conclusively the existence of a mitochondrial 'malic enzyme' in bovine adrenal cortex. The NADPH required for steroid 11β-hydroxylation could be accounted for by the activity of this enzyme which catalyzes the reaction:



$$\text{Malate} + \text{NADP}^+ \xrightleftharpoons{\text{Mn}^{++} \text{ or } \text{Mg}^{++}} \text{Pyruvate} + \text{NADPH} + CO_2$$

The bimodal distribution of the enzyme together with the different kinetic properties of the mitochondrial and cytosol enzymes led to the postulation of a 'malate shuttle' (Cammer *et al*., 1968; Simpson and Estabrook, 1969) as a mechanism of transferring reducing equivalents into the mitochondria.

Peron *et al*. (1966) and Guerra *et al*. (1966) using rat adrenal mitochondria showed that succinate— and Malate— supported 11β-hydroxylation was inhibited by the respiratory uncoupler, dinitrophenol. Harding *et al*. (1965), again using rat adrenal mitochondria, showed that antimycin A and cyanide at concentrations which inhibited respiration, abolished succinate-supported 11β-hydroxylation. These results prompted the suggestion that $NADP^+$ might be reduced by NADH via an emergy-linked transhydrogenase and that reduction of NAD^+ by succinate might occur via reversed electron transport in the NADH dehydrogenase part of the respiratory chain.

Thus reducing equivalents for mitochondrial monooxygenases arise from the action of mitochondrial dehydrogenases which transfer electrons from Krebs cycle intermediates in part via an energy controlled nucleotide transhydrogenase and in part by malate decarboxylation and reduction of $NADP^+$ by the malic enzyme. This is illustrated in Fig. 1.4.

Oxygen Utilization by Steroid Mono-oxygenases

Steroid mono-oxygenases by definition utilize molecular oxygen. Very little is known about the nature of oxygen activation and incorporation. It has been postulated that the perferryl ion Fe(II)-O_2 is the so-called active oxygen. However Sih (1969) has suggested, since such complexes are stable towards oxidation of unactivated carbon-hydrogen bonds, that the active form is the hydroperoxo-complex of ferrous ion $[Fe(II)\text{-}O\text{-}OH]^-$, a much more reactive species. Studies of Bray and co-workers (Knowles et al. 1969) have shown the presence of $O_2^{-\cdot}$ in the xanthine oxidase-catalyzed oxidation of xanthine - a mixed-function oxidation. However it must be remembered that $O_2^{-\cdot}$ is one of the more stable oxygen radicals. Corey and Gregariou (1959) suggested that the species of active oxygen in the hydroxylating intermediate is in a positively charged electrophilic form such as HO^+. The existence of HO^+ now appears unlikely (Samuel, 1968). Staudinger (1966) presented evidence concerning the attacking oxygen species and showed that hydroxylation may be mediated by the generation of an oxygen atom rather than free hydroxyl radicals. The oxygen atom ($\cdot\overline{\underline{O}}\cdot$) is regarded as a very powerful oxidant, capable of cleaving C-H bonds directly. Other speculations on the chemical nature of the active oxygen intermediate have been made - reviewed by Diner (1964) - but its true nature is still unknown.

Recent reviews on the nature of steroid hydroxylations and their associated enzymes include those by Simpson et al. (1969) and Harding et al. (1969).

THE CHOLESTEROL SIDE-CHAIN CLEAVAGE REACTION

Quantitatively the most important catabolic fate of cholesterol in mammalian systems is its degradation to bile acids which occurs in the liver (Danielsson et al., 1963). However, a very important physiological event is the degradation of cholesterol to produce steroid hormones. This reaction which occurs in endocrine tissue such as the adrenals, the gonads and the placenta, involves the removal of a C_6 unit from the alkyl side-chain of cholesterol to produce the C_{21} steroid, pregnenolone (Staple et al., 1956). There are reports (Shimizu et al., 1965; Jungman, 1968) that cholesterol can be converted directly to dehydroepiandrosterone which involves the simultaneous production of 2-methylheptan-6-one. Such a pathway would allow the direct production of androgens without the intermediary formation of C_{21} steroids. The relative importance of such a pathway compared to the classical pathway of C_{19}-steroid biosynthesis, i.e. cholesterol ⟶ pregnenolone ⟶ androgens, is not yet known.

Gurin and his collaborators (Lynn et al., 1954; Staple et al., 1956) first demonstrated that a six-carbon fragment is cleaved from cholesterol during the process by which the sterol is converted to pregnenolone.

The enzyme system responsible for the cleavage of the cholesterol side-chain between carbon atoms 20 and 22 has been most studied in adrenal cortex and is located in the mitochondria. Halkerston et al. (1961) using acetone powders of such mitochondria, demonstrated that the enzyme system required

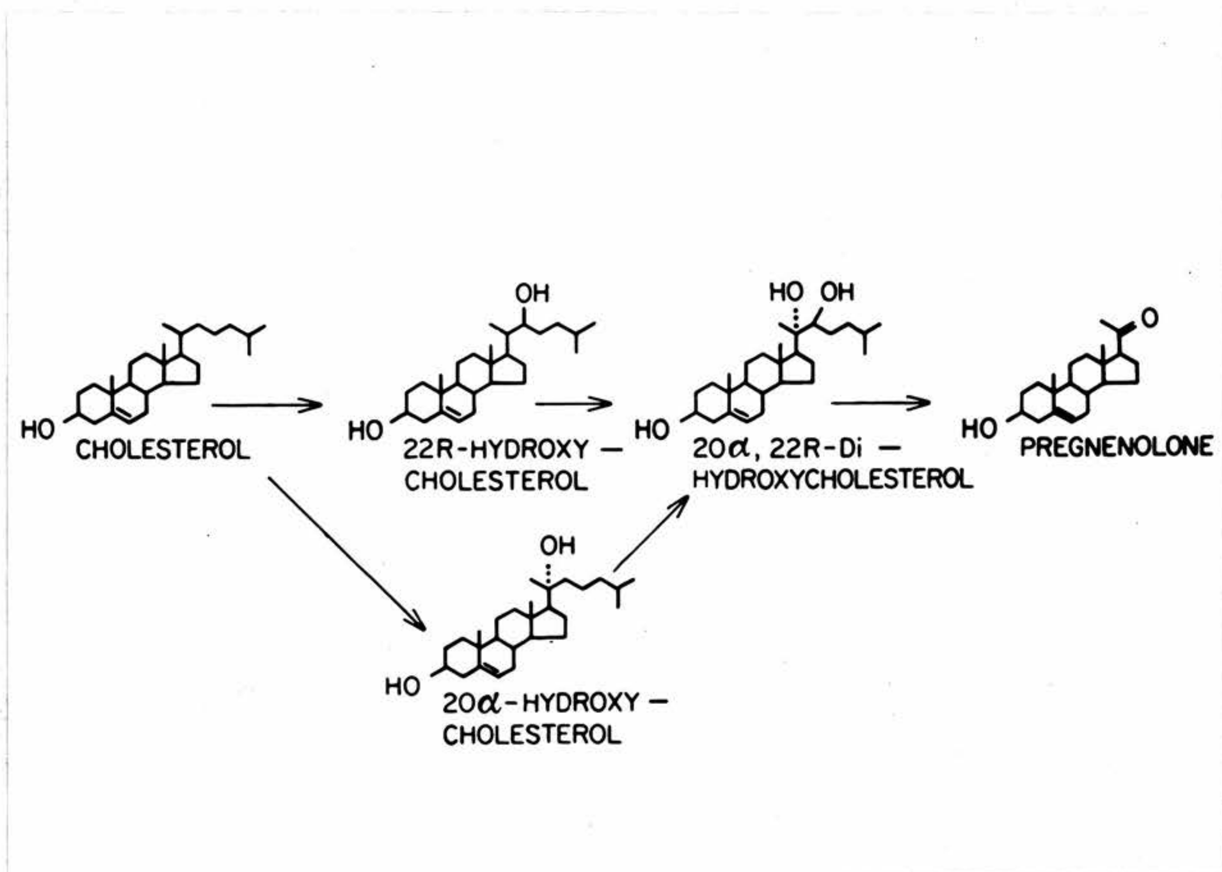


Fig. 1.5. The postulated pathways of the cholesterol side-chain cleavage reaction in endocrine tissue.

molecular oxygen and NADPH and thus partially fulfilled the requirement to be considered as a mixed-function oxidase or mono-oxygenase.

The most commonly postulated pathway (Fig. 1.5) for the conversion of cholesterol to pregnenolone in steroid-hormone producing organs entails 20α-hydroxycholesterol and 20α,22R-dihydroxycholesterol as intermediates (Shimizu et al., 1961; Constantopoulos and Tchen, 1961; Shimizu et al., 1962). This hypothetical scheme was advanced on the basis that (i) cholesterol is cleaved between carbons 20 and 22 giving rise to a six-carbon fragment (Staple et al., 1956; Constantopoulos et al., 1962), (ii) 20α-hydroxycholesterol is formed from cholesterol-4-^{14}C by cow adrenal cortex homogenates in the presence of various trapping agents (Solomon et al., 1956), and (iii) 20α-hydroxycholesterol and 20α,22R-dihydroxycholesterol are more efficiently transformed to pregnenolone than is cholesterol (Shimizu et al., 1961; Shimizu et al., 1962).

The question of the transformation of cholesterol into 20α-hydroxycholesterol or 20α,22R-dihydroxycholesterol has been controversial. Thus Solomon et al.(1956) claimed the formation of 20α-hydroxycholesterol from cholesterol, under certain trapping conditions, in the presence of 20α-hydroxycholesterol. Constantopoulos et al.(1962) described the accumulation of 20α,22R-dihydroxycholesterol with a bovine adrenocortical preparation in the presence of pregnenolone and progesterone. Ichii et al. (1963) described the formation of both sterols from cholesterol with bovine corpora lutea when pregnenolone was added to the medium. On the other

hand, Koritz and Hall (1964), Hall and Koritz (1964) and Simpson and Boyd (1967a) were unable to observe the formation of these sterols from cholesterol. Simpson and Boyd (1967a) also cast doubt upon the significance that can be attributed to the observation that 20α-hydroxycholesterol can inhibit the side-chain cleavage of cholesterol, since 25-hydroxycholesterol, 26-hydroxycholesterol and, especially, 24-hydroxycholesterol also inhibited the cleavage. Furthermore, the mechanism of inhibition produced by these hydroxylated cholesterol derivatives was apparently the same as that produced by 20α-hydroxycholesterol, so that the inhibition evoked by the latter compound could not be taken as evidence for its intermediacy.

22R-Hydroxycholesterol has also been shown to give rise to pregnenolone in bovine adrenocortical acetone-dried mitochondrial preparations (Chaudhuri et al., 1962) in a yield exceeding that realised from cholesterol, but this alternative pathway has been largely ignored. However, recently, Burstein and Gut (1969) have proposed 22R-hydroxylation as the initial step in the cholesterol side-chain cleavage reaction, since the formation of 20α-hydroxycholesterol was considerably slower than the formation of 22R-hydroxycholesterol. Furthermore, the overall rate of disappearance of 22R-hydrocycholesterol and the appearance (from it) of pregnenolone was relatively much higher than that obtained with 20α-hydroxycholesterol.

The problem is further complicated by the observation of Shimizu (1968) that the side-chain cleavage of 20α,22R-

dihydroxycholesterol has a requirement for molecular oxygen and NADPH, i.e. the cleavage of the dihydroxysterol is apparently a mixed-function oxidation.

From these reports, it is clear that the question of the identity of the intermediates between cholesterol and pregnenolone remains unclear. The evidence presented for the presence of these intermediates must be considered indirect such as mobility on chromatograms and recrystallization with authentic carrier. No-one has succeeded in isolating any of the products in well-characterized crystalline form.

Involvement of cytochrome P-450 in the cholesterol side-chain cleavage reaction

Simpson and Boyd (1966) demonstrated that the cholesterol side-chain cleavage activity in adrenocortical mitochondrial extracts was inhibited by carbon monoxide. Furthermore, the partition constant for the carbon monoxide inhibition was found to be about unity, and similar to the partition constant of cytochrome P-450 with carbon monoxide. In 1967, Simpson and Boyd showed that the carbon monoxide inhibition was light reversible, and the photochemical action spectrum for the light reversal showed a maximum at 450 nm, similar to the optical spectrum of cytochrome P-450 (Omura *et al.*, 1965). Thus cytochrome P-450 appeared to be involved in the cholesterol side-chain cleavage reaction in a similar capacity (i.e. as the oxygen-activating site) as had been reported for other steroid mixed-function oxidases (Estabrook *et al.*, 1963; Omura *et al.*, 1965).

Fractionation of the Cholesterol side-chain cleavage Enzyme System

Following the fractionation of the adrenal mitochondrial steroid 11β-hydroxylase system into three components - cytochrome P-450, a flavoprotein and a non-haem iron protein - (Omura *et al.*, 1966), Simpson and Boyd (1967b) partially resolved the adrenocortical mitochondrial cholesterol side-chain cleavage system into the three similar components. Thus the steroid 11β-hydroxylase and the cholesterol side-chain cleavage enzyme system appeared to be very similar, substrate specificity being determined by either separate cytochrome P-450s or some other factor. Bryson and Sweat (1968) confirmed the resolution of the adrenal cholesterol side-chain cleavage system using an ammonium sulphate fractionation technique. In 1968, Sulimovici and Boyd (1968b) showed that a similar three component system of NADPH diaphorase, non-haem iron protein and cytochrome P-450 was operative in the case of the rat ovarian cholesterol side-chain cleavage enzyme.

Precursor pool of cholesterol

Armstrong *et al.* (1964), using rat ovarian slices, observed that the specific activity of ^{14}C-progesterone biosynthesised from acetate-1-^{14}C was higher than the specific activity of the ^{14}C-cholesterol in the same incubation. The authors concluded that there was a lack of homogeneity of the ovarian cholesterol pool, with only part of the total sterol being drawn upon for steroid synthesis. The same concept that the precursor cholesterol might be a small portion

of the 'total cholesterol' in the adrenal gland had been previously proposed by Hayano _et al_. (1956). The studies of Koritz and Peron (1958) have also suggested that ACTH makes a steroid precursor 'available' in rat adrenal gland. Solod _et al_. (1966) proposed that in rabbit ovary the cholesterol laid down most recently, whether derived from plasma cholesterol or synthesised _in situ_ from acetate, was the first to be used for pregnenolone formation. When the rate of steroid secretion is increased by luteinizing hormone cholesterol deposited in the tissue at an earlier time is available for pregnenolone synthesis. The precursor pool of cholesterol for steroidogenesis might therefore be only a small fraction of the total cholesterol within the tissue. Such a situation appears feasible as cholesterol has an important structural role in the lipoprotein complexes of the subcellular organelles, as well as the quantitatively less important role as the steroid hormone precursor.

PLACENTAL STEROIDOGENESIS

Shortly after the isolation of pregnanediol from human pregnancy urine (Marrian, 1929; Dingemanse _et al_., 1930), it was realized that it is a major metabolite of progesterone (Browne _et al_., 1937) and that its excretion increased with progressing pregnancy (Venning, 1938). It was soon apparent that large amounts of progesterone were elaborated during pregnancy and that it was the placenta which was the site of origin. Similar evidence led to the conclusion that the large amounts of oestrogens elaborated

during pregnancy were also formed in the placenta. The early evidence has been reviewed by Diczfalusy and Troen (1961).

Conversion of acetate to cholesterol has been demonstrated in both perfused placenta (Levitz *et al.*, 1962) and in placental tissue minces (Van Leusden and Villee, 1965). However, Villee *et al.* (1966) have concluded that the rate of cholesterol synthesis from mevalonate is much less than the rate of progesterone production. In late pregnancy, the placenta is capable of producing over 250 mg. of progesterone per day (Pearlman, 1957). It is possible, therefore, that the circulating plasma cholesterol (as lipoprotein) could act as the precursor of progesterone. It is generally accepted that the placenta is an "incomplete" endocrine organ that does not synthesize complex steroids and sterols from acetate in amounts sufficient to explain the large steroid output during pregnancy. Preformed precursors of maternal and foetal origin, such as dehydroepiandrosterone sulphate (Siiteri and MacDonald, 1963) and 16α-hydroxydehydroepiandrosterone sulphate (Magendantz and Ryan, 1964) reach the placenta via the foetal and maternal blood and are converted to oestradiol, oestrone and oestriol by the placental aromatizing system. Placental tissue contains very small amounts of steroid 17-20 lyase (desmolase), as demonstrated by the conversion of progesterone to androst-4-ene-3,17-dione (Little *et al.*, 1963; Warren and Cheatum, 1964) but the pathway appears insignificant. This enzyme system is much more active in the foetal adrenal - illustrating the function of the complete "foetal-placental

unit".

The hydroxysteroid dehydrogenases occur in considerable amounts in placental tissue. The most extensively studied placental enzyme is probably the 17β-oestradiol dehydrogenase. This soluble enzyme has been studied principally by Engel and collaborators (see Karavolas et al., 1969) and Talalay et al., 1958). Considerable information is available about the placental 3β-hydroxysteroid dehydrogenase and the Δ^5 to Δ^4-isomerase system. The reaction is rapid (Pion et al., 1966). Koide and Torres (1965) provide evidence that the enzyme is present in both particulate fractions of the tissue, mitochondria and microsomes.

Several steroid hydroxylases are present in placental tissue and as far as is known require both NADPH and molecular oxygen. The most important (quantitatively, at least) are the mitochondrial cholesterol side-chain cleavage system (described in the next section)and the microsomal aromatising system.

The mechanisms involved in aromatization have not yet been fully elucidated, but it is generally accepted that 19-hydroxylation of a Δ^4-3-oxo-structure is an early step in the procedure (Wilcox and Engel, 1965). However, the enzyme appears to be carbon monoxide insensitive (Meigs and Ryan, 1968) and therefore possibly does not involve cytochrome P-450. Recently Shaw et al. (1969) showed that the enzyme system was also present in placental mitochondria, and provided an interesting example of a bimodal distribution of a steroid hydroxylase.

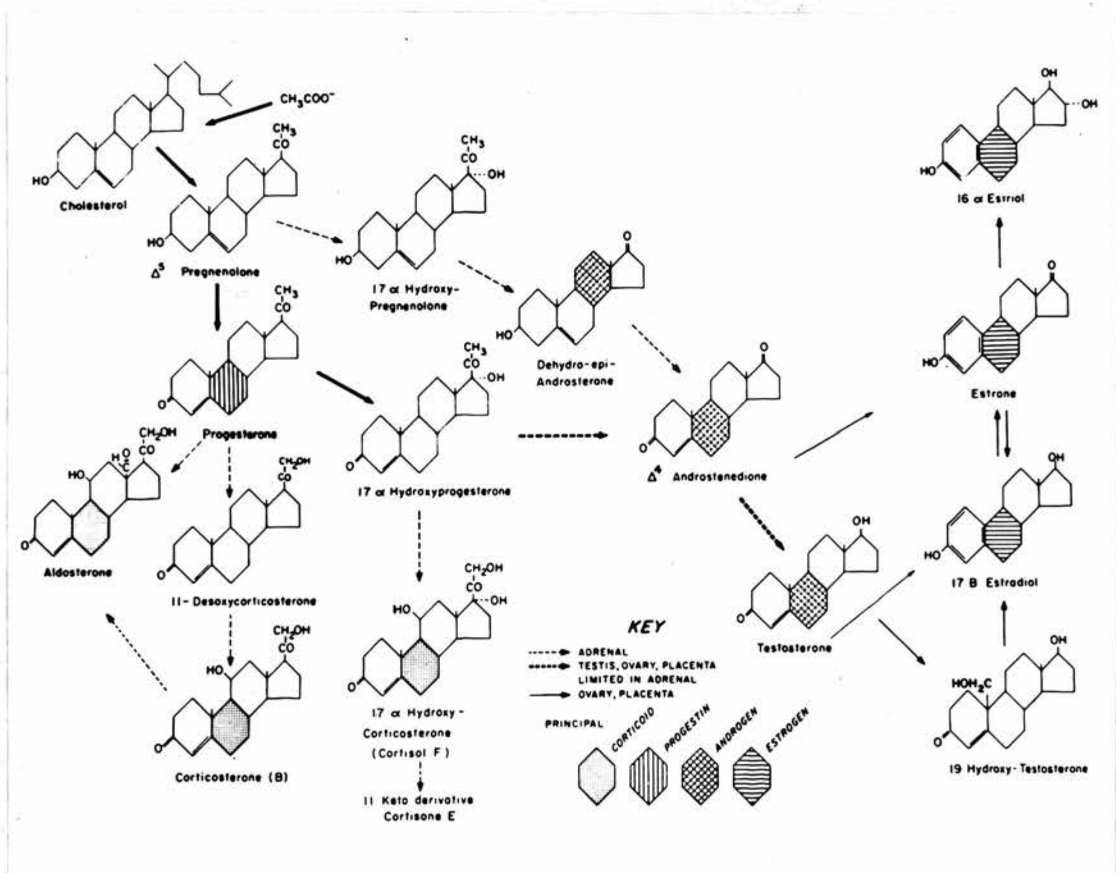


Fig. 1.6. Steroid biosynthesis occurring in the placenta and in other endocrine tissues that produce steroids. After McKerns (1969).

Hydroxylation of oestrone, oestradiol and oestriol at C-2 catalyzed by a placental microsomal enzyme has been described (Lucis, 1965; Fishman and Dixon, 1967).

Other placental steroid hydroxylation reactions have been reviewed by Mitchell (1967) and Hagerman (1969).

Fig. 1.6 illustrates the pathways of steroidogenesis in placental tissue, and compares these pathways with those known to occur in adrenal and ovarian tissue.

The Placental Cholesterol Side-chain Cleavage Enzyme System

In 1945, Bloch found that in late pregnancy blood-borne deuteriated cholesterol was converted to pregnanediol, presumably via progesterone. Placental perfusion experiments (Solomon et al., 1954, 1960) indicated that radioactive cholesterol was converted to progesterone. Shimizu et al. (1961) demonstrated that 20α-hydroxycholesterol was converted to progesterone using a placental homogenate.

However, an active cholesterol side-chain cleavage enzyme system was not isolated from placental tissue until 1965 (Morrison et al., 1965). These workers found that, as in the other endocrine tissues the enzyme was located in the mitochondria. A so-called 'solubilized' preparation of the enzyme was obtained from acetone powder extracts of placental mitochondria. Attempts reported in this study to fractionate the enzyme system and identify the components were unsuccessful. Ryan et al. (1966) reported that native placental mitochondria converted radioactive cholesterol to progesterone,

with a small yield (0.6%). However, a net synthesis of progesterone by placental mitochondria was observed.

In 1968, Meigs and Ryan showed the cholesterol side-chain cleavage activity of placental mitochondrial acetone powders was inhibited by carbon monoxide; this inhibition being light reversible. In the same study, the presence of cytochrome P-450 in both placental mitochondria and microsomes was first reported, but at a much lower concentration (about one tenth) than in adrenal cortex tissue.

Our knowledge of the placental cholesterol side-chain cleavage systems is based on these few reports.

Aims of the present study

This study has been concerned with a particular sterol mono-oxygenase - the cholesterol side-chain cleavage enzyme system of human term placenta. The preceding pages show that by contrast with the mono-oxygenases of other tissues, this enzyme has received less attention than it deserves, especially when the importance of the placenta as a source of C_{21} metabolites of cholesterol is taken into consideration. Moreover, the physiological circumstance of its rapid postpartum extrusion provides an exceptional opportunity for the study of a normal human tissue. It was hopedthat isolation and purification of the placental enzyme system would allow comparison with the much studied adrenal cortex and ovarian sterol and steroid hydroxylases.

CHAPTER 2

EXPERIMENTAL PROCEDURE

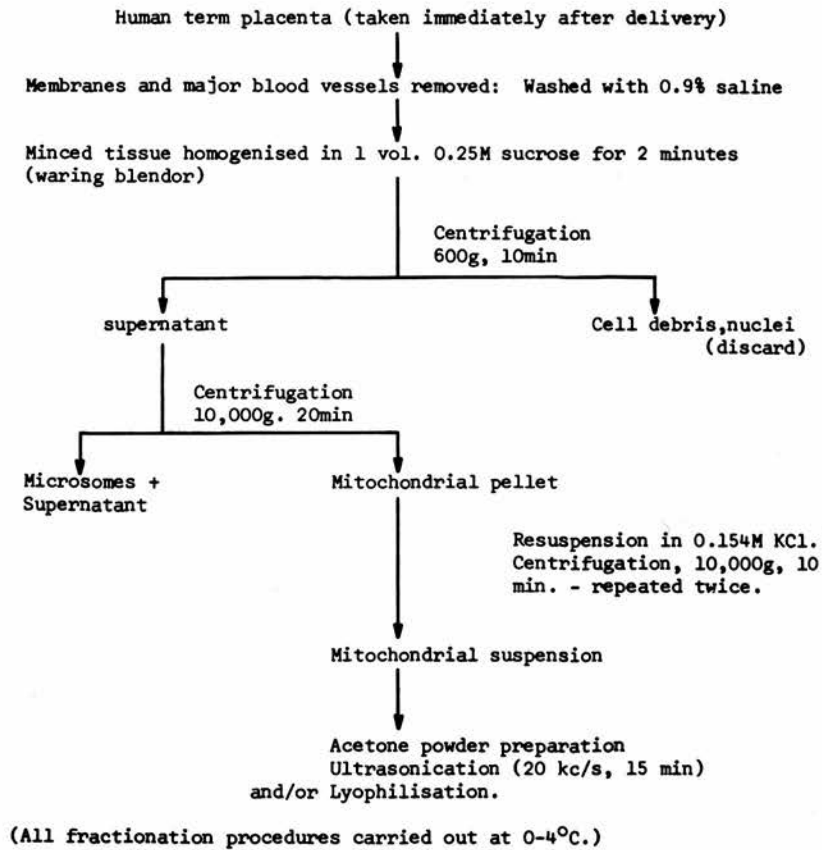


Fig. 2.1. Subcellular fractionation procedure for a placental homogenate.

EXPERIMENTAL PROCEDURE

Preparation of Tissue and Isolation of Subcellular fractions

Human term placentae were obtained after normal delivery and placed on ice. The preparation of the tissue was commenced within one hour of delivery. The membranes and major blood vessels were removed. The tissue was washed with either 0·25 M sucrose or 0·9% sodium chloride solutions in an attempt to remove as much of the blood as possible. The washed tissue was then minced using a domestic meat grinder. The minced tissue was then homogenised in 1 vol. 0·25 M sucrose for 2 minutes using a top drive Waring Blender (MSE).

The homogenate was centrifuged at 600 x g. for 10 minutes to sediment the nuclei and cell debris. The supernatant was decanted and retained while the sediment was rehomogenised in 1 vol. 0·25 M sucrose and centrifuged at 600 x g. for a further 10 minutes. The combined supernatants were centrifuged at 10,000 x g. for 20 minutes to sediment the mitochondria. The 10,000 x g. supernatant was further centrifuged at 105,000 x g. for 60 minutes. The mitochondrial fraction was suspended in 0·154 M potassium chloride and centrifuged at 10,000 x g. for 10 minutes. This washing procedure was repeated twice. All the described operations were carried out between 0 - 5°C. The subcellular fractionation procedure is outlined in Fig. 2.1.

Bovine adrenal cortex mitochondria were prepared in a similar manner to the placental mitochondria, except that the tissue was homogenised in 2 vols. 0·25 M sucrose.

Method of assay

An enzymic assay has to be reproducible, rapid, sensitive and simple. Spectrophotometric and fluorometric methods are generally the techniques of choice and such methods are employed in the study of the enzymes involved in steroidogenesis. In such cases the interpretation of the results is complicated by the possibility that the steroids determined colorimetrically could arise either from de novo synthesis or from the release of preformed stored steroids. In the case of the cholesterol side-chain cleavage reaction, such methods are not useful because pregnenolone, the product of the reaction, has no dominant chromophore either in the visible or the ultra-violet region of the spectrum. The problem is further complicated, particularly in the case of the subcellular fractions, by the fact that the added cholesterol substrate will be chemically indistinguishable from the endogenous cholesterol and will equilibrate with it to an unknown extent. For these reasons, it was decided to use a radioactive method of assay, using [4-^{14}C] cholesterol (Radiochemical Centre, Amersham) of high specific activity (60 mc/mmole). Methods of separating and quantitating radioactive steroids were available in the laboratory. These procedures had been developed for studies on the cholesterol side-chain enzyme systems of bovine adrenal cortex and rat ovarian mitochondria (Simpson and Boyd, 1967a; Sulimovici and Boyd, 1968a).

The enzymic assay used in this study was based on the conversion of added [4-^{14}C] cholesterol to radioactively

labelled C_{21} steroids. Radioactive product formation over a standard period of time was used as a measure of the enzymic activity. The concentration of unesterified endogenous cholesterol in native mitochondria was found to be about 300 μg. per incubation. Each incubation contained mitochondria from approximately 18 g. wet weight placental tissue. Similarly, approximately 10 μg. cholesterol per incubation was found when a lyophilized mitochondrial extract preparation was employed as the enzyme source, again derived from approximately 18 g. wet weight tissue. The cholesterol estimations were performed using the Liebermann-Burchard reaction described later in this section. As it is difficult to obtain conclusive evidence concerning the equilibration of the added $[4-^{14}C]$ cholesterol with the endogenous cholesterol of the mitochondria, it was decided to calculate the enzymic activity as the rate of conversion of the exogenous $[4-^{14}C]$ cholesterol into C_{21} radioactive metabolites. Throughout this work, the cholesterol side-chain cleavage activity is expressed as the percentage conversion of $[4-^{14}C]$ cholesterol to radioactively labelled C_{21} steroids.

The $[4-^{14}C]$ cholesterol was chromatographed by thin-layer chromatography (TLC) on Silica-gel H (developing solvent; di-isopropyl ether-petroleum spirit, B.Pt., 60 - 80°C - acetic acid, 70:30:2) to remove possible autoxidised cholesterol contaminants. The region corresponding to a standard cholesterol reference sample (Organon) was eluted from the plate with redistilled acetone. Each batch of $[4-^{14}C]$ cholesterol was "cleaned" in such a way immediately before use.

It is possible (see also pp. 104-105) that the radioactive technique described cannot be regarded as providing an accurate estimate of the enzymic activity in all circumstances, but it was felt that valuable comparative information would be obtainable by its use in suitably designed experiments.

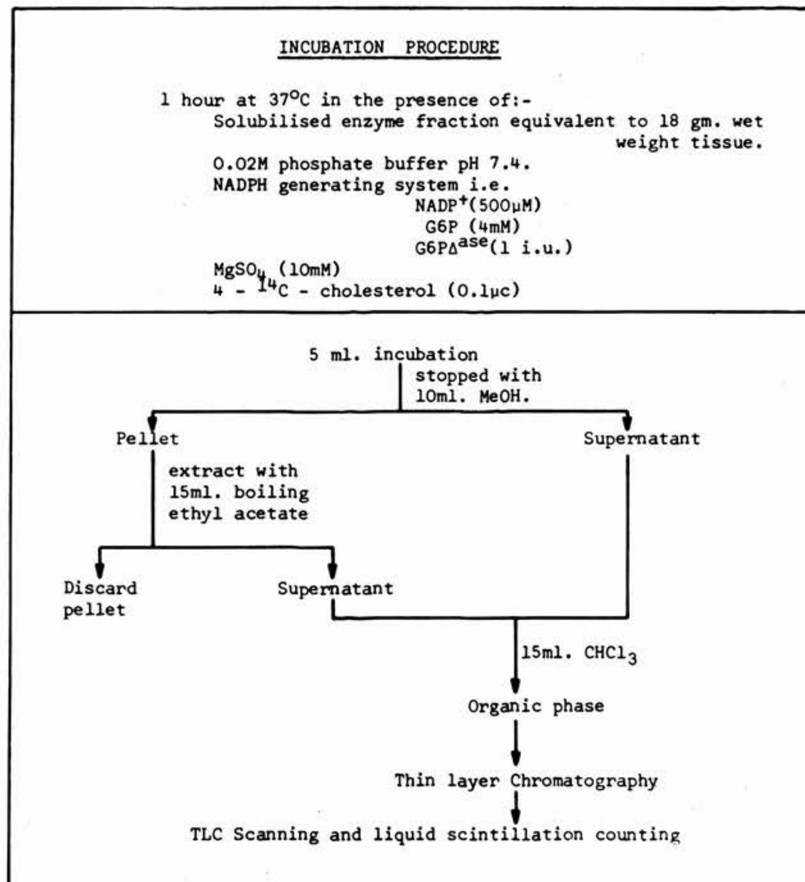


Fig. 2.2. The incubation and extraction procedures for the cholesterol side-chain cleavage assay.

Incubation procedure

The various mitochondrial enzyme preparations (equivalent originally to 20 mg. mitochondrial protein) were incubated with $4\text{-}^{14}C$-cholesterol (0·1 μC) in the presence of an NADPH generating system (5 μmol $NADP^{+}$, 50 μmol glucose-6-phosphate, 1·0 i.u. of glucose-6-phosphate dehydrogenase). The incubations were carried out in a total volume of 5·0 ml. at 37°C in air, using 25 ml. Erlenmeyer flasks and a Dubnoff incubator with constant shaking. The incubation period varied from 15 - 60 minutes depending on the activity of the particular enzyme preparation. Incubations were commenced by the addition of the radioactive substrate in 0·05 ml. acetone.

Extraction procedure

The incubations were terminated by the addition of 10 ml. methanol. The protein precipitate was centrifuged down and re-extracted with 15 ml. boiling ethyl acetate. 15 ml. chloroform was added to the combined extracts and the mixture centrifuged to separate the organic and aqueous phases. On removal of the aqueous phase, the organic phase was taken to dryness using a current of air on a warm water bath. Figure 2.2 summarises the extraction procedure.

Separation using thin-layer chromatography

The residue was dissolved in 0·3 ml. chloroform and transferred to a thin-layer plate (20 cm. x 5·2 cm.) coated with silica gel H (Merck). This process was repeated three times for each residue resulting in 95-98% of the initial

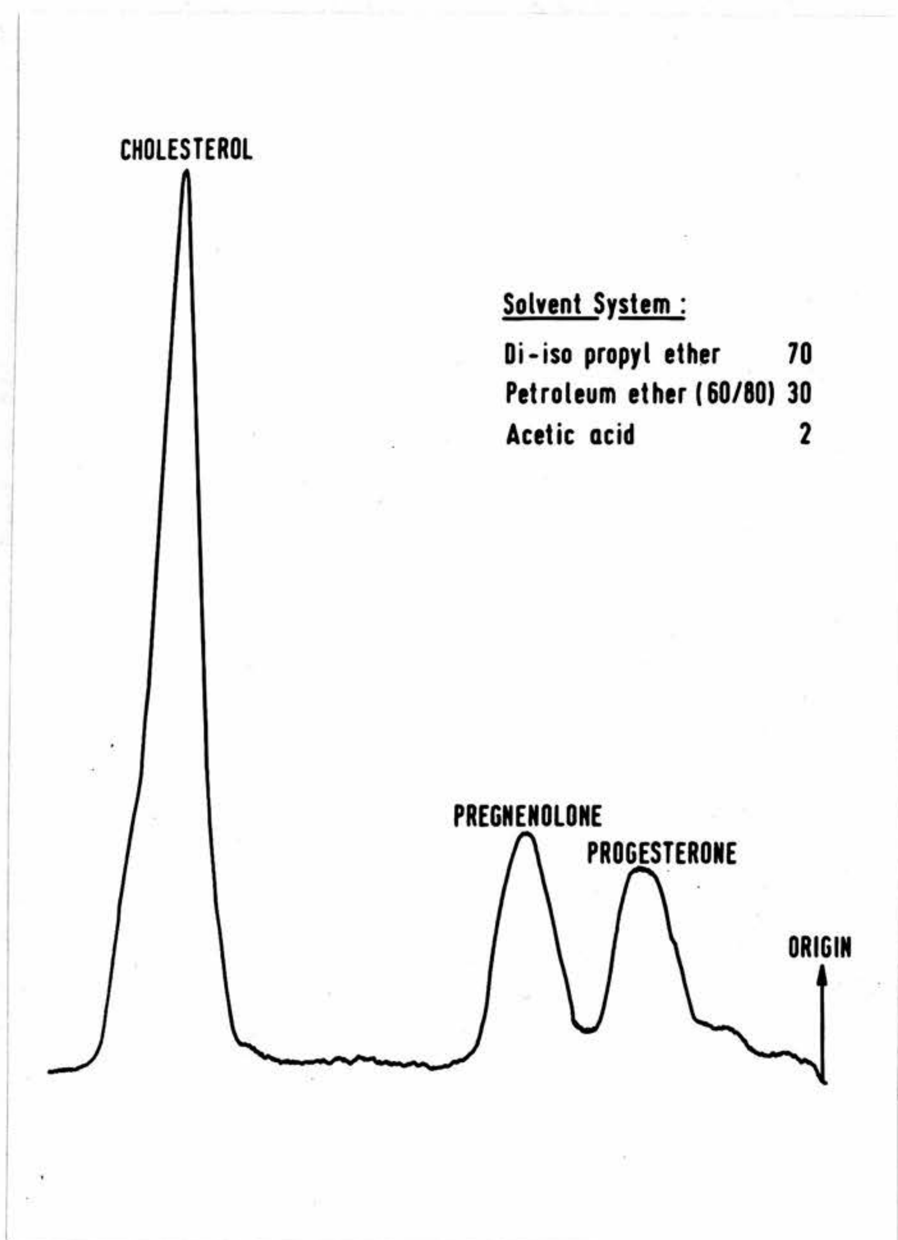


Fig. 2.3. Thin-layer radiochromatogram showing the substrate and products after incubation of supernatant of a placental mitochondrial sonicate with [4-^{14}C] cholesterol and NADPH.

radioactivity being transferred to the plate. Standard non-radioactive steroids were also applied at the origin. The thin-layer plates were developed in the solvent system, di-isopropyl ether-petroleum spirit (60-80°C, b.pt.) - acetic acid (70:30:2, v/v). The plates were developed twice in the same direction in this solvent system. After chromatography the standards were visualised by spraying with a 1:4 dilution of the scintillation liquid (see next section) with methanol and viewing under an ultraviolet lamp. The plates were scanned in a gas flow thin-layer scanner (Ravenhill and James, 1967). Figure 2.3 shows a typical scan obtained on such a scanner after incubation of a lyophilized placental mitochondrial extract with [4-^{14}C] cholesterol. The two radioactive products had the same mobility as the standard pregnenolone and progesterone. The solvent system also had the advantage of separating the postulated intermediates of the cholesterol side-chain cleavage reaction (namely, 20α,22R-dihydroxy-cholesterol) from cholesterol and the C_{21} steroids (Simpson and Boyd, 1967a).

Measurement of radioactivity

The regions on the TLC plates corresponding to the radioactive and standard steroids were directly scraped into liquid scintillation vials. The scintillation liquid employed was 4 g./litre 2,5-diphenyloxazole (PPO) and 30mg./ litre 1,4-bis-(5-phenyl-oxazolyl-2)-benzene (POPOP) in dry toluene containing 5% (v/v) methanol. 5.0 ml. scintillation fluid was added to each vial and the radioactivity was determined using a Packard Tri-carb Model 314-EX liquid

scintillation spectrometer. Under the conditions of operation carbon-14 was counted with an efficiency of 72%. No quenching was observed. Mitton (1967) showed that addition of silica gel to the counting vial did not affect the counting efficiency.

Table 2.1 shows the recovery of radioactivity throughout all the procedures for a typical incubation.

Assay for 3β-hydroxysteroid dehydrogenase activity

The enzymic assay for the 3β-hydroxysteroid dehydrogenase activity was based on the cholesterol side-chain cleavage enzyme assay described in the earlier sections of this chapter. [4-^{14}C] pregnenolone (Radiochemical Centre, Amersham) replaced the [4-^{14}C] cholesterol as the substrate and NAD^+ (0·2 mM) was added as the co-enzyme in place of the NADPH. The radioactive steroids were analysed and quantitated as before except that the TLC development was carried out in chloroform-ethyl acetate (80:20, v/v).

Protein determinations

Protein concentrations were determined routinely by the modified biuret method of Layne (1957). Determinations of small protein concentrations were performed using the method of Lowry et al. (1951). In both cases, bovine serum albumin (Sigma, London) was used as the protein standard.

Estimation of haem iron

This was measured by a modification of the method of Adler and George (1965). All glassware for the iron deter-

TABLE 2.1

RECOVERY OF RADIOACTIVITY DURING THE ASSAY PROCEDURE

Radioactivity added as [4-^{14}C] cholesterol	Radioactivity recovered (c.p.m.)		Percentage radioactivity recovered
200,090 c.p.m.	In incubation flask	20	0·01
	After extraction	1,114	0·6
	After spotting the extract	995	0·5
	On T.L.C. plate	196,257	98·0
		TOTAL	99·11

minations were soaked overnight in strong hydrochloric acid and washed thoroughly with distilled water before use. To a 1·5 ml. aliquot, 0·5 ml. concentrated hydrochloric acid and 0·5 ml. 25% trichloroacetic acid were added. After standing for 10 minutes the samples were centrifuged. The supernatant was analysed for non-haem iron (see next section) and the precipitate was incubated for 30 minutes in a boiling water bath with 0·1 ml. hydrogen peroxide and 0·1 ml. perchloric acid. After cooling, 0·1 ml. 10% aqueous hydroxylamine hydrochloride was added and the samples were allowed to stand for 5 minutes. Then 1 ml. pyridine and 0·1 ml. bathophenanthroline sulphonate solution (1 mg./ml.) were added to each sample. After 10 minutes the solutions were read at 535 nm. using a Unicam S.P. 600 spectrophotometer. The acid non-extractable iron was taken as a measure of haem iron. The iron determinations were made quantitative using a calibration curve constructed using a freshly prepared ferrous ammonium sulphate solution.

Estimation of non-haem iron

This was measured by a modification of the method of Massey (1957). The supernatant remaining after the acid precipitation (see previous section) was decanted. To a 1·5 ml. aliquot of this fraction was added 1·0 ml. pyridine followed by 0·1 ml. ascorbic acid solution (5 mg./ml.) and 0·1 ml. bathophenanthroline sulphonate solution (1 mg./ml.). After standing for 10 minutes, the solutions were read at 535 nm. using a Unicam S.P. 600

spectrophotometer. The acid extractable iron was taken as a measure of the non-haem iron.

Estimation of labile sulphide

The inorganic sulphide (labile sulphide) of the non-haem iron protein was determined by an adaption of the method of Fogo and Popowsky (1949). The sample to be analysed (in 1·0 ml. of solution) was placed in 0·3 ml. of 4% zinc acetate in a tube 10 x 75 mm., and 0·05 ml. of 12% sodium hydroxide was added. The tubes were stoppered and 0·25 ml. of 0·5% N, N-dimethylphenylenediamine hydrochloride and 0·05 ml. of 0·023 M ferric chloride (in 1·2 N HCl) were added to each tube; the stopper was replaced on the tubes rapidly after each addition. After 20 minutes, 0·85 ml. of water was added to each tube and the absorbance was determined at 670 nm. Using such an assay system, Lovenberg et al. (1963) found that 1 μmole of sodium sulphide gave an absorbance of 11·4 at 670 nm. This extinction coefficient was used to calculate the inorganic sulphide content. These workers also found that cysteine,oxidised and reduced glutathione, insulin and bovine serum albumin did not react in this test, thus indicating that this method is specific for inorganic sulphide.

Estimation of the cholesterol (non-esterified) content of mitochondrial preparations

The cholesterol content of mitochondrial preparations were estimated by the Liebermann-Burchardt reaction. Chloroform-methanol (2:1, v/v) extracts of the preparations

were taken to dryness under a stream of nitrogen after removal of the aqueous phase. Chloroform was used as the solvent for the dried extract. Redistilled acetic anhydride containing 5% v/v. of concentrated sulphuric acid was prepared in a flask immersed in an ice-bath and then allowed to warm to room temperature at which point it was added to the chloroform phase. The colour was developed at 25°C for 30 minutes and the optical density of the solution estimated at 625 nm. using 1 cm. light path glass cells and a Unicam S.P. 600 spectrophotometer. Suitable blank solutions and solutions of known cholesterol content were run with each group of estimations (Boyd, 1962).

The estimation of mitochondrial phospholipid

The method employed for mitochondrial phospholipid determinations was based on that used by Scholan (1969) to estimate microsomal phospholipid. Methanol (10 ml.) was added to a suitable aliquot of the mitochondrial phospholipid and the suspension refluxed. After centrifugation, chloroform (20 ml.) was added to the supernatant followed by water (7·5 ml.). The resultant system was allowed to undergo surface dialysis overnight. Samples were taken from the chloroform layer, evaporated to dryness and 60% perchloric acid (2 ml.) added. The solution was digested until clear. After cooling and diluting, 2·0 ml. of the amidol/sodium metabisulphite reducing solution (1 g. amidol + 20 g. sodium metabisulphite in 100 ml. water) followed by 1 ml. of 8% ammonium molybdate solution.* The solution was diluted to 15 ml. and the optical density recorded at 570 nm. after

* Allen, (1940).

10 minutes. A calibration curve for phosphorus content was prepared using potassium phosphate solutions. The value for the inorganic phosphorus content was multiplied by 25 in order to obtain the value for the phospholipid content of the mitochondrial fraction.

CHAPTER 3

PRELIMINARY STUDIES ON THE CHOLESTEROL SIDE-CHAIN CLEAVAGE ENZYME OF HUMAN PLACENTA

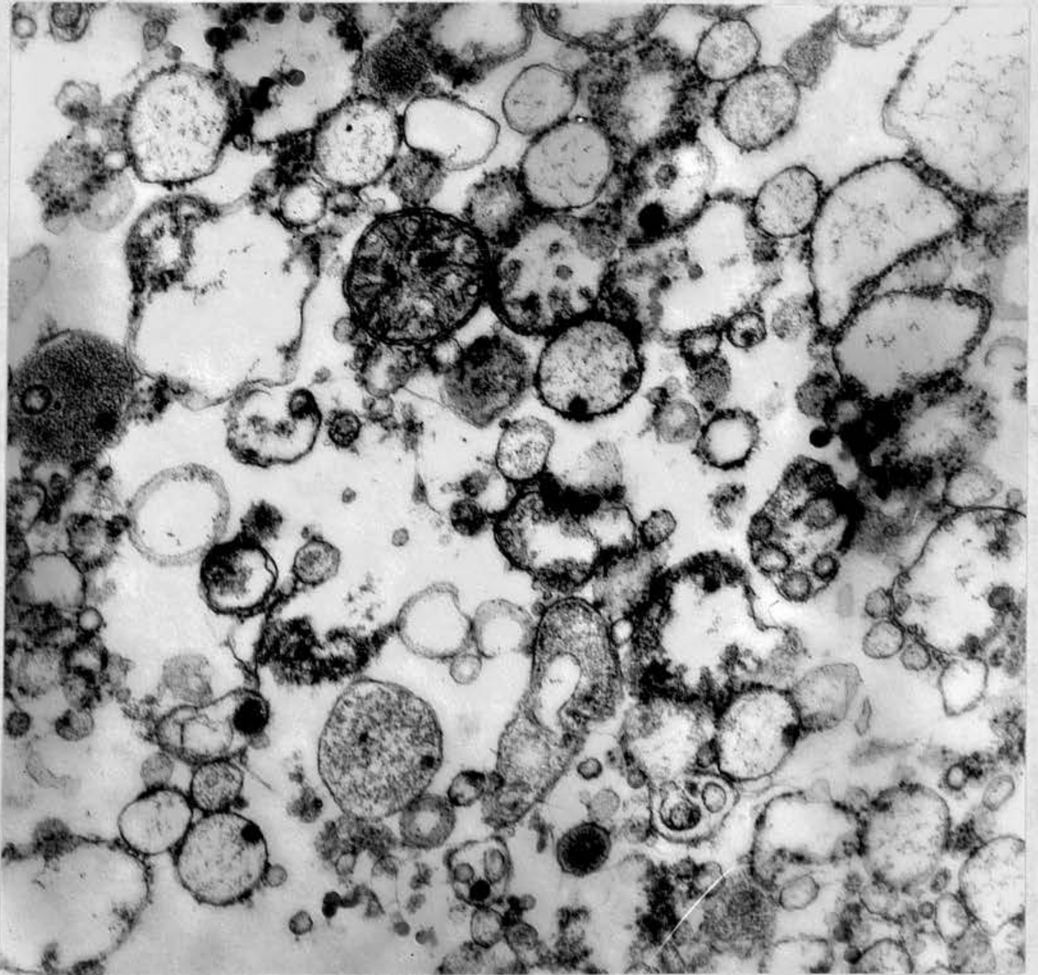


Fig. 3.1. Electron micrograph of a placental mitochondrial fraction. (magnification x 32,000).

(A)

PRELIMINARY STUDIES ON THE CHOLESTEROL SIDE-CHAIN CLEAVAGE ENZYME SYSTEM OF NATIVE PLACENTAL MITOCHONDRIA

Distribution of the enzyme

Experiments were carried out to establish in which cell fraction the cholesterol side-chain cleavage activity of human term placenta was located. Incubations were set up, as shown in Table 3.1, containing mitochondria or microsomes equivalent to 18 g. wet weight placental tissue. Incubations were performed at $37^{o}C$ for 60 minutes. The results for separate experiments are shown in Table 3.2. It was found that the cholesterol side-chain cleavage activity was located only in the mitochondrial fraction. These results are in agreement with those obtained by Morrison et al. (1965). It is also in accord with most results on the location of the cholesterol side-chain cleavage activity in other endocrine tissues (e.g. Halkerston et al. (1961), Hall and Koritz (1964)).

An electron microscopic examination (kindly performed by the Dept. of Pathology, University of Edinburgh) of the isolated so-called mitochondrial fraction confirmed the view that the preparation was of mitochondrial origin (see Figure 3.1). However, many of the mitochondria appeared damaged by the homogenisation procedure and the preparation did contain some endoplasmic reticulum.

When native placental mitochondria (equivalent to 18 g. wet weight tissue) were incubated with $[4-^{14}C]$ cholesterol and the NADPH generating system using the standard assay technique described in Chapter 2, the only radioactive metabolite detected was progesterone. In one hour incubations

TABLE 3.1

TYPICAL INCUBATION MIXTURE

	Amount	Volume ml.
Mitochondria	20 mg. protein/ml.	1·00
OR		
Solubilised mitochondrial extract	4 mg. protein/ml.	
$NADP^+$	5 µmoles	0·50
D-Glucose-6-phosphate	50 µmoles	0·50
D-Glucose-6-phosphate dehydrogenase	1 i.u.	0·10
Magnesium sulphate	50 µmoles	0·25
[4-^{14}C] Cholesterol	0·1 µC	0·05

All incubations were performed in a total volume of 5.0 ml in the presence of 20 mM phosphate buffer, pH 7.4.

TABLE 3.2

PERCENTAGE CONVERSION OF [4-^{14}C] CHOLESTEROL TO ^{14}C-PREGNENOLONE, ^{14}C-PROGESTERONE AND TOTAL CONVERSION IN MITOCHONDRIA, MICROSOMES AND 105,000 g. SUPERNATANT FROM HUMAN PLACENTAL TISSUE

Subcellular fraction	Pregnenolone	Progesterone	Total Conversion
Washed mitochondrial. 1.	0·3	7·6	7·9
(20 mg. protein/ml. 2.	0·2	4·9	5·1
Washed microsomes 1.	0·1	0·2	0·3
(22 mg. protein/ml.) 2.	0·1	0·1	0·2
105,000 g. supernatant 1.	0·1	0·1	0·2
(19 mg. protein/ml.) 2.	0·1	0·2	0·3

at 37°C, the conversions of [4-^{14}C] cholesterol to progesterone varied between 4-8%. Pregnenolone, the usually accepted product of the cholesterol side-chain cleavage reaction was not detected. This means that the mitochondrial preparation contained an active 3β-hydroxysteroid dehydrogenase-Δ^{4-5} isomerase system. This enzyme system is normally assumed to be of microsomal origin. The electron-microscopic examination revealed that there were contaminating microsomes present in the mitochondrial preparation.

Sources of reducing power for the cholesterol side-chain cleavage reaction in placental mitochondria

The immediate source of reducing power for the cholesterol side-chain cleavage reaction in bovine adrenal cortex is NADPH (Halkerston et al., 1961). This is in accord with the classification of the enzyme as a mixed-function oxidase (Mason, 1957) i.e. it has a requirement for reduced pyridine nucleotide and molecular oxygen. It has been of considerable interest to determine the source of the NADPH required for mitochondrial steroid hydroxylation (see Simpson et al., 1969). Simpson and Estabrook (1969) have presented evidence suggesting an important role for intramitochondrial 'malic enzyme' as a means of supplying NADPH. In bovine adrenocortical mitochondria there is good correlation between corticosterone formation from 11-deoxycorticosterone (11β-hydroxylation) and pyruvate formation from malate. However, Peron and co-workers (1966) who have studied the same problem in rat adrenocortical mitochondria (i.e. 11β-hydroxylation) suggest that the energy-

linked NADH-$NADP^+$ transhydrogenase is important in intramitochondrial NADPH generation. A species difference could explain these conclusions.

In the present study, various Krebs tricarboxylic acid cycle intermediates were incubated separately with native placental mitochondria in the absence of the NADPH generator system. The results are summarised in Table 3.3. It is seen that only succinate (5mM) and to a lesser extent, isocitrate could support the cholesterol side-chain cleavage reaction. Malate and fumarate did not support the cleavage reaction. It, therefore, seems unlikely that intramitochondrial NADPH generation in placental mitochondria proceeds via 'malic enzyme' activity. This is in agreement with the observation of Dr. E.R. Simpson (personal communication) that placental mitochondria contain very little detectable 'malic enzyme' activity. Isocitrate presumably supports the cleavage reaction via the mitochondrial $NADP^+$-linked isocitrate dehydrogenase. It was of interest to observe that when succinate was used to supply the NADPH reducing equivalents the detected product of the incubation was pregnenolone and not progesterone. This indicated that the endogenous NAD^+ was reduced in the presence of succinate and no oxidised NAD^+ was available for the dehydrogenase reaction to convert pregnenolone to progesterone. These results were indicative of NADH-$NADP^+$ energy-linked transhydrogenase activity coupled with reverse electron flow from succinate to NAD^+.

Table 3.4 summarises the data obtained when amounts of NADPH greater than that (1 mM) normally employed in the

TABLE 3.3

CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY OF NATIVE PLACENTAL MITOCHONDRIA IN THE PRESENCE OF VARIOUS OXIDISABLE SUBSTRATES AS POSSIBLE SOURCES OF REDUCING EQUIVALENTS

Additions	Percentage conversion of [4-^{14}C] cholesterol in 60 min. at 37°C to:		
(20 mg. protein/ml.)	^{14}C-pregnenolone	^{14}C-progesterone	Total
None	0·1	0·2	0·3
NADPH generating system	1·0	8·7	9·7
Malate, 5mM	0·2	0·5	0·7
Succinate, 5mM	6·6	1·0	7·6
Fumarate, 5mM	0·2	0·5	0·7
Isocitrate, 5mM	0·2	4·0	4·2

standard enzymic assay were used. Pregnenolone was found to accumulate at the higher NADPH levels. The experimental data appeared compatible with the presence of an NADPH-NAD^+ transhydrogenase in the placental mitochondrial preparation, which reduced the level of the endogenous NAD^+. However, even at the highest NADPH concentration used (4 mM), progesterone was produced in considerable amounts, which means that all the endogenous NAD^+ is not reduced.

It can be seen (from Table 3.4) that extramitochondrially produced NADPH (produced by the generating system) can be utilized by the cholesterol side-chain cleavage enzyme system. Since NADPH only penetrates the mitochondrial membrane at a very slow rate, the enzymic activity is presumably a reflection of the intactness, or rather lack of intactness, of the mitochondrial preparation. The Waring blendor method of homogenisation is not noted for the preparation of 'good' i.e. intact, mitochondria. However, the more usual homogenisation technique employing a teflon pestle and a glass homogeniser vessel is not very useful when dealing with placental tissues due to the considerable amount of connective and vascular tissue present.

Identity of the products

After extraction of each incubation, the concentrated extract was applied on to a TLC plate and the chromatoplate developed in the di-isopropyl ether-petroleum spirit-acetic acid (70:30:2) system. The three radioactive peaks obtained on scanning the plates using the gas-flow TLC scanner corresponded to standards of progesterone, pregnenolone and

TABLE 3.4.

EFFECT OF NADPH ON THE CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY OF NATIVE PLACENTAL MITOCHONDRIA

NADPH (mM)	Percentage conversion of [4-^{14}C] cholesterol at 37°C for 60 min. to:		
	^{14}C-Pregnenolone	^{14}C-Progesterone	Total
1·0	2·8	10·4	13·2
1·5	4·9	5·1	10·0
2·0	6·5	4·9	11·4
2·5	7·1	4·3	11·4

NADPH generator system used. 20 mg. mitochondrial protein/ml.

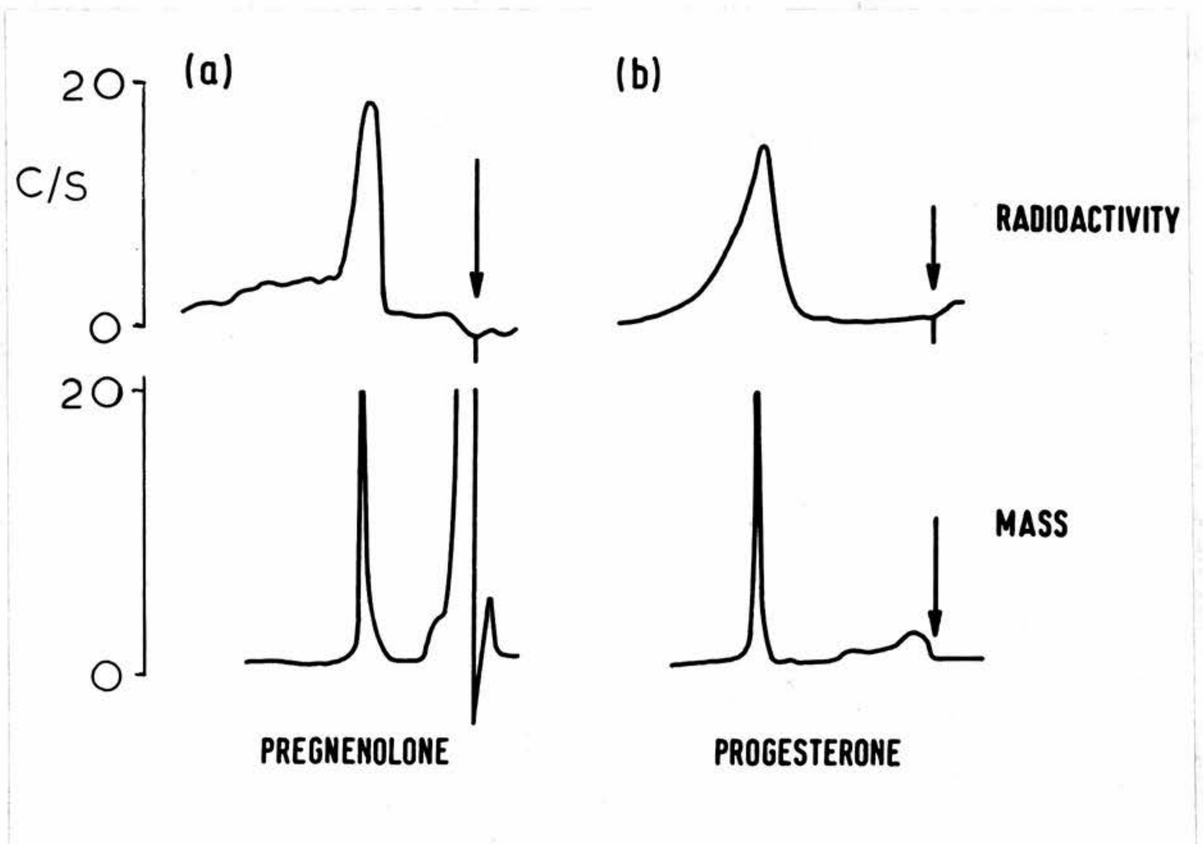


Fig. 3.2. Gas-liquid radiochromatograms of metabolites.
(a) Standard non-radioactive pregnenolone and radioactive product from incubation, identified as pregnenolone.
(b) Standard non-radioactive progesterone and radioactive product from incubation identified as progesterone.

cholesterol respectively. The radioactive material corresponding to pregnenolone and progesterone also behaved as these compounds in the solvent systems chloroform-ethyl acetate (8:2, v/v) and benzene-ethyl acetate-acetone (6:1:1, v/v).

For further identification, the radioactive samples eluted from the thin-layer plates were analysed by gas-liquid chromatography (GLC). Gas-liquid chromatography was carried out by means of a Pye Argon Gas Radiochromatograph with a ^{90}Sr detector. The glass column (125 cm. x 0·5 cm. internal diameter) contained 3% OV-17 on Gas Chrom P (Applied Science Laboratories, State College) prepared as described by Holmes and Stack (1962). The column was pretreated for 24 hours at 250°C. Operation temperature was 230°C with the flash heater at 260°C. The argon flow rate was 40 ml./min. Gas flow through the stream splitter and to the mass detector was adjusted so that about 95% of the sample went to the radioactive detector and 5% went to the mass detector. Samples were introduced on to the column using a solid injector system (Menini _et al_., 1965).

The retention times (t_R) of the substances injected in relation to cholesterol (internal standard t_R = 15 minutes) were identical with the relative retention times of standard pregnenolone (0·50) and progesterone (0·75). The radioactive and mass detector traces are shown in Fig. 3.2.

(B)

SOLUBILISATION OF THE PLACENTAL MITOCHONDRIAL CHOLESTEROL SIDE-CHAIN CLEAVAGE SYSTEM

As one of the primary aims of this work was to study the cholesterol side-chain cleavage enzyme system from a mechanistic standpoint rather than in relation to the cell as a whole, methods of solubilising the enzyme system were examined. Solubility in this study is defined in a strictly operational manner i.e. presence of enzymic activity in a 105,000 g. x 60 min. supernatant. Because of the particulate nature of at least one of the enzyme components (cytochrome P-450), prolonged centrifugation at 105,000 g. will undoubtedly sediment most of the component (see Cooper et al., 1965). However, the so-called 'soluble' preparation does permit some preliminary fractionation studies to be performed, and so in that sense, it might be considered 'soluble'.

(a) Ultrasonication

Cooper et al. (1965) observed that when a sonicate of adrenal cortex mitochondria was centrifuged at 105,000 g. x 30 mins., steroid 11β-hydroxylase activity was found in the supernatant, although a large proportion of the activity was still associated with the particulate material. Simpson and Boyd (1966), using a similar procedure to Cooper, reported that cholesterol side-chain cleavage activity could be found in the 105,000 g. x 30 min. supernatant of a sonicate of bovine adrenocortical mitochondria.

The preparation of ultrasonicated placental mitochondria followed the method of these authors. The mitochondrial

pellet was resuspended by gentle hand homogenisation in 2 volumes of ice-cold distilled water. The suspension was subjected to ultrasonication (20 kc/s) for 3 periods of 5 minutes duration with intervals for cooling, using a M.S.E. Ultrasonic Disintegrator. The suspension was cooled in ice throughout the sonication process. When this sonicate was centrifuged at 105,000 g. for 30 minutes, and the supernatant and the pellet assayed for cholesterol side-chain cleavage activity, the results shown in Table 3.5 were obtained. The data showed that the cholesterol side-chain cleavage activity resided in the supernatant of the placental mitochondrial sonicate. It was seen that the sonicate pellet had no activity and that when combined with the supernatant resulted in an almost complete inhibition of the enzymic activity. It was also noted that the activity of the mitochondrial sonicate was less than the activity of the native mitochondrial preparation (from equivalent amounts of wet weight tissue). This was in contrast to the effect of ultrasonication on the bovine adrenocortical and rat ovarian mitochondrial cholesterol side-chain cleavage enzyme systems (Simpson and Boyd, 1967a; Sulimovici and Boyd, 1968b). In both these cases, sonication of the native mitochondrial preparations resulted in a considerable enhancement of the cholesterol side-chain cleavage activity. It was apparent that there was material present in the 105,000 g. sonicated mitochondrial pellet that was inhibitory to the enzyme system. However, this inhibition could be released merely by high speed centrifugation of the sonicate (105,000 g., 45 min.) to

TABLE 3.5

THE CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY OF NATIVE PLACENTAL MITOCHONDRIA, MITOCHONDRIAL SONICATE AND 105,000 g. x 45 MIN. SUPERNATANT OF THE MITOCHONDRIAL SONICATE

Enzyme Source	Percentage conversion of [4-^{14}C] cholesterol in 60 min. at 37°C to:		
	^{14}C-Pregnenolone	^{14}C-Progesterone	Total
Native Mitochondria (20)	0·4	4·4	4·8
Mitochondrial sonicate (20)	0·2	2·5	2·7
105,000 g. x 45 min. supernatant (S_1) of sonicate (13)	13·2	6·7	19·9
105,000 g. x 45 min. pellet (P_1) of sonicate (7)	0·4	4·0	4·4
S_1 + P_1	0·9	3·8	4·7

Protein concentrations (mg. protein/ml.) are recorded in parenthesis.

produce a supernatant which possessed enhanced cholesterol side-chain cleavage activity (2-3 fold that of native mitochondria). These results indicated that the placental mitochondrial cholesterol side-chain cleavage enzyme could be 'solubilised' and secondly, that material inhibitory to the enzyme activity was associated with the particulate fraction after sonication.

(b) Ultrasonication and Lyophilization

Sulimovici (1968) used various methods (ultrasonication, acetone powder preparation, lyophilization, freezing and thawing, detergents, etc.) in an attempt to solubilise the cholesterol side-chain cleavage enzyme system present in immature rat ovarian mitochondria. This author observed that only when a combination of ultrasonication and lyophilization was used could solubilisation of the enzyme be achieved.

A placental mitochondrial preparation suspended in 2 volumes distilled water was sonicated (as described in the previous section) and the sonicate then lyophilized. The lyophilized mitochondria were taken up in 0·1M KCl (30 mg. lyophilized material per ml. of solution) using hand homogenisation. The suspension was centrifuged at 105,000 g. for 30 mins. The resulting supernatant and pellet fractions were assayed for cholesterol side-chain cleavage activity using the normal assay technique. The results obtained are tabulated in Table 3.6. Cholesterol side-chain cleavage activity was only found in the supernatant fraction. The pellet material inhibited the cholesterol side-chain cleavage activity of the supernatant very strongly. On an equivalent

TABLE 3.6

THE CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY OF LYOPHILIZED PLACENTAL MITOCHONDRIA, THE 105,000 g. x 60 MIN. SUPERNATANT AND PELLET OF THE LYOPHILIZED PLACENTAL MITOCHONDRIAL SUSPENSION

Enzyme Source	Percentage conversion of $[4\text{-}^{14}C]$ cholesterol in 60 min. at $37^{o}C$ to:		
	^{14}C-Pregnenolone	^{14}C-Progesterone	Total
Native mitochondria (21)	0·4	4·4	4·8
30 mg. lyophilized mitochondrial suspension (15)	0·3	0·9	1·2
105,000 g. x 60 min. supernatant* (S_1) of lyophilized mitochondria (4)	15·1	4·6	19·7
105,000 g. x 60 min. pellet* (P_1) of lyophilized mitochondria (11)	0·1	0·2	0·3
$S_1 + P_1$	0·6	0·5	1·1

* equivalent to 30 mg. lyophilized mitochondria. Protein concentrations (mg./ml.) are recorded in parenthesis.

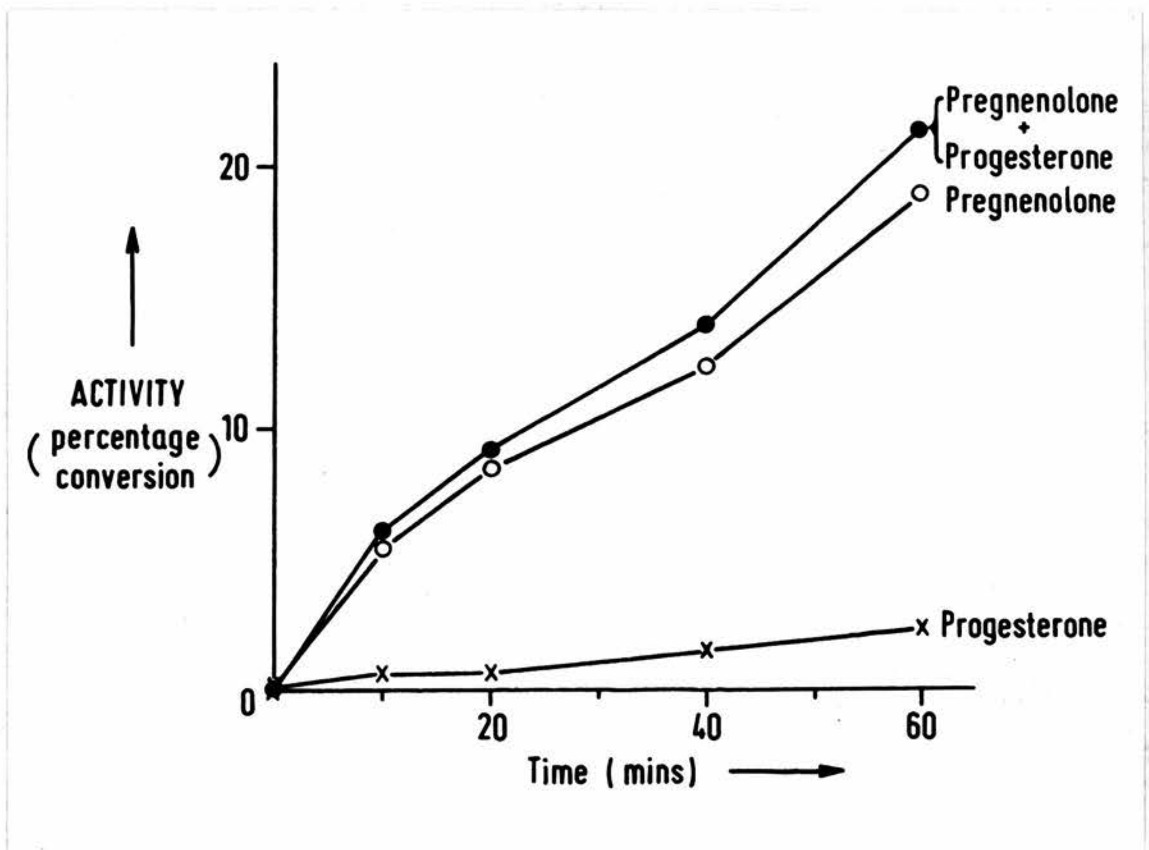


Fig. 3.3. Time course of the formation of [^{14}C] pregnenolone and [^{14}C] progesterone from [4-^{14}C] cholesterol by a supernatant of lyophilized placental mitochondria.

wet weight tissue basis, the lyophilized supernatant was more active than the sonicated supernatant preparation (compare Tables 3.5 and 3.6). This probably reflected further extraction of the enzyme from the particulate phase - where its presence could not be demonstrated due to the presence of inhibitory material. A combination of ultra-sonication and lyophilization facilitated solubilisation of the enzyme. It was observed after such a treatment that the particulate material was inhibitory towards cholesterol side-chain cleavage activity. Figure 3.3 shows a time course of the product formation when the lyophilized mitochondrial supernatant was incubated with [4-^{14}C] cholesterol and the NADPH-generator system. The rate of product formation appeared linear over a 60 minutes incubation period.

The lyophilized mitochondria could be stored several weeks at -20°C with very little loss of activity. 500 gm. of placental tissue on average yielded from 0·8 - 1·2 g. of lyophilized mitochondria.

(c) Acetone powder preparation

Treatment of cellular and subcellular fractions with acetone at around -20°C has commonly been used in attempts to solubilise membrane-bound enzyme systems. It also has the advantage of depleting the endogenous substrate(s) present especially when these are of a lipid nature.

Halkerston *et al*. (1961) reported the solubilisation of the cholesterol side-chain cleavage enzyme from an acetone powder of bovine adrenocortical mitochondria. The activity in the acetone-dried fraction could be solubilised by

extraction with 0·067 M phosphate buffer pH 6·8, followed by removal of particulate material by centrifuging at 105,000 g. for 60 minutes.

In the present study, washed mitochondrial suspensions (in 2 volumes distilled water) were added dropwise to 20 volumes of acetone (at -40°C) with continuous stirring. Filtration was carried out using a Buchner apparatus. The acetone-precipitated material was washed with 3 volumes of acetone (at -20°C) followed by 3 volumes of ether (at -20°C) Acetone and ether were stored during the operation in an acetone-solid carbon dioxide (Cardice) bath. The material was finally dried in a vacuum desiccator. One placenta (approx. 400 g. tissue) yielded on average 1-1·2g. mitochondrial acetone powder.

300 mg. placental mitochondrial acetone powder were suspended in 10 ml. 0·1 M KCl using hand homogenisation. The suspension was centrifuged at 105,000 x g. for 60 min. When the resulting supernatant and pellet were assayed for the presence of the cholesterol side-chain cleavage enzyme, activity was found only in the supernatant. The pellet material was inhibitory towards activity. The results are shown in Table 3.7. The activity of the mitochondrial acetone powder extract was observed to be three or four fold that of the lyophilized preparation. Presumably at least part of this enhancement was due to removal of endogenous cholesterol substrate present in the mitochondrial preparation.

As with the sonicated and the lyophilized preparations, the particulate material was found to be inhibitory towards

TABLE 3.7

THE CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITIES OF A PLACENTAL ACETONE POWDER SUSPENSION, THE 105,000 g. x 60 MIN. SUPERNATANT AND PELLET OF THE ACETONE POWDER SUSPENSION

Enzyme Source	Percentage Conversion of [4-^{14}C] cholesterol in 60 min. at 37°C to:		
	^{14}C-Pregnenolone	^{14}C-Progesterone	Total
30 mg. placental acetone powder suspension (16)	0·3	0·7	1·0
105,000 g. x 60 min. supernatant (S_1) of 30 mg. acetone powder (4)	43·7	10·4	54·1
105,000 g. x 60 min. pellet (P_1) of 30 mg. acetone powder (12)	0·1	0·2	0·3
S_1 + P_1	0·3	0·4	0·7

Protein concentrations (mg./ml.) are recorded in parenthesis.

cholesterol side-chain cleavage activity.

Of the three methods of "solubilisation" investigated (i.e. presence of activity in a 105,000 x g, 30 min. supernatant), all were successful in obtaining a 'solubilised' enzyme preparation. The enzyme appeared more readily extractable than the enzyme of rat ovarian mitochondria (Sulimovici, 1968). However, it was apparent in all three cases that there was inhibitory material associated with the insoluble mitochondrial particles. The presence of the inhibitory substance was only made apparent when the native mitochondria were subjected to some disruptive procedure, since native mitochondria were enzymatically more active than, for example, the mitochondrial sonicate. (compare Tables 3.2 and 3.5).

The next section will deal with investigations into the nature and characteristics of this inhibitory substance.

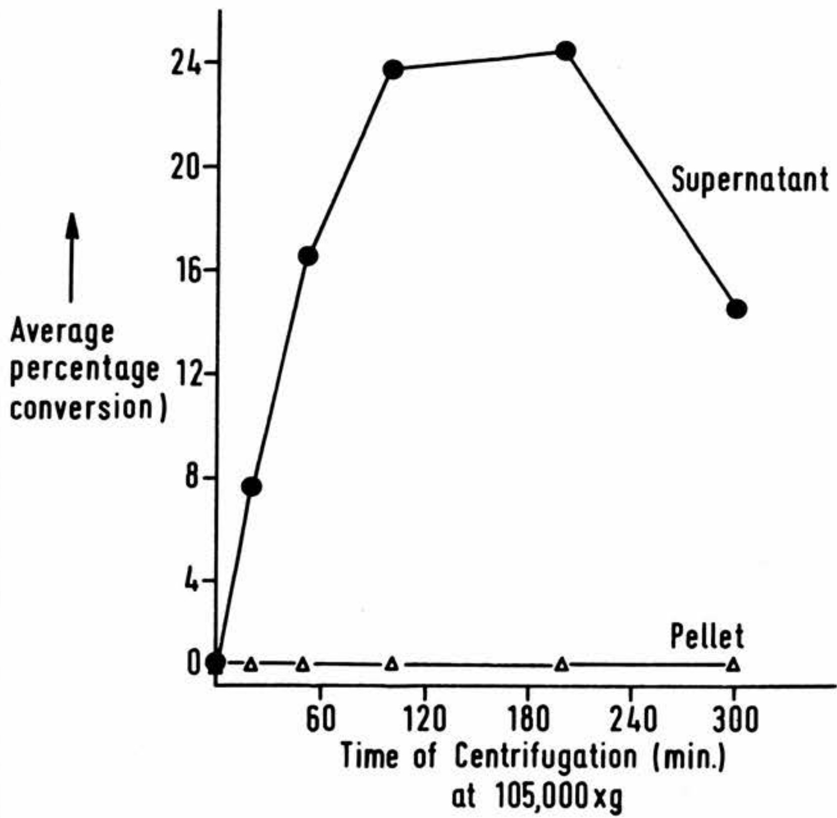


Fig. 3.4. Effect of time of high speed centrifugation on the cholesterol side-chain cleavage activity of the supernatant of lyophilized placental mitochondria.

(C)

NATURE OF THE ENDOGENOUS INHIBITOR

It was apparent from the results of the attempts to solubilise the placental cholesterol side-chain cleavage system that there was, associated with the mitochondrial preparation, material that was inhibitory towards the cholesterol side-chain cleavage activity. The inhibitory property was only observed when the mitochondria were disrupted in some way, e.g., ultrasonication or lyophilization. It appeared that in the intact mitochondrion, the components of the enzyme system (including here the substrate and the cofactor(s)) were protected from the inhibitor, presumably by the ordered structure of the organelle. It was found that by means of a purely physical technique, ultracentrifugation, the enzyme could be separated from the inhibitor (see Table 3.5). Figure 3.4 shows the time course of the sedimentation of the inhibitor at 105,000 x g. from a suspension of lyophilized placental mitochondria. This was achieved by taking an aliquot of the supernatant at a given time, the remainder of the supernatant being further centrifuged to obtain the next aliquot. It was found that maximal activity was not achieved until after at least 90 mins. centrifugation at 105,000 x g. Thus the inhibitory component must be associated with particulate material of wide size range. After a plateau region of activity, this then began to decrease. This presumably could be due to partial sedimentation of a component (s) of the enzyme system - possibly the cytochrome P-450 as reported by Cooper _et al_. (1965).

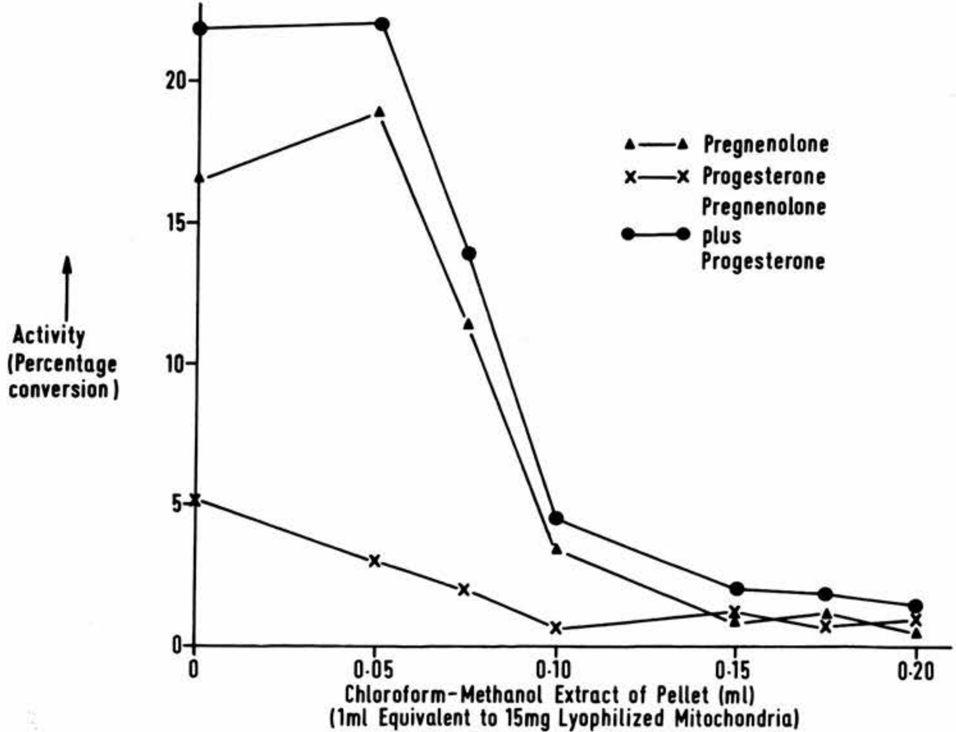


Fig. 3.5. Effect of chloroform-methanol extract of mitochondrial pellet on the cholesterol side-chain cleavage activity of the 105,000 g x 60 min supernatant of lyophilized placental mitochondria

Effect of heating

The inhibitory activity was still maintained after heating the pellet suspension (in distilled water) for 15 min. in a boiling water bath. It was also, after this treatment, still firmly associated with the particulate fraction. The results are summarised in Table 3.8.

Extraction of pellet with chloroform-methanol (2:1, v/v)

The evidence suggested the inhibitor to be of lipoidal character. The 105,000 x g. pellet of lyophilized mitochondria was resuspended in 20 vols. chloroform-methanol (2:1 v/v) and refluxed for 60 min. in an atmosphere of nitrogen. After centrifugation (10,000 x g, 10 mins.) of this suspension, inhibitory activity was found in the organic extract (see Fig. 3.5). This confirmed that the inhibitor was of lipid origin.

Thin-layer chromatography of the chloroform-methanol extract on silica gel H TLC plates developing the plates in the system hexane-diethyl ether-acetic acid (75:24:1, v/v), showed that the detectable components of the extract consisted of cholesterol, phospholipids and cholesterol esters. Detection was accomplished either by spraying with phosphomolybdate or by immersion in an iodine atmosphere. Samples of standard phospholipid (remained at origin), Cholesterol (R_f, 0·10), triglyceride (0·40), cholesterol ester (0·95), were run on the same plate.

Each of the three components (together with a control area of silica gel from behind the origin) was extracted from the plates with chloroform-methanol (2:1, v/v) and after

TABLE 3.8

EFFECT OF HEATED ($95^{o}C$, 15 MIN.) 105,000 g. x 60 MIN. PELLET OF LYOPHILIZED PLACENTAL MITOCHONDRIA ON THE CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY OF THE 105,000 g. x 60 MIN. SUPERNATANT OF THE LYOPHILIZED MITOCHONDRIAL PREPARATION. THE HEATED PELLET SUSPENSION WAS CENTRIFUGED AT 1000g. x 15 MIN.

	Percentage Conversion of [4-^{14}C] cholesterol for 60 min. at $37^{o}C$ to:		
	^{14}C-Pregnenolone	^{14}C-Progesterone	Total
105,000 g. x 60 min. supernatant (S_1)	19·0	2·3	21·3
S_1 + Supernatant of heated pellet suspension	11·7	0·6	12·3
S_1 + Pellet of heated pellet suspension	2·7	0·6	3·3

All preparations were present in amounts equivalent to 30 mg. lyophilized mitochondria.

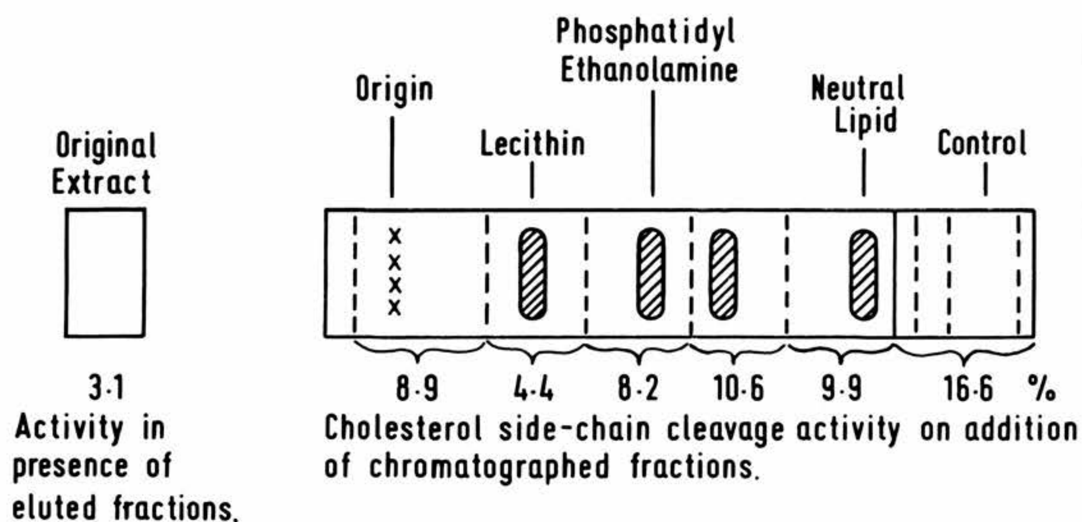


Fig. 3.6. The inhibition of cholesterol side-chain cleavage activity by eluted fractions after TLC of the $CHCl_3$-MeOH extract of the lyophilized mitochondrial pellet.

removal of solvent each fraction was assayed for inhibitory activity. The most inhibitory component was found to be the origin (phospholipid) fraction (see Table 3.9). Each assay was carried out with amounts of each fraction equivalent to 30 mg. mitochondrial powder.

The phospholipid fraction was further resolved by TLC using the development system, chloroform-methanol-water (65:35:8, v/v) using basic silica gel H plates as described by Skipski _et al._ (1962). Figure 3.6 shows the resolution of the phospholipid fraction into two main components - lecithin and phosphatidyl ethanolamine. There was also apparently cardiolipin present in the neutral lipid fraction at the solvent front, as well as smaller amounts of other unidentified components. Each fraction was eluted from the silica gel with chloroform-methanol (2:1, v/v). After removal of the solvent, each fraction (equivalent to 3 mg. mitochondrial powder) was assayed for inhibitory activity in the standard cholesterol side-chain cleavage enzymic assay, using supernatant from a lyophilized mitochondrial preparation as the enzyme source. The results are shown in Fig. 3.6.. It was observed that although most of the fractions showed inhibitory activity, the most potent inhibitor was the lecithin fraction. Qualitatively, the amounts of lecithin and phosphatidyl ethanolamine were approximately equal with smaller amounts of cardiolipin. Fleischer _et al._ (1967) analysed highly purified mitochondria from bovine heart and kidney and found them to contain diphosphatidyl glycerol (cardiolipin), phosphatidyl ethanolamine and lecithin as major lipid components approximately in the molecular ratio 1:4:4.

TABLE 3.9

EFFECT OF ELUTED FRACTIONS ON CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY AFTER T.L.C. CHROMATOGRAPHY OF A LIPID EXTRACT OF LYOPHILIZED PLACENTAL MITOCHONDRIA USING THE SOLVENT SYSTEM, HEXANE-DIETHYL ETHER-ACETIC ACID (75:24:1)

	Control	Origin (Phospholipid)	'Cholesterol' region	Remainder of T.L.C. plate
Percentage Conversion	15·0	4·1	11·0	12·3

A chloroform-methanol extract of lyophilized placental mitochondria equivalent to 3 mg. mitochondrial powder was applied on to the T.L.C. plate. The enzyme source was a 105,000 g. x 60 min. supernatant of lyophilized mitochondria.

Further investigation of acetone treatment of placental mitochondria

It is well known that acetone treatment of mitochondria results in a depletion of mitochondrial lipid - more than 90% of which is phospholipid (Rouser et al., 1968).

Since the phospholipid of placental mitochondria appeared to be involved in the inhibition of cholesterol side-chain cleavage activity on disruption of the mitochondria, it was decided to re-investigate the acetone treatment of placental mitochondria.

Placental mitochondria prepared using the normal procedure were resuspended in 2 volumes distilled water and added dropwise into 20 volumes of acetone (at -20°C) with continuous stirring. Slow stirring was continued for 10 minutes during which time the temperature rose to about -5°C. The resulting material was filtered using a Buchner apparatus. The precipitated material was finally washed with 3 volumes ice-cold ether before drying in a vacuum desiccator. When this acetone-dried mitochondrial powder was assayed for cholesterol side-chain cleavage activity in the normal manner, i.e. suspension of the powder (30 mg./ml.) in 0·1 M KCl, followed by centrifugation at 105,000 x g. for 60 min, the 105,000g. pellet did not inhibit the enzymic activity of the supernatant. The results are summarised in Table 3.10. The results also demonstrate that there is very considerable steroid 3β-hydroxysteroid dehydrogenase-isomerase activity

TABLE 3.10

EFFECT OF PROLONGED ACETONE TREATMENT (-10°C, 10 MIN.) ON THE REMOVAL OF THE ENDOGENOUS INHIBITOR OF CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY OF PLACENTAL MITOCHONDRIA

Enzyme Source	Percentage Conversion of [4-^{14}C] cholesterol in 60 min. at 37°C to:		
	^{14}C-Pregnenolone	^{14}C-Progesterone	Total
30 mg. mitochondrial acetone powder suspension	4·5	53·3	57·8
105,000 g. x 60 min. supernatant (S_1) from 30 mg. acetone powder suspension	49·5	5·8	55·3
105,000 g. x 60 min. pellet (P_1) from 30 mg. acetone powder suspension	0·3	0·2	0·5
S_1 + P_1	1·4	58·7	60·1

associated with the pellet fraction since in the presence of the pellet fraction progesterone was virtually the sole product. It was also observed that there was very little increase in the total cholesterol side-chain cleavage activity on addition of the pellet, suggesting that almost all the cholesterol side-chain cleavage enzyme was 'solubilised'. This is in marked contrast to the solubilisation studies on the steroid 11β-hydroxylase of bovine adrenocortical mitochondria when most of the 11β-hydroxylase activity was still associated with the particulate fraction (Cooper et al., 1965). It therefore appeared that the inhibitory substance was removed in the acetone filtrate. The acetone-ether filtrate was dried over anhydrous Na_2SO_4 and then concentrated to dryness on a rotary evaporator. The residue was dissolved in chloroform-methanol (2:1 v/v) and assayed for inhibitory activity. The solvent was removed prior to the addition of the other components of the assay system. The results are shown in Table 3.11. The filtrate thus contained the inhibitory substance. Examination of the filtrate by TLC using the solvent system hexane-diethyl ether-acetic acid (75:24:1, v/v) revealed two detectable components - phospholipid and cholesterol. Elution of the origin region (phospholipid) with $CHCl_3$-MeOH (2:1, v/v) and redevelopment in the chloroform-methanol-water (65:35:8) system (using basic silica gel H plates - prepared using 0·01 M Na_2CO_3) revealed two main phospholipid components - lecithin and phosphatidyl ethanolamine, which chromatographed with their respective standards - together with a smaller amount of a component

TABLE 3.11

EFFECT OF ACETONE-ETHER EXTRACT OF PLACENTAL MITOCHONDRIA ON THE CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY OF A 105,000 g. x 60 MIN. SUPERNATANT (S_1) OF 30 mg. PLACENTAL MITOCHONDRIAL ACETONE POWDER SUSPENSION

Amount of acetone extract added (given in amounts equivalent to mg. acetone powder)	Percentage conversion of [4-^{14}C] cholesterol in 60 min. at 37°C to:		
	^{14}C-Pregnenolone	^{14}C-Progesterone	Total
None	32·0	8·6	40·6
25	6·2	0·5	6·7
50	4·9	0·3	5·2
100	4·2	0·7	4·9

present at the solvent front - probably cardiolipin.

Addition of 5% n-butanol to the acetone (at -20°C) was also found to facilitate removal of the inhibitory lipid from the mitochondrial preparations. It was, however, difficult to remove the butanol, due to its relatively high boiling point, to yield a dry mitochondrial powder.

It was apparent that phospholipid and possibly cholesterol were involved in the inhibitory process. To attempt to eliminate the involvement of cholesterol, the effect of exogenous cholesterol on the cholesterol side-chain cleavage activity of a supernatant of a mitochondrial acetone powder suspension was investigated.

<u>Effect of cholesterol</u>

Due to the nature of the standard cholesterol side-chain cleavage assay - using tracer ^{14}C-cholesterol - it was possible that at least part of the inhibition could arise from dilution of the tracer by the endogenous cholesterol. Cholesterol side-chain cleavage assays were therefore performed in the presence of varying amounts of exogenously added 'cold' cholesterol. This cholesterol was added in a small volume (0·05 ml. acetone) with the tracer [4-^{14}C] cholesterol (assumed to have negligible mass). The results are summarised in Table 3.12. The cholesterol content of a lyophilized mitochondrial preparation was estimated to be about 300 μg. per 30 mg. mitochondrial powder, using the Liebermann-Burchard reaction (see Chapter 2). It was, therefore, apparent that the endogenous cholesterol could not alone account for the inhibition. It must, however, be remembered that the endogenous

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TABLE 3.12

EFFECT OF EXOGENOUS CHOLESTEROL ON THE CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY OF THE 105,000 g. x 60 MIN. SUPERNATANT OF 30 mg. LYOPHILIZED PLACENTAL MITOCHONDRIAL SUSPENSION

Amount of cholesterol added to 5 ml. incubation (μg)	Percentage conversion of [4-^{14}C] cholesterol to ^{14}C-pregnenolone plus ^{14}C-progesterone
20	24·6
50	23·0
200	17·9
400	13·4

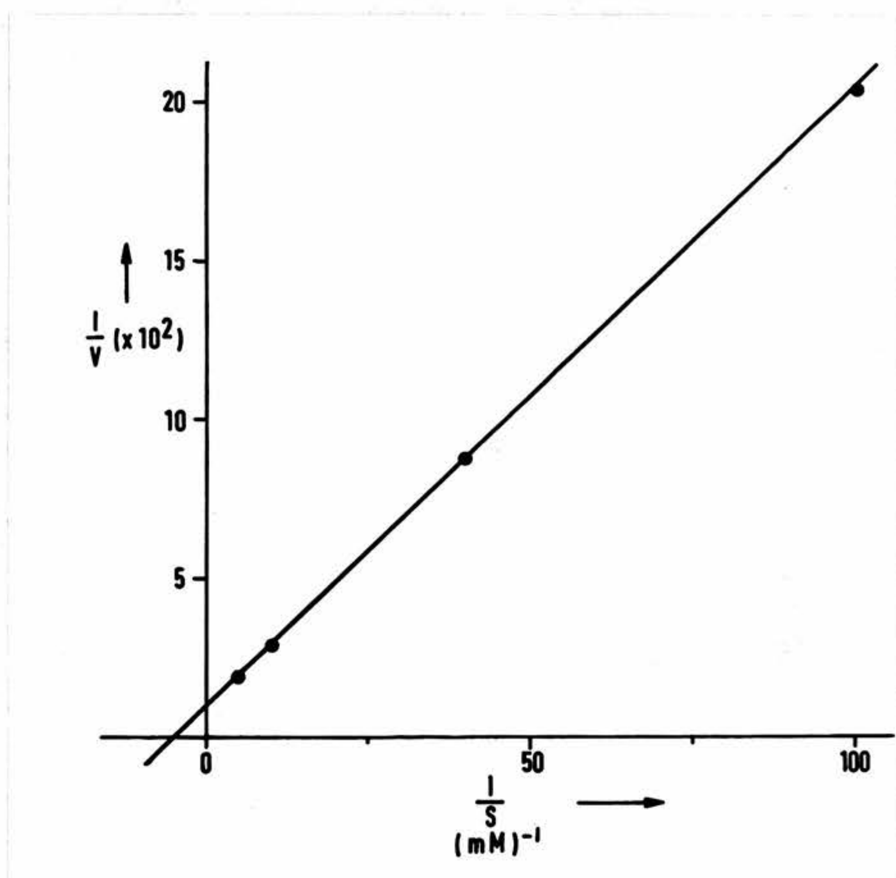


Fig. 3.7. A Lineweaver-Burk plot showing the effect of added cholesterol (S) on the cholesterol side-chain cleavage activity of a supernatant of lyophilized placental mitochondria (v = percentage conversion).

cholesterol is presumably mitochondrial lipoprotein cholesterol, while the nature of the added cholesterol would be presumably 'free' cholesterol.

The data was also used to calculate the apparent Michaelis constant, K_m, for the enzyme and the cholesterol substrate. Figure 3.7 shows a Lineweaver-Burk plot (Lineweaver and Burk, 1934). The K_m was found to be about 200 μM, which is very high indeed. However, it must be remembered that the enzyme source was a mitochondrial acetone powder and so would be depleted in cholesterol. It seems very likely that on readdition of cholesterol, it is bound to sites not involved in the cholesterol side-chain cleavage activity. Using a supernatant from lyophilized mitochondria as the source of the enzyme, the K_m was found in some cases to be in the region of 40-60 μM. However there was considerable variation from one preparation to another. The lower Michaelis constants for lyophilized mitochondrial preparations presumably reflects endogenous cholesterol binding to the cholesterol binding sites. Until the problems of equilibration between added cholesterol and the cholesterol binding sites, both structural and enzymic and the state of cholesterol in mitochondrial lipoproteins are understood, a K_m will not be meaningful.

This was further evidence for the involvement of phospholipid in the inhibitory process. It was therefore decided to investigate the effect of authentic highly purified phospholipid.

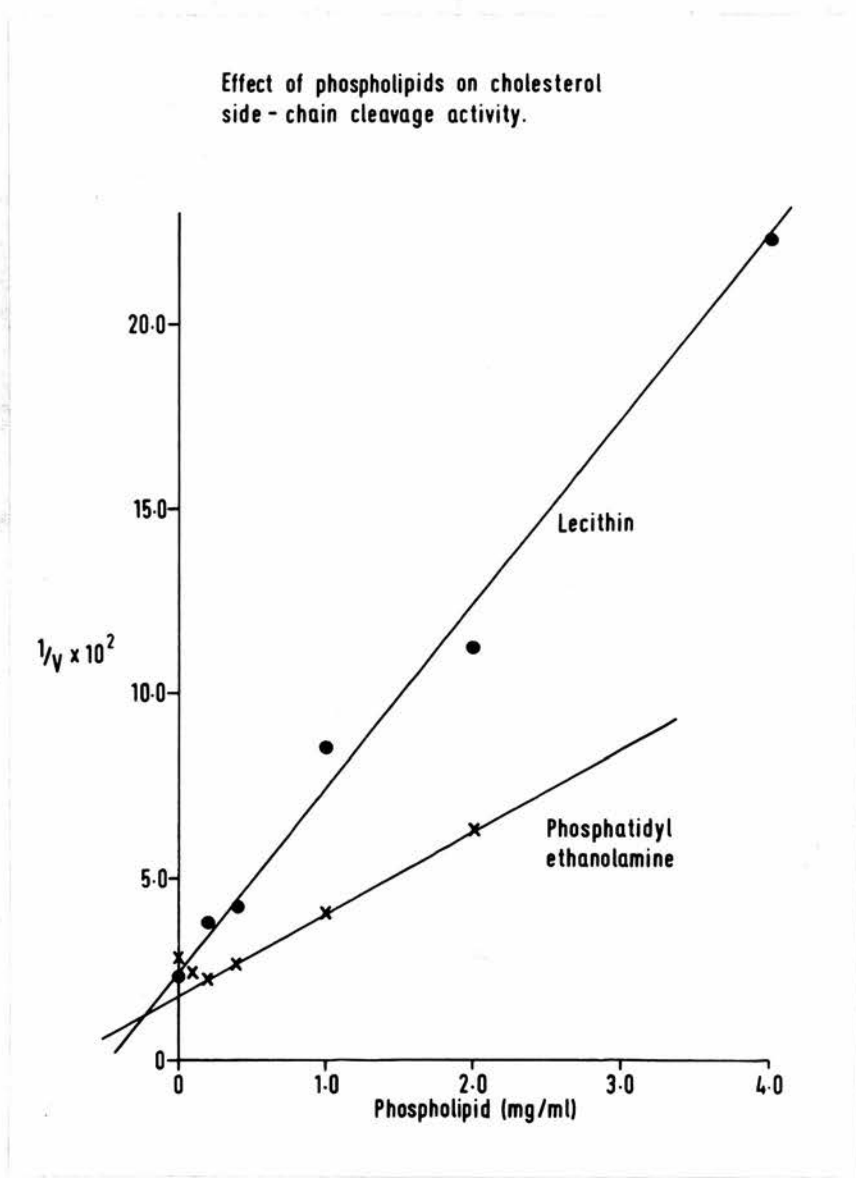


Fig. 3.8. The effect of authentic phosphatidyl ethanolanine and lecithin on the cholesterol side-chain cleavage activity of a supernatant (105,000 g. x 60 min.) of placental mito-chondrial acetone powder.
(v = percentage conversion)

Effect of authentic phospholipid

Authentic samples of lecithin and phosphatidyl ethanolamine were obtained from Lipid Products (Epsom). Both phospholipid samples were obtained dissolved in hexane. They were both colourless solutions - a good visual criterion for the purity of phospholipid samples. The purities of the samples were checked by thin-layer chromatography using the development system chloroform-methanol-water (65:35:8) and deactivated (0·01 M Na_2CO_3) silica gel H chromatoplates. Both samples gave single spots - visualisation was by immersion in an iodine atmosphere.

The hexane solvent was removed in a current of nitrogen. The phospholipid residue was weighed and then suspended in distilled water by means of ultrasonication (20 Kc/s, 10 sec.). This procedure was found to produce a very good dispersion of the phospholipid in water. The effect of varying amounts of the particular phospholipid upon the cholesterol side-chain cleavage activity of a 105,000 g. x 60 min. 0·1 M KCl extract of a placental mitochondrial acetone powder was investigated using the standard assay procedure. The results are shown in Fig. 3.8. plotted according to Dixon (1953). It was observed that lecithin was a potent inhibitor of cholesterol side-chain cleavage activity. However, phosphatidyl ethanolamine at low concentrations (0·1 - 0·4 mg./ml.) stimulated the enzymic activity, but at higher concentrations inhibition was observed (60% inhibition at 2 mg./ml.). Since the actual substrate (cholesterol) concentration could not be determined due to the probable presence of several binding

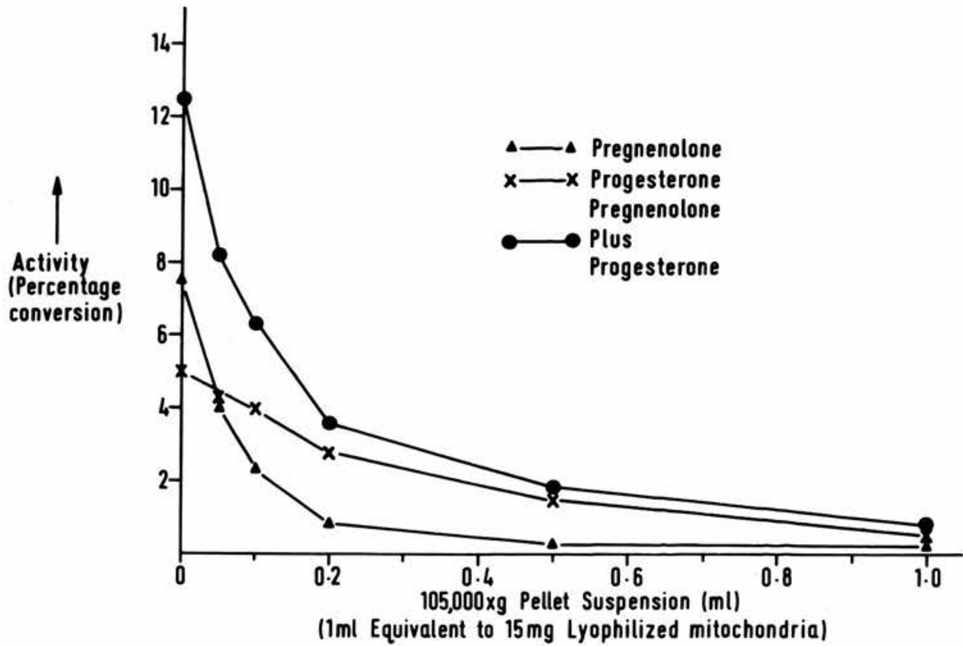


Fig. 3.9a. Effect of varying amounts of lyophilized placental mitochondrial pellet (105,000 g. x 60 min.) on the cholesterol side-chain cleavage activity of the supernatant of lyophilized mitochondria.

sites for cholesterol, no attempt was made to calculate an inhibitor constant, K_i, for the inhibition.

<u>Effect of varying amounts of the 105,000 g. pellet of extracted lyophilized mitochondria</u>

30-40% of the dry weight of mitochondria is lipid, of which about 95% is phospholipid (see Rouser <u>et al</u>., 1968). The effect of varying amounts of the 105,000 g. x 60 min. pellet from a lyophilized mitochondrial suspension on the cholesterol side-chain cleavage activity of the supernatant was investigated to determine whether there was correlation between the inhibition produced by endogenous phospholipid in the pellet and the authentic phospholipids. The results are shown in Fig. 3.9a. The data of Fig. 3.9a was replotted according to Dixon (1953) as shown in Fig. 3.9b. Comparison of Figs. 3.8 and 3.9b show that the pellet is a stronger inhibitor of the enzymic activity than is the pure phospholipid. 50% inhibition was observed when a pellet suspension equivalent to 0·3 mg./ml. lyophilized mitochondria was present i.e. about 0·1 mg./ml. phospholipid. The amount of lecithin required for 50% inhibition was 0·4 mg./ml. It seems likely, therefore, that phospholipid, especially lecithin, is closely involved in the inhibitory process and that the state of phospholipid is probably important. It must be remembered that the purified lecithin was added as a dispersion in water whereas the endogenous phospholipid of the mitochondria was in a lipoprotein-type structure.

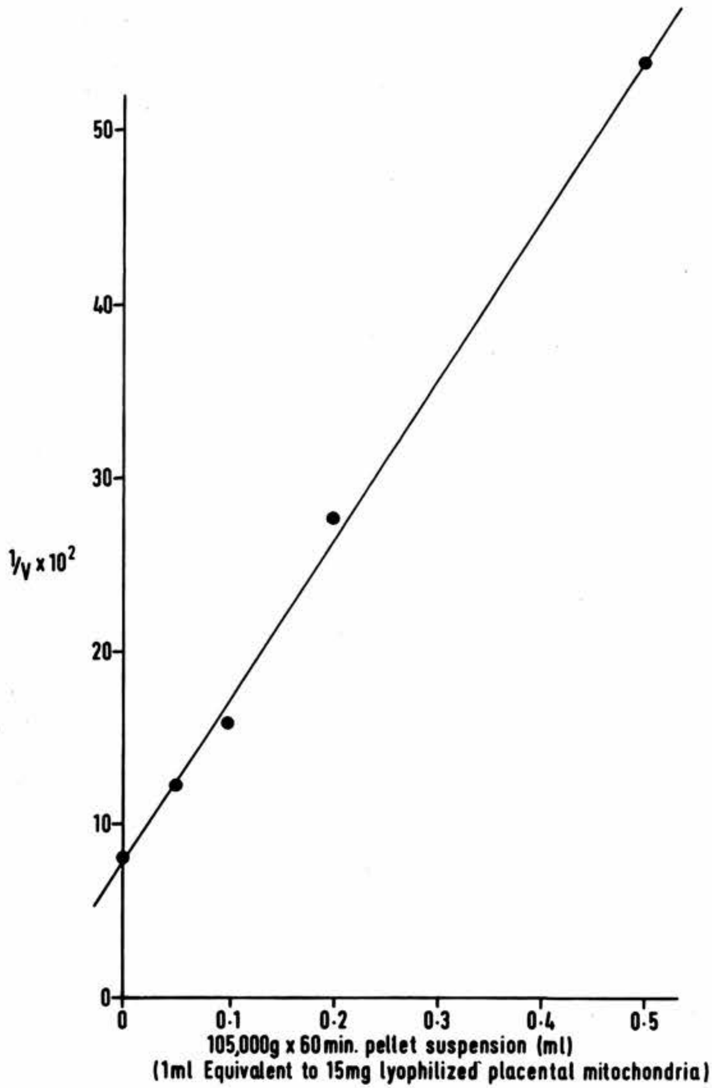


Fig. 3.9b. The data of Fig. 3.9a plotted according to Dixon (1953).
(v = percentage conversion)

Specificity of the endogenous inhibitor

The best studied mitochondrial steroid hydroxylase has been the steroid 11β-hydroxylase of adrenocortical mitochondria that hydroxylates 11-deoxycorticosterone (DOC) to produce corticosterone (see Simpson et al., 1969). Placental mitochondrial acetone powders (and also lyophilized preparations) were assayed for steroid 11β-hydroxylase activity using the method described by Simpson and Estabrook (1969). Steroid 11β-hydroxylase activity was not observed in either the mitochondrial suspensions or the 105,000 g. x 60 min. supernatants. This was in agreement with the observations of Billiar and Little (1966). Thus it was not possible to study the action of the endogenous inhibitor on a placental 11β-hydroxylase.

It was seen earlier in this chapter that placental mitochondrial preparations contained an active 3β-hydroxysteroid dehydrogenase-Δ^{4-5}isomerase system. Mitochondrial acetone powder preparations from which the inhibitor had been removed had a very active dehydrogenase system associated with the 105,000 g. x 60 min. pellet (Table 3.10) and were used as the enzyme source. Lyophilized placental mitochondria were suspended in 0·1 M KCl, centrifuged at 105,000 g. x 60 min. as usual and the pellet resuspended in distilled water (20 ml. pellet suspension from 300 mg. lyophilized mitochondria). Varying amounts of the pellet suspension were added to the dehydrogenase assay system (see Chapter 2) which employed [4-^{14}C] pregnenolone as the substrate and NAD^+ as the cofactor. It was observed (Table 3.13) that addition of large amounts

TABLE 3.13

EFFECT OF THE 105,000 g. x 60 MIN. PELLET (P_1) OF LYOPHILIZED PLACENTAL MITOCHONDRIA ON THE CONVERSION OF [4-^{14}C] PREGNENOLONE TO [^{14}C] PROGESTERONE IN THE 105,000 g. x 60 MIN. SUPERNATANT (S_1) OF LYOPHILIZED MITOCHONDRIA

Amount of pellet suspension added to S_1 (equivalent to mg. lyophilized mitochondria)	Percentage conversion in 15 min. at 37°C
None	41·6
1·2	82·2
2·4	92·5
6·0	92·3
12·0	92·0
24·0	92·3

Concentration of S_1 equivalent to 24 mg. lyophilized mitochondria per incubation.

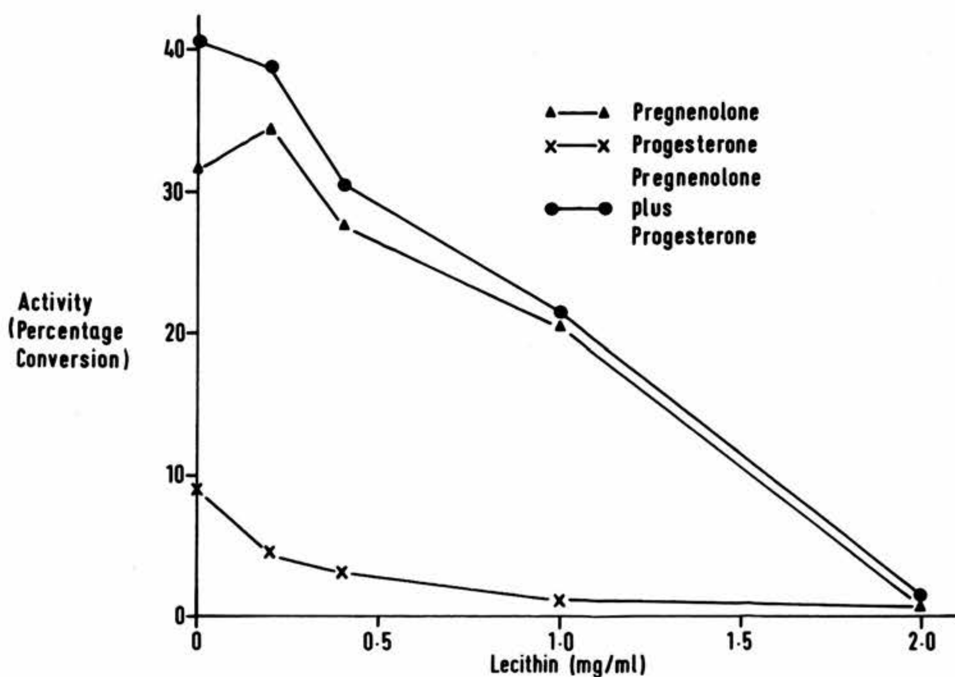


Fig. 3.10. Effect of authentic lecithin on the cholesterol side-chain cleavage activity of a 105,000 g. x 60 min. supernatant of lyophilized bovine adrenocortical mitochondria.

of the pellet suspension resulted in greatly enhanced dehydrogenase activity - due to the dehydrogenase being present in the pellet suspension. Thus the inhibitor did not interfere in the metabolism of pregnenolone by the dehydrogenase system. Presumably there was no interaction between the inhibitor and pregnenolone.

Absence of the endogenous inhibitor in the mitochondria of other steroid-producing tissues

Simpson (1967) in studies with bovine adrenocortical mitochondria and Sulimovici and Boyd (1968b) using immature rat ovarian mitochondria both observed enhanced cholesterol metabolism to the steroid hormones after ultrasonication of the mitochondria. They attributed the increased activity to the possibly easier penetration of the exogenous tracer cholesterol to the substrate binding site of the enzyme. Placental mitochondria appear, therefore, to be different in some way to the other tissue mitochondria capable of converting cholesterol to the steroid hormones. If the inhibitory substance is phospholipid, this poses a problem since the mitochondria of these other tissues have reasonably similar phospholipid compositions. The effect, therefore, of a pure phospholipid-lecithin - upon the cholesterol side-chain cleavage activity of a 105,000 g. x 60 min. supernatant of lyophilized adrenocortical mitochondria was investigated. The procedure was similar to that used earlier for the placental enzyme. The results are summarised in Fig. 3.10. Though the adrenal enzyme was sensitive to the presence of lecithin, a lecithin concentration of 1·0 mg./ml. was required to produce 50%

inhibition, whereas 0·4 mg./ml. produced a similar degree of inhibition of the placental enzyme.

It seemed possible that the difference in sensitivity of the placental and adrenal supernatant enzymes to a pure phospholipid and their respective "pellet" fractions might just be a reflection of the enzyme concentration in the mitochondria. Adrenocortical mitochondria are certainly more active than a similar amount of placental mitochondria when cholesterol side-chain cleavage activity is compared (Simpson, 1967 and present study). To test this hypothesis, 150 mg. lyophilized adrenocortical mitochondria were suspended in 10 ml. 0·1 M KCl and centrifuged at 105,000 g. for 30 minutes. 0·5 ml. aliquots of the resulting supernatant (1 ml. equivalent to 15 mg. lyophilized mitochondria; protein concentration, 1·7 mg./ml.) were incubated with varying amounts of the residual pellet which was suspended in water (1 ml. equivalent to 15 mg. lyophilized mitochondria; protein concentration, 4·4 mg./ml.) The standard cholesterol side-chain cleavage assay was used, employing a 30 minutes incubation period. The pellet suspension was heated in a boiling water bath for 10 minutes prior to the incubations, in order to destroy residual cholesterol side-chain cleavage activity. This procedure permitted a study of the effect of large amounts of 'pellet' upon relatively small amounts of the adrenal enzyme. The results are shown in Table 3.14. It was seen that the adrenal cholesterol side-chain cleavage enzyme was inhibited by particulate mitochondrial material under such conditions.

It thus appeared that the relatively small amount of

TABLE 3.14

EFFECT OF 105,000 g. x 60 MIN. PELLET OF A LYOPHILIZED ADRENOCORTICAL MITOCHONDRIAL PREPARATION ON THE CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY OF A LOW CONCENTRATION OF THE ENZYME PRESENT IN THE 105,000 g. x 60 MIN. SUPERNATANT (S_1) OF THE MITOCHONDRIAL PREPARATION

Amount of adrenal lyophilized 105,000 g. x 60 min. pellet added (equivalent to mg. lyophilized mitochondria)	Percentage conversion of [4-^{14}C] cholesterol to C_{21} products
None	24·2
7·5	9·1
22·5	4·8
45·0	5·3

Amount of S_1 present was equivalent to 7·5 mg. lyophilized mitochondria.

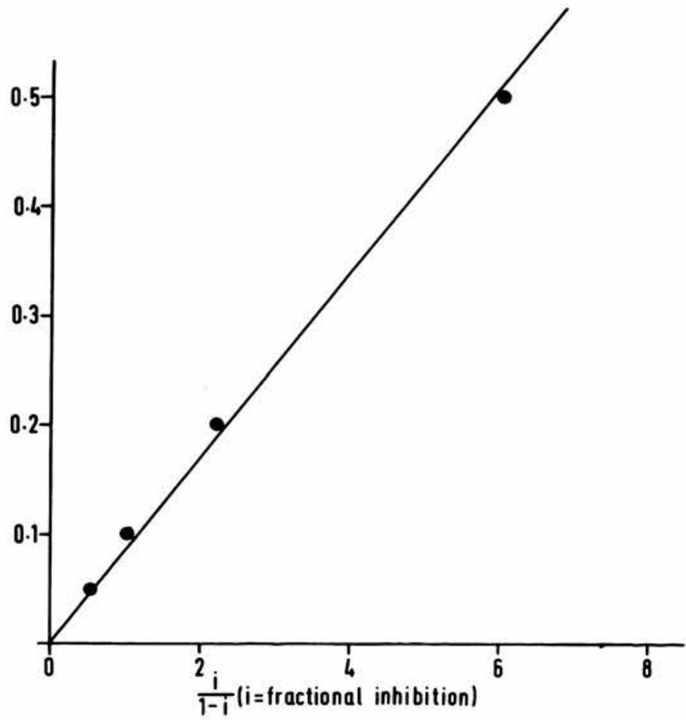


Fig. 3.11. The data of Fig. 3.9a. plotted according to Reiner (1969). (i = fractional inhibition)

the cholesterol side-chain cleavage enzyme played an important role in the inhibitory process. The results suggested a phospholipid-cholesterol-enzyme interaction, possibly involving micelle formation. In the placental situation, a critical micellar concentration is reached, resulting in an inhibition of activity for some reason. However, in the adrenal situation, the enzyme concentration is higher and the critical micellar concentration is surpassed with relation to protein concentration. An 'inhibitory' micelle is therefore not formed and enzymic activity is observed.

Further evidence for the critical involvement of the enzyme in the inhibitory process was obtained by plotting the data of Fig. 3.9a. according to Reiner (1969). The plot is $\frac{i}{1 - i}$ versus the inhibitor concentration, where i is the fractional inhibition, and is shown in Fig. 3.11. The plot was found to be a straight line, which is indicative of an enzyme-substrate-inhibitor interaction to produce an inactive complex. A purely substrate-inhibitor interaction to produce an inactive complex is expected to give a curved plot. This result confirmed the involvement of the enzyme in the inhibitory process.

The mechanism of the inhibition will be considered more fully in the Discussion.

CHAPTER 4

CHARACTERISTICS OF THE 'SOLUBILISED' PLACENTAL CHOLESTEROL SIDE-CHAIN CLEAVAGE ENZYME SYSTEM

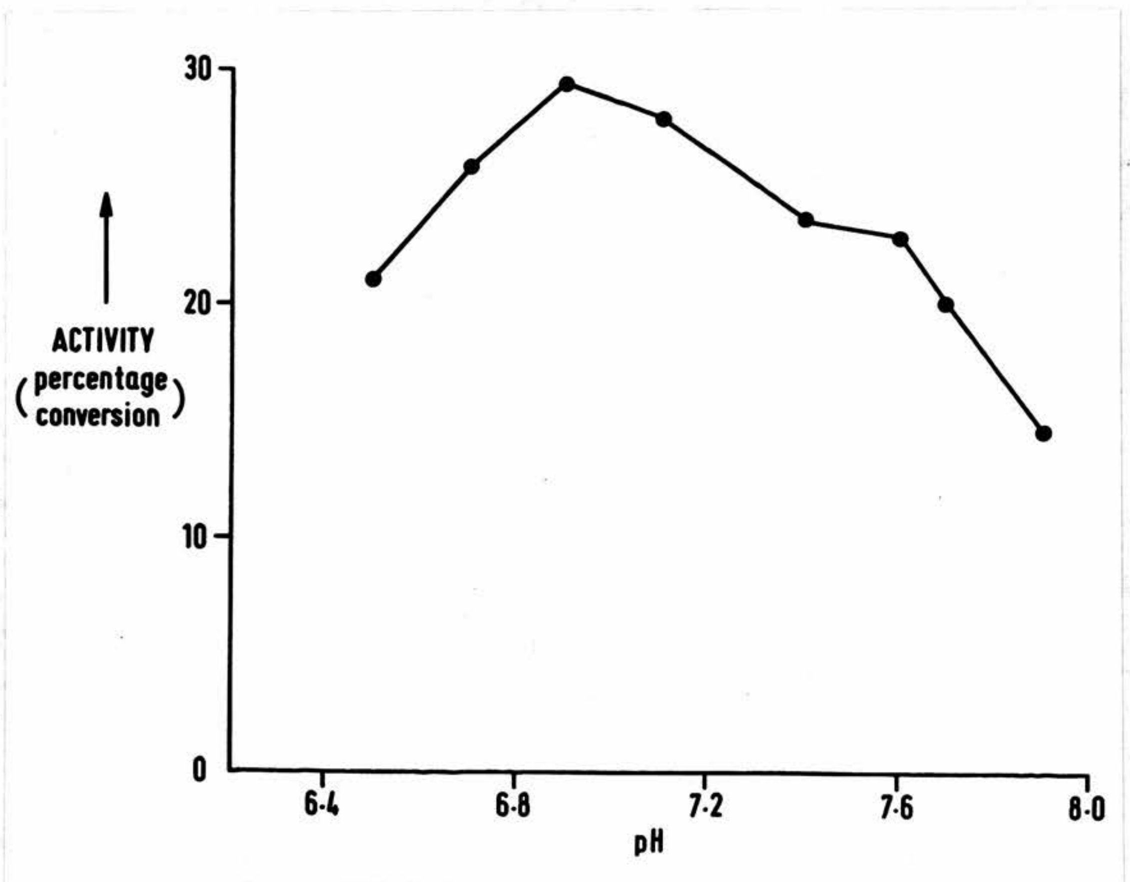


Fig. 4.1. pH-activity profile of a 105,000 g. x 60 min. supernatant of lyophilized placental mitochondria. The buffer employed was phosphate.

CHARACTERISTICS OF THE 'SOLUBILIZED' PLACENTAL CHOLESTEROL SIDE-CHAIN CLEAVAGE ENZYME SYSTEM

Effect of pH

The cholesterol side-chain cleavage enzyme assays were performed as described in Chapter 2, using phosphate buffers of varying pH. The enzyme source was a 105,000 g. x 60 min. supernatant of a lyophilized placental mitochondrial suspension in 0·1 M KCl. The results are shown in Fig. 4.1. The pH-activity profile was found to have quite a broad maximum with a maximal activity in the region of pH 7. Thus the enzyme activity is not very sensitive to the pH between 6·5 and 8·0. Routine assays were carried out at pH 7·4., close to the physiological pH.

Effect of NADPH

A characteristic of mammalian steroid mixed-function oxidases is that they appear to have a specific requirement for NADPH. It was found that the placental cholesterol side-chain enzyme appeared to have a specific requirement for NADPH, since NADH (at concentrations up to 1 mM) did not support cholesterol side-chain cleavage activity.

The NADPH concentration (produced using the NADPH generator system) used in the routine assay was 0·5 mM. However, very much lower concentrations of NADPH were required to produce any effect on the enzymic activity. Figure 4.2 shows the effect of NADPH on the cholesterol side-chain cleavage activity of a 105,000g. x 60 min. supernatant of lyophilized placental mitochondria (protein concentration, 4 mg./ml.) plotted according to Lineweaver and Burk (1934).

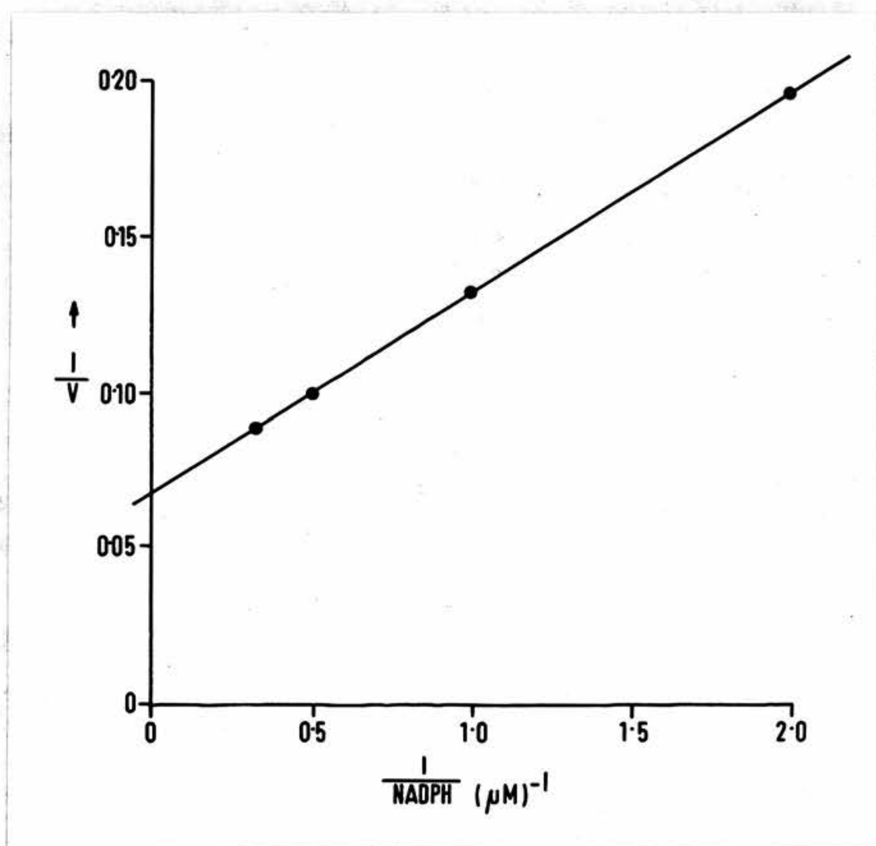
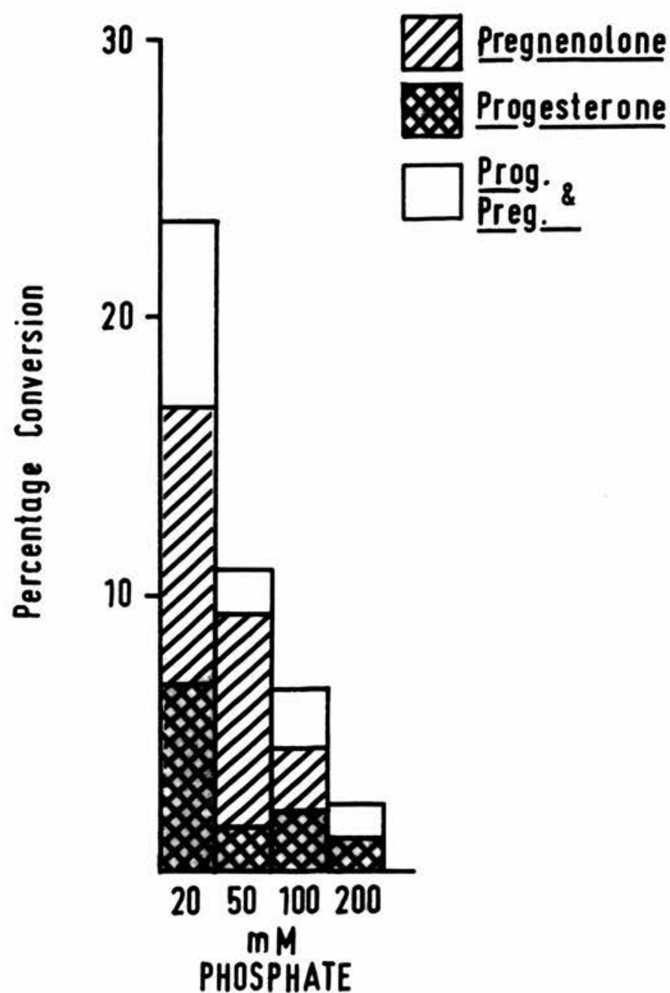


Fig. 4.2. A Lineweaver-Burk plot to determine the K_m for NADPH of the cholesterol side-chain cleavage enzyme of supernatant of lyophilized placental mitochondria.
(v = percentage conversion)



Effect of phosphate on the conversion of cholesterol-4-^{14}C to pregnenolone-4-^{14}C and progesterone-4-^{14}C by a lyophilised placental mitochondrial preparation

Fig. 4.3. Effect of (sodium) phosphate buffer, pH $7 \cdot 4$, on the cholesterol side-chain cleavage activity of supernatant of lyophilized placental mitochondria.

The intercept on the abscissa allowed the calculation of an apparent Km value for NADPH. It was found to be about 1 μM. The assays were performed in the presence of 200 μg of added cholesterol in addition to the tracer cholesterol to ensure saturating levels of the substrate. The NADPH concentration was varied by addition of different amounts of $NADP^+$ plus 10 μmoles of glucose-6-phosphate and 0·25 i.u. of glucose-6-phosphate dehydrogenase.

Concentrations of NADPH from 2 μM to 0·5 mM did not have much effect on the cholesterol side-chain cleavage activity, in disagreement with the observations of Satoh et al. (1966) who observed that 0·5 mM NADPH strongly inhibited the adrenocortical cholesterol side-chain cleavage enzyme. NADPH concentrations greater than 0·5 mM did, however, slightly inhibit the enzymic activity.

Effect of salt concentration

It was observed that variations in the phosphate buffer concentration of the standard assay system for the solubilised enzyme produced a marked effect on the cholesterol side-chain cleavage activity. The observations are recorded in Fig. 4.3. Increasing concentrations of phosphate were found to produce an increasing inhibition of the cholesterol side-chain cleavage activity of the lyophilized mitochondrial extract. 200 mM phosphate almost completely abolished the cholesterol side-chain cleavage activity. Satoh et al. (1966) reported a similar effect of phosphate on the cholesterol side-chain cleavage activity of an acetone powder extract of bovine adrenocortical mitochondria. Since there was a

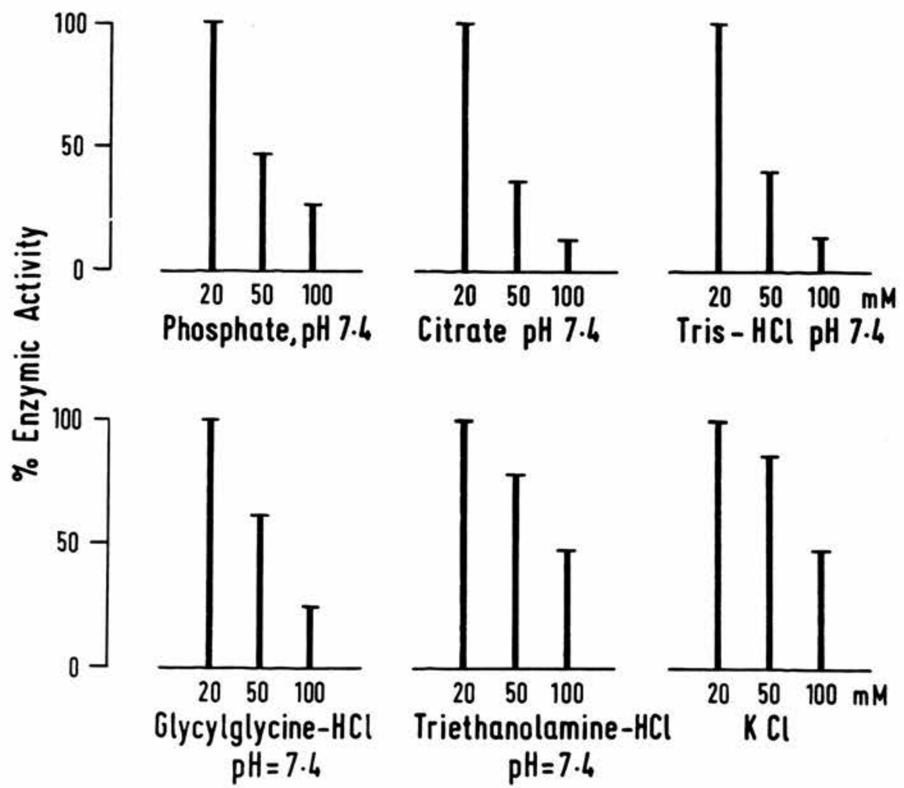


Fig. 4.4. Effect of various buffer salts at pH 7·4 on the cholesterol side-chain cleavage activity of a lyophilized placental mitochondrial extract.

requirement for NADPH by the enzyme system, the possibility existed of competition between the phosphate groups of the cofactor and the buffer salt at the coenzyme binding site. However, doubling the NADPH concentration did not produce any increase in enzymic activity; in fact, increasing the NADPH concentration to 1 mM produced a slight decrease in the enzymic activity. Table 4.1 presents the data obtained when the NADPH concentration was doubled at increasing phosphate concentrations.

Several other buffer systems were then used to investigate the specificity of the phosphate inhibition. These included triethanolamine-HCl, Tris-HCl, glycylglycine-HCl and citrate, all at pH 7·4. Cholesterol side-chain cleavage assays were carried out at varying concentrations of these buffers. The results are summarised in Fig. 4.4. It was seen that inhibition of enzymic activity occurred with increasing concentrations of all the buffers investigated. Assays were also conducted at a fixed phosphate concentration (20 mM) but with an increasing salt concentration using potassium chloride (see also Fig. 4.4). This confirmed that the inhibition was a function of the salt concentration. Thus the solubilised cholesterol side-chain cleavage enzyme activity was sensitive to the salt concentration rather than to a specific ion such as phosphate.

In order to investigate whether the salt inhibitory effect was reversible or not, the following experiment was carried out.

300 mg. lyophilized placental mitochondria were taken

TABLE 4.1

EFFECT OF PHOSPHATE ON THE CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY AT TWO DIFFERENT NADPH CONCENTRATIONS

Phosphate pH 7·4 (mM)	NADPH 0·5 mM	NADPH 1·0 mM
10	35·7	30·5
50	26·0	24·3
190	9·0	10·9

The enzymic activity is expressed as the percentage conversion of [4-^{14}C] cholesterol to C_{21} products in 60 min. at 37°C. The enzyme source was a 105,000 g. x 60 min. supernatant of a lyophilized mitochondrial preparation.

up in 10 ml. 0·1 M phosphate pH 7·4 and centrifuged at 105,000 g. x 60 min. The supernatant was divided into two portions. To one portion was added NaCl (final concentration 1 M) and the portion was left at 4°C for 1 hour. After this time, both portions were dialysed against 0·04 M phosphate, pH 7·4, for 3 hours (3 changes). Cholesterol side-chain cleavage activity assays were then performed in the normal manner using 1·0 ml. aliquots of the two portions. The results are shown in Table 4.2. It appears that the salt effect was reversible; in fact, a small increase in activity was observed. It is very possible that the state of aggregation (or disaggregation) of the enzyme complex is important with regard to enzymic activity. One must conclude from these results that maximal enzymic activity is associated with a minimal ionic strength of the assay medium. However, the salt (buffer) concentration must be sufficient to maintain the hydrogen ion concentration near the optimum pH of the enzyme. The inhibitory effect of high salt concentrations will have to be taken into consideration when the effect of such compounds as thiols on cholesterol side-chain cleavage activity is studied. However, the inhibitory effect of high salt concentrations on the enzymic activity does appear reversible.

<u>Effect of phosphate concentration upon the cholesterol side-chain cleavage activity of native placental mitochondria</u>

Since high phosphate concentrations were inhibitory to the activity of the solubilised enzyme system, the effect on the enzymic activity of native placental mitochondrial

TABLE 4.2

EFFECT OF EXPOSURE TO A HIGH SALT CONCENTRATION (1 M NaCl) ON THE CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY OF A 105,000 g. x 60 MIN. SUPERNATANT OF A LYOPHILIZED PLACENTAL MITOCHONDRIAL PREPARATION

	Percentage conversion of [4-^{14}C] cholesterol to C_{31} products in 60 min. at 37°C
Control	10·7
Sample exposed to 1 M NaCl for 60 min., then dialysed	13·3

Each incubation contained equivalent to 30 mg. lyophilized mitochondria.

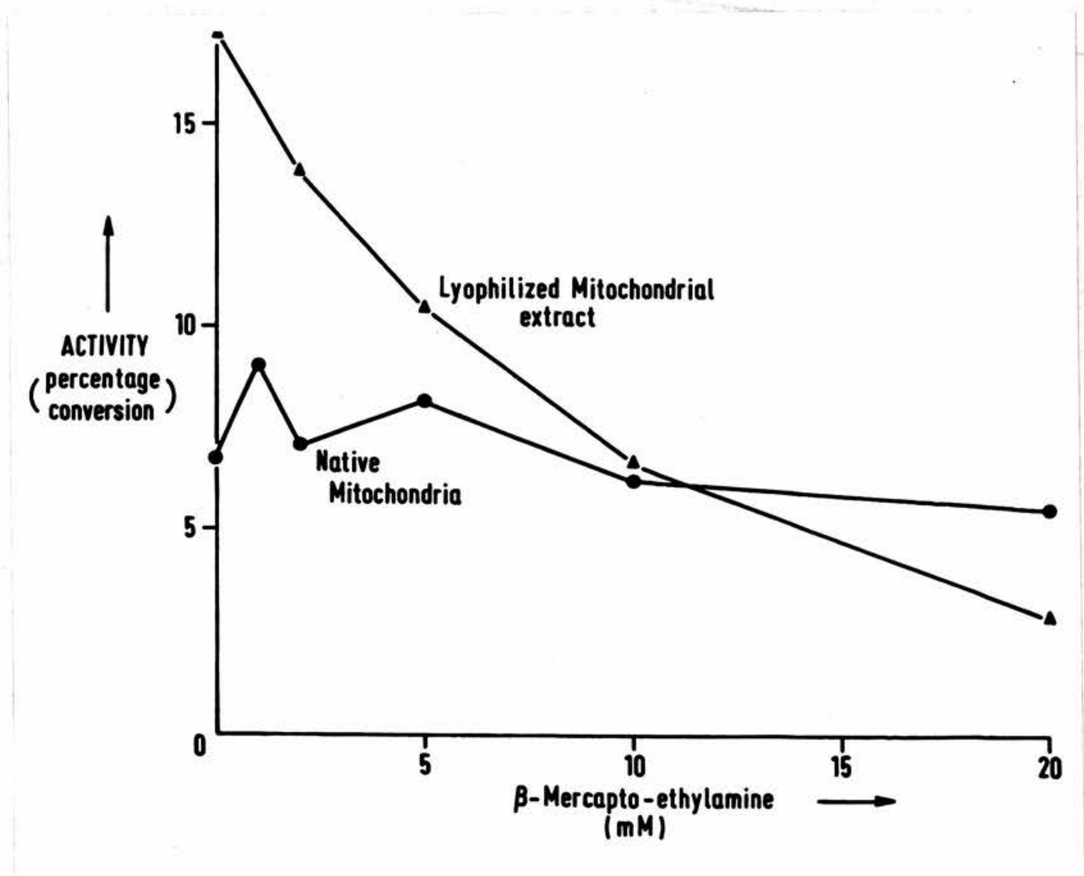


Fig. 4.5. Effect of β-mercapto-ethylamine on the cholesterol side-chain cleavage activities of placental native mitochondria and lyophilize mitochondrial extracts.

preparations was investigated. The results, displayed in Table 4.3, show that increasing phosphate concentrations have little effect on the cholesterol side-chain cleavage activity of native mitochondria. However, the levels of enzymic activity in the native mitochondria were very much smaller than in the solubilised preparations and significant differences were less readily seen.

Effect of thiol compounds

There is an increasing amount of evidence supporting the importance of sulphydryl groups - both of the cytochrome P-450 and of the non-haem iron protein - in the structure and catalytic activity of hydroxylases similar to the cholesterol side-chain cleavage enzyme system (Mason *et al.*, 1965). Thiol compounds such as β-mercaptoethylamine appear especially important for cholesterol 7α-hydroxylase activity (Scholan, 1969). In this instance, β-mercaptoethylamine appears to direct the oxygen attack to the 7α position, whilst suppressing free radical type reactions which give rise to a wide range of autoxidative products. Sulimovici and Boyd (1968a) observed that β-mercapto-ethylamine (1-10 mM) produced some stimulation of the cholesterol side-chain cleavage activity of native rat ovarian mitochondria. Higher concentrations of the thiol were found to be inhibitory.

Figure 4.5 illustrates the effect of varying concentrations of β-mercapto-ethylamine on the cholesterol side-chain cleavage activity of native placental mitochondria. At low concentrations of the β-mercapto-ethylamine (1-5 mM), some stimulation of cholesterol side-chain cleavage activity

TABLE 4.3

EFFECT OF PHOSPHATE CONCENTRATION ON THE CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY OF NATIVE PLACENTAL MITOCHONDRIA

Phosphate, pH 7·4 (mM)	Percentage conversion of [4-^{14}C] cholesterol in 60 min. at 37°C to:		
	^{14}C-Pregnenolone	^{14}C-Progesterone	Total
20	0·8	2·1	2·9
50	0·5	1·9	2·4
100	0·8	1·8	2·6
200	0·5	1·5	2·0

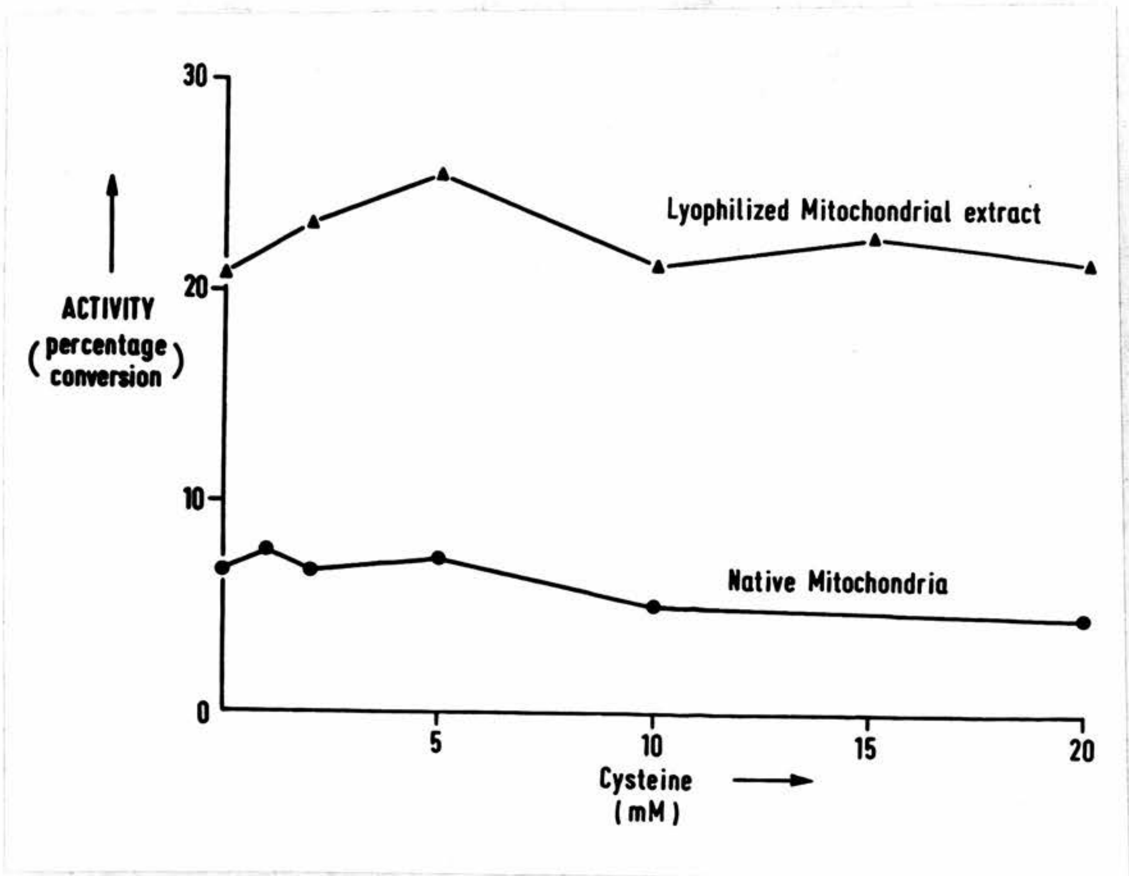


Fig. 4.6. Effect of cysteine on the cholesterol side-chain cleavage activities of placental native mitochondria and lyophilized mitochondrial extracts.

was observed; higher concentrations were inhibitory. Figure 4.5 also shows the effect of β-mercapto-ethylamine on the solubilised enzyme (from lyophilized mitochondria) when the thiol was found to be inhibitory; 8 mM β-mercapto-ethylamine produced about 50% inhibition.

Figure 4.6 illustrates the observations made on the cholesterol side-chain cleavage activities of native and solubilised mitochondrial preparations in the presence of increasing amounts of cysteine. The cysteine solution was brought to pH 7·4 with KOH prior to addition to the incubations. In both preparations, cysteine had no significant effect on the cholesterol side-chain cleavage activity.

The effect of reduced glutathione on the cholesterol side-chain cleavage activities of native and solubilised placental mitochondrial preparations is shown in Fig. 4.7. Glutathione at low concentrations around 0·5 mM did stimulate the native mitochondrial activity; otherwise glutathione had no significant effect on the enzymic activity.

In the absence of NADPH, none of the three thiols supported the enzymic activity. This is in contrast to the oxygenase involved in the biosynthesis of prostaglandins which utilizes glutathione (reduced)(Nugteren et al., 1966).

Other thiol compounds such as β-mercapto-ethanol, homocysteine, and dithiothreitol were also tested. β-mercapto-ethanol and homocysteine were found to have little effect on the enzyme activity in the concentration range (0-20 mM) investigated. Dithiothreitol inhibited the solubilised enzyme (50% inhibition at 10 mM).

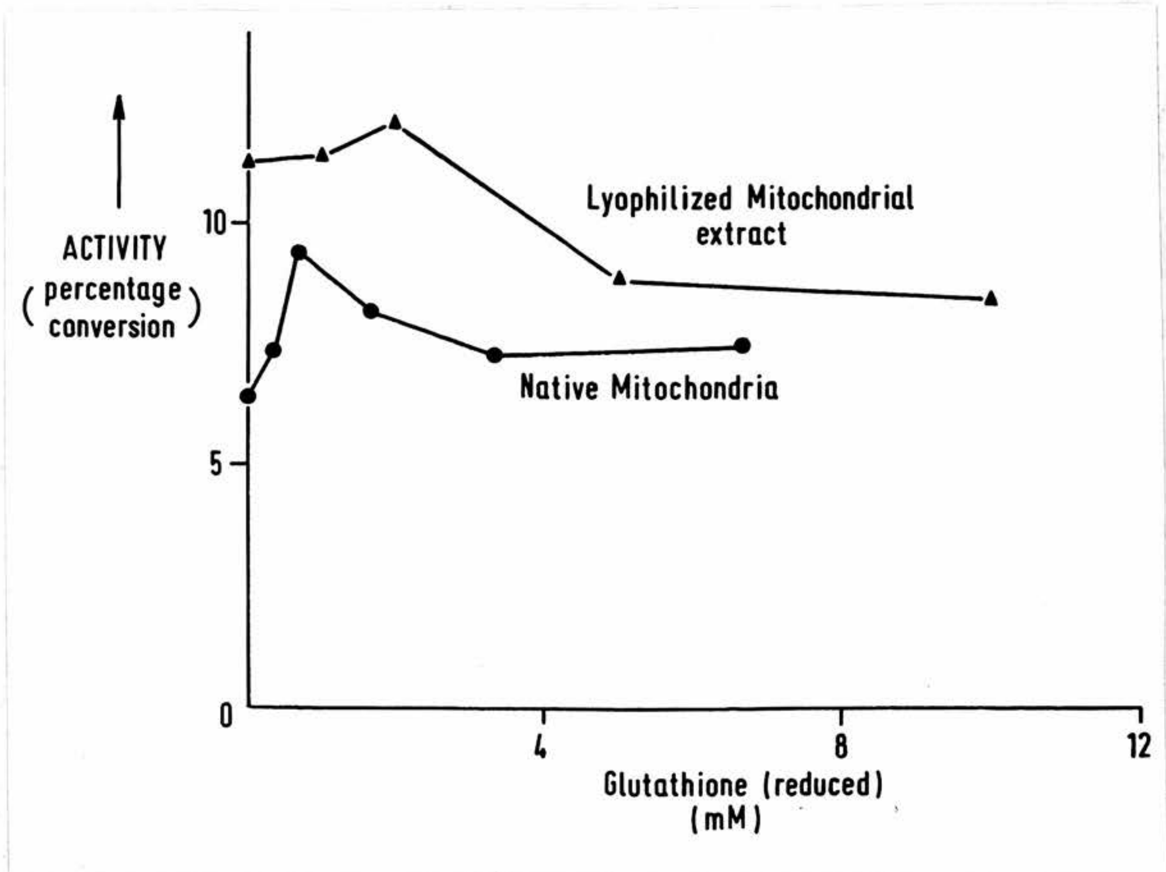


Fig. 4.7. Effect of glutathione (reduced) on the cholesterol side-chain cleavage activities of placental native mitochondria and lyophilized mitochondrial extracts.

In general, none of the thiol compounds studied had a marked stimulatory effect on the cholesterol side-chain cleavage activity. Some stimulation was observed at low concentrations using native mitochondrial preparations. In these instances, the observed effect could possibly be attributed to an effect on the permeability of the mitochondria to NADPH. i.e. an indirect effect on the supply of reducing equivalents rather than a direct involvement at the enzyme level.

NADPH was always required and no evidence was obtained for the substitution of NADPH as the electron donor by any of the thiol compounds. The thiol compounds apparently had no protective effect on the enzyme system as measured by any significant increase in enzymic activity.

Activation Energy of the cholesterol side-chain cleavage reaction in placental mitochondria.

Sterol mono-oxygenases such as the Steroid 11β-hydroxylase and the cholesterol side-chain cleavage enzyme are concerned in reactions which involve both oxygenation and oxidation. The enzymes catalyse the introduction of molecular oxygen into the substrate molecule. They also require the presence of an external electron donor. In the case of most mammalian mono-oxygenases the donor is NADPH. Due to the dual requirement for molecular oxygen and an electron donor, Mason (1957) termed these enzymes as "mixed-function oxidases". The cholesterol side-chain cleavage reaction involves the oxidation of both cholesterol and NADPH. i.e. the coupling of two exergonic reactions. The

overall redox potential arising from the transfer of electrons from NADPH to molecular oxygen along the mitochondrial respiratory chain is over 50 Kcal mole^{-1}. Thus the oxidation of NADPH in a hydroxylase reaction is energetically expensive. It seemed therefore likely that considerable enrgy was required in an activation step - either activation of the substrate or the oxygen. Since molecular oxygen is a comparatively inert substance, the activation of the oxygen into a reactive species could be the important energy requiring process. It was decided, therefore, to determine the activation energy of the cholesterol side-chain cleavage reaction. It was thought that this value could be meaningful in any energetic consideraction of the reaction mechanism.

The procedure for the determination of the activation energy of the reaction was as follows: Placental mitochondrial acetone powder extracts (prepared as described earlier) were incubated with [4-^{14}C] cholesterol and NADPH using the normal assay system. Incubations were performed in duplicate at varying temperatures between 24° - 37°C (increments of 2-3°C). The incubation periods were such that the initial velocity could be expressed validly as a function of the enzymic activity. The normal incubation period was 10 minutes and the rate of production of metabolites was linear over this time period. The temperature of the incubation bath was taken at 2 minute intervals during the incubation. This permitted a mean temperature value to be obtained. The influence of temperature on the reaction velocity can be

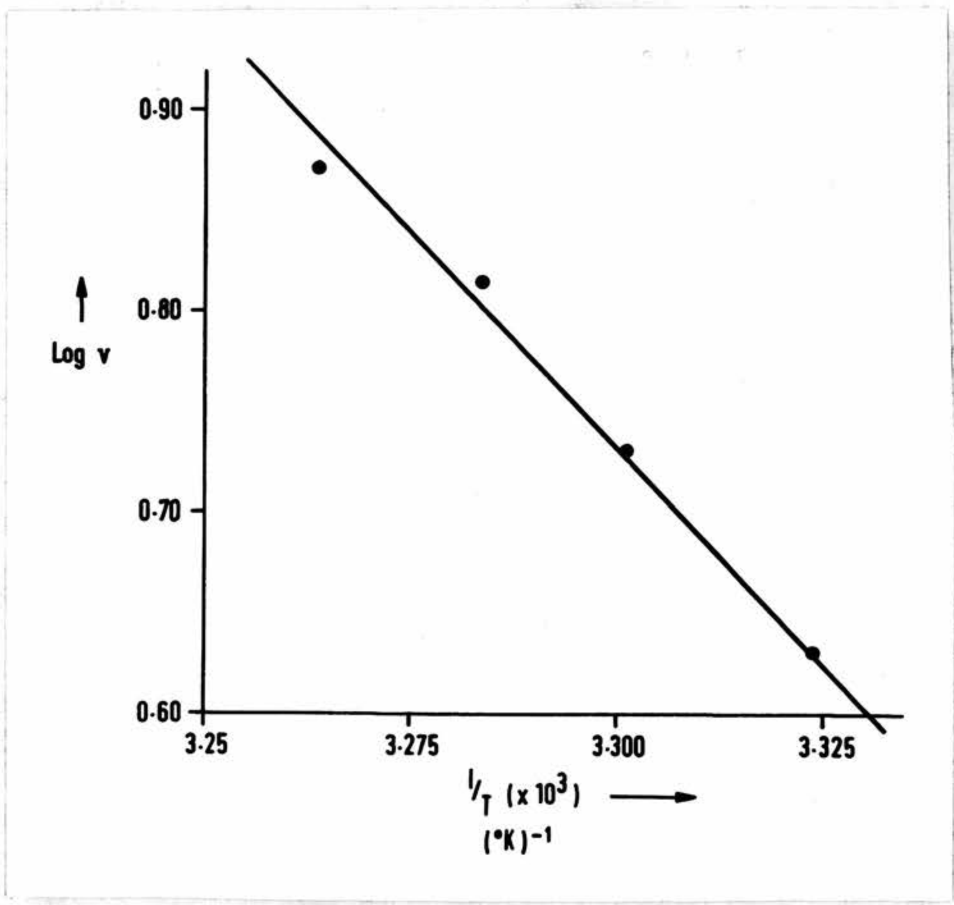


Fig. 4.8. An Arrhenius plot to show the effect of temperature on the rate of the cholesterol side-chain cleavage reaction (v = percentage conversion in 10 minutes, x 10).

expressed in the form of the Arrhenius equation, given below.

$$k = Ae^{-E/RT} \qquad (C)$$

where k is the specific reaction rate, $e^{-E/RT}$ is the Boltzmann factor, A is a constant, sometimes called the frequency factor and E is the energy of activation of the reaction.

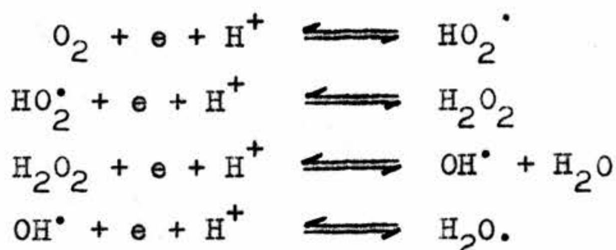
Taking logarithms of equation (C), it is found that

$$\log k = \log A - \frac{E}{2 \cdot 303RT}$$

This requires a plot of log k against 1/T to be linear, the gradient being equal to - E/2·303R. The activation energy, E, which is a constant and a characteristic of the reaction, can thus be calculated from the value of this slope.

The enzymic activity at each temperature was taken as a measure of the specific reaction rate, k. Hence there was a need for short incubation periods to ensure a linear relationship between k and the enzymic activity. Figure 4.8 shows a typical plot of log ν against 1/T for the cholesterol side-chain cleavage reaction occurring in placental mitochondrial extracts. The mean value of E obtained was -18·4 ± 2·5 kcal $mole^{-1}$ (4 separate experiments). This is a relatively high activation energy and is comparable to the activation energy of the liver microsomal cholesterol 7α-hydroxylase which was found to be -22 ± 4 kcal $mole^{-1}$ (Boyd, 1970). It appears possible that there is a common activation step in the two enzymic reactions. Unfortunately these two values of activation energies could not be compared with those for other mixed function oxidases, since the literature appeared devoid of such estimations. However, George and Stratmann (1952) and very recently Brown and Mebine (1969)

have determined the activation energy of the reaction involving the autoxidation of oxymyoglobin to metmyoglobin. Activation energies obtained with myoglobins from different sources ranged from 24-27 kcal $mole^{-1}$. One can speculate that the activation of oxygen by these enzymes could be the important energy-requiring process. Although in its ground state molecular oxygen has two unpaired electrons (triplet state) and is paramagnetic, it does not behave chemically as a biradical. Molecular oxygen is fairly inert, its apparent reactivity being due to the activated species $O^{\bullet}$, $OH^{\bullet}$, $RO_2^{\bullet}$, able to initiate chain reactions. Free radical species derived from oxygen have frequently been proposed as intermediates in biochemical reactions (Norman and Radda, 1962; Yamazaki and Piette, 1963; Staudinger and Ullrich, 1964). Oxygen reduction can, in principle, occur in four single electron steps:



$$O_2 + e + H^+ \rightleftharpoons HO_2^{\bullet}$$
$$HO_2^{\bullet} + e + H^+ \rightleftharpoons H_2O_2$$
$$H_2O_2 + e + H^+ \rightleftharpoons OH^{\bullet} + H_2O$$
$$OH^{\bullet} + e + H^+ \rightleftharpoons H_2O.$$

The redox potentials at pH 7 for these reactions have been calculated as about, -0·6, + 1·1, + 0·4, and + 2·3 V respectively (George, 1965; Mason, 1965). Thus quite strong reducing conditions are required to add the first electron to oxygen, and the two free radicals, $HO_2^{\bullet}$ and $OH^{\bullet}$, are oxidising agents, the latter a powerful one. There is also the possibility of oxygen atoms being formed in the reduction,

by the O—O bond breaking in the second of the above reactions to give water and $O^{\cdot}$, instead of H_2O_2 (George, 1965).

The formation and reactions of a number of oxygen free radicals have been quite extensively studied by physical chemists. Both $HO_2^{\cdot}$ and $OH^{\cdot}$ can undergo acid dissociations, giving $O_2^{\cdot -}$ and O^- respectively, with pK values of 4·5 and 11·9 (Schested, Rasmussen and Fricke, 1968; Rabani and Matheson, 1964). Thus at pH values of biochemical interest $O_2^{\cdot -}$ and $OH^{\cdot}$ might occur; HO_2, also being a possible species in the lower pH range. There appears to be no conclusive evidence for the existence of the species OH^+ (Samuel, 1968). Radiation work has given data on the lifetimes of the oxygen radicals. In the neutral pH range, both $O_2^{\cdot -}$ and $HO_2^{\cdot}$ have half-lives of tens of milliseconds (Czapski and Dorfman, 1964). In the alkaline region, the stability of $O_2^{\cdot -}$ is markedly increased. e.g. it has a half-life of about 200 ms at pH 10 (see Bray, 1970). $O_2^{\cdot -}$ has been observed by Knowles _et al_. (1969) using electron paramagnetic resonance, during xanthine oxidase-catalysed oxidation of substrates by oxygen at pH 10. It must be remembered, however, that $O_2^{\cdot -}$ is the most stable oxygen radical in the alkaline region. From the work of Komai _et al_. (1969) it appears that it is the flavin of the reduced enzyme molecule that reacts with oxygen in producing this radical.

$OH^{\cdot}$ is the active hydroxylating species of Fenton's reagent (Norman and Lindsay-Smith, 1965). However, direct evidence on the possible involvement of this radical must

await the evolution of new methods.

The nature of the active oxygen species still remains to be elucidated. The value of the activation energy obtained for the cholesterol side-chain cleavage mixed function oxidation partially explains the requirement for the powerful reducing agent NADPH.

CHAPTER 5

COMPONENTS OF THE PLACENTAL MITOCHONDRIAL CHOLESTEROL SIDE-CHAIN CLEAVAGE ENZYME SYSTEM

COMPONENTS OF THE PLACENTAL MITOCHONDRIAL CHOLESTEROL SIDE-CHAIN CLEAVAGE ENZYME SYSTEM

The presence of cytochrome P-450 in placental mitochondria

Adrenocortical mitochondria possess a very active cholesterol side-chain cleavage enzyme system and have considerable amounts of cytochrome P-450 (Simpson and Boyd, 1966, 1967a). These workers showed that cytochrome P-450 was involved in the cholesterol side-chain cleavage enzyme on the basis of carbon monoxide inhibition studies together with the release of this inhibition by light, maximal release of the inhibition occurring with light of wavelength 450 nm. However, other endocrine tissues which are known to possess an active cholesterol side-chain cleavage enzyme system appear to contain much smaller amounts of cytochrome P-450 compared to the adrenal cortex. Cooper (1970) has reported that the cytochrome P-450 content of rat ovarian mitochondria is about one tenth that of rat adrenal mitochondria. The rat ovarian mitochondria possess a very active cholesterol side-chain cleavage enzyme system (Sulimovici and Boyd, 1968a). Rat testis mitochondria are capable of cleaving the cholesterol side-chain (Menon et al., 1965) but the cytochrome P-450 content of testis mitochondria is very low (Machino et al., 1969).

In a study of placental drug metabolising systems, Juchau et al. (1968) failed to detect cytochrome P-450 in placental preparations; this contrasts with the liver in which cytochrome P-450 is frequently involved in drug

metabolism. They suggested, since certain steroid hydroxylation reactions had been demonstrated in placental tissue, that the electron transport chain for such hydroxylations might utilise a different cytochrome system. However, Meigs and Ryan (1968) demonstrated the presence of cytochrome P-450 in both placental mitochondria and microsomes. The mean cytochrome P-450 contents reported by these authors were 0·123 nmole per mg. mitochondrial protein and 0·042 nmole per mg. microsomal protein. The absolute amounts of cytochrome P-450 in placental particles are, therefore, approximately one tenth those calculated from date on the adrenal cytochrome P-450 content (Harding and Nelson, 1966).

In the present study the search for spectroscopic evidence for the presence of cytochrome P-450 in placental mitochondria was made very difficult by the presence of contaminating haemoglobin. Carboxyhaemoglobin has a maximum absorption peak at 420 nm. which is similar to that of cytochrome P-420. Since placental tissue has a very good blood supply, this made it very difficult to remove all traces of haemoglobin prior to homogenisation. However, considerable amounts were removed by 'washing' the tissue with either 0·9% NaCl solution or 0·25 M sucrose. Repeated 'washing' of the mitochondrial fraction also aided the removal of some of the remaining haemoglobin. However, there was always a trace of haemoglobin that remained firmly associated with the mitochondria. The presence of this haemoglobin complicated the spectral search for the small amounts of cytochrome P-450 present. Figure 5.1(a) shows a typical

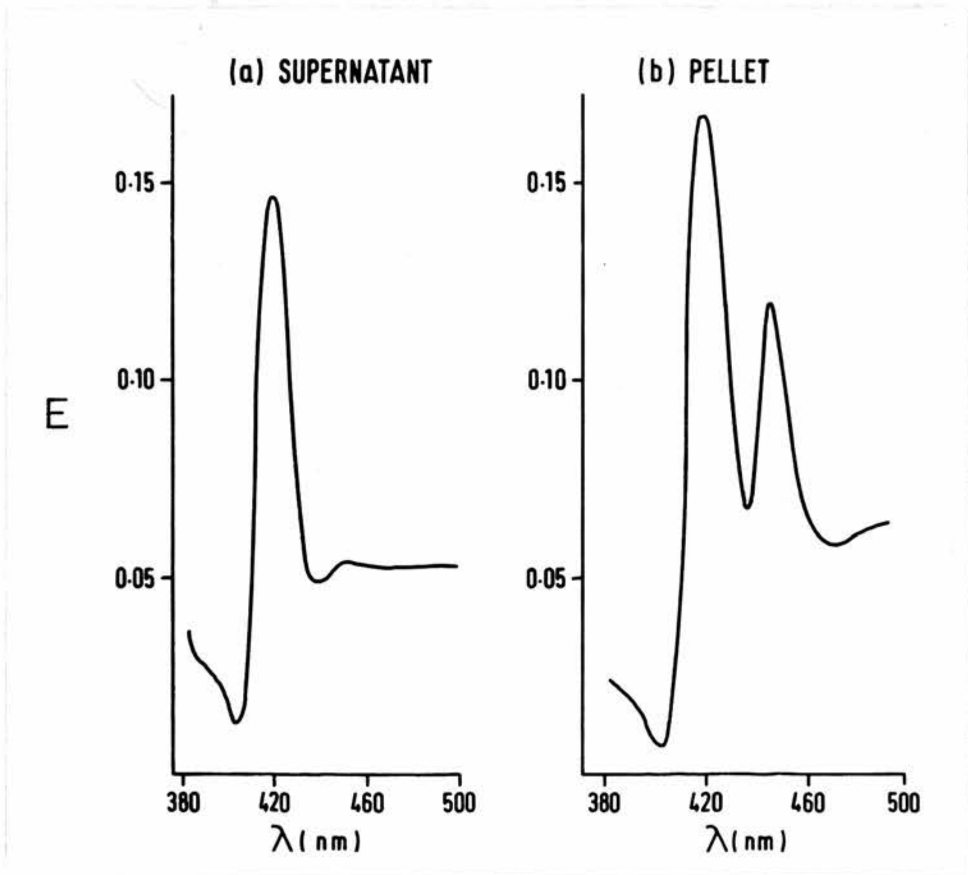


Fig. 5.1. The reduced carbon monoxide difference spectrum of (a) the 105,000 g. x 60 min. supernatant of lyophilized placental mitochondria (b) the 105,000 g. x 60 min. pellet of the same mitochondrial preparation. A reaction cuvette containing dithionite was gassed with CO; a reference cuvette contained dithionite.

reduced (dithionite) carbon monoxide difference spectrum of the 105,000 g. x 60 min. supernatant from a lyophilized placental mitochondrial suspension in 0·1 M phosphate buffer pH 7·4 (equivalent to 2 mg.mitochondrial protein per ml.). The 105,000 g. x 60 min. pellet was resuspended in 0·1 M phosphate, pH 7·4, and a similar reduced, carbon monoxide difference spectrum obtained (9 mg. mitochondrial protein per ml.). This is shown in Fig. 5.1(b). The spectra were obtained using a Unicam S.P. 800 recording spectrophotometer with a scale expansion accessory. As can be seen in Fig. 5.1, both the supernatant and the pellet contained material which gave a maximum absorption at 420 nm - certainly almost entirely due to contaminating haemoglobin. The difference in absorbance readings at 450 nm and 490 nm obtained from the CO difference spectrum of dithionite reduced preparations was used to calculate the cytochrome P-450 content with the extinction coefficient of 91 $mM^{-1}cm^{-1}$ (Omura and Sato, 1964b). The cytochrome P-450 content of the 105,000 g. x 60 min. pellet was found to be 0·05 nmole per mg. mitochondrial protein. Due to the contaminating haemoglobin, the cytochrome P-420 content could not be assessed. It could be speculated that there was a very small amount of cytochrome P-450 in the supernatant preparation from the mitochondria - too small to estimate. However, it should be noted that the cholesterol side-chain cleavage activity was located only in the supernatant fraction from a mitochondrial preparation (see Chapter 3). It appeared therefore that the amount of cytochrome P-450 involved in placental cholesterol side-chain cleavage activity was very small.

Effect of Carbon Monoxide on the cholesterol side-chain cleavage reaction in human placenta

Simpson and Boyd (1966) observed that the cholesterol side-chain cleavage reaction in bovine adrenocortical mitochondria was sensitive to carbon monoxide. Previously Harding et al. (1965) had shown that steroid 11β-hydroxylase activity in the same tissue was inhibited by carbon monoxide. The haemoprotein, cytochrome P-450, was believed from these studies to be the carbon monoxide sensitive component. These observations were presented as evidence for the involvement of cytochrome P-450 in the 11β-hydroxylation of deoxycorticosterone and the cholesterol side-chain cleavage reaction. The effect of carbon monoxide on the cholesterol side-chain cleavage reaction of human placental mitochondria was therefore investigated.

These studies were carried out in a similar manner to those described by Simpson and Boyd (1966). The apparatus used in this investigation is described below.

Carbon monoxide was prepared by the action of conc. sulphuric acid on formic acid (both Analar grade). The gas was bubbled through water to remove acid vapours, then through chromous chloride to remove oxygen and finally through potassium hydroxide pellets to dry the gas. Nitrogen was also bubbled through chromous chloride solution to ensure it was oxygen free. Appropriate gas mixtures were prepared in one litre burettes. Incubations were carried out in standard 25 ml. Erlenmeyer flasks. The flasks were fitted with rubber stoppers drilled to permit insertion of hypodermic syringe needles - one needle being connected to the gas burette

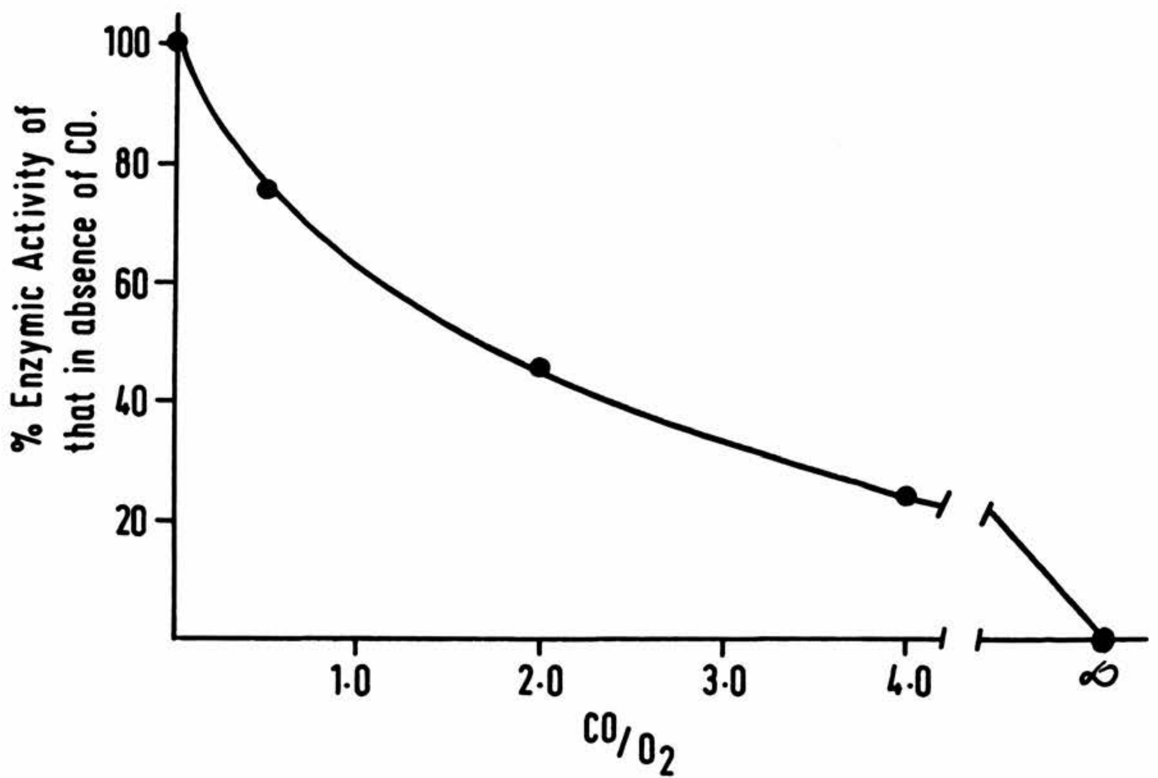


Fig. 5.2. Effect of carbon monoxide/oxygen ratio on the rate of the cholesterol side-chain cleavage reaction in supernatant of a placental mitochondrial sonicate.

by means of rubber tubing, the second shorter needle being used to control the gas outflow from the flask. Gas mixtures were bubbles through the flasks by maintaining a constant head of pressure. The flasks were contained in a shaking incubation bath maintained at 37°C. This system is an open one, in contrast to the closed system of Warburg (1949) and Estabrook _et al_. (1963).

The standard incubation mixture of Table 3.1 was made up as usual, but omitting the cholesterol-4-^{14}C substrate. The enzyme source was a 105,000 g. x 60 min. supernatant from sonicated placental mitochondria. A period of 5 min. after the commencement of the bubbling of the appropriate gas mixture was allowed for the contents to reach equilibrium with respect to the temperature, the gas composition and the state of reduction of the NADP. The [4-^{14}C] cholesterol tracer was then added using an Agla syringe by removing the outlet tube and inserting the syringe through the hole in the rubber stopper. The incubation was allowed to proceed for one hour and then terminated with the addition of methanol and extracted in the normal manner.

The numerical results are shown in Fig. 5.2. As the carbon monoxide content of the gas phase was increased there was a corresponding decrease in the activity of the cholesterol side-chain cleavage enzyme system.

Calculation of the Partition Constant

Warburg (1949) derived the following expression to account for the inhibition of cellular respiration by carbon monoxide. In the case of the cholesterol side-chain

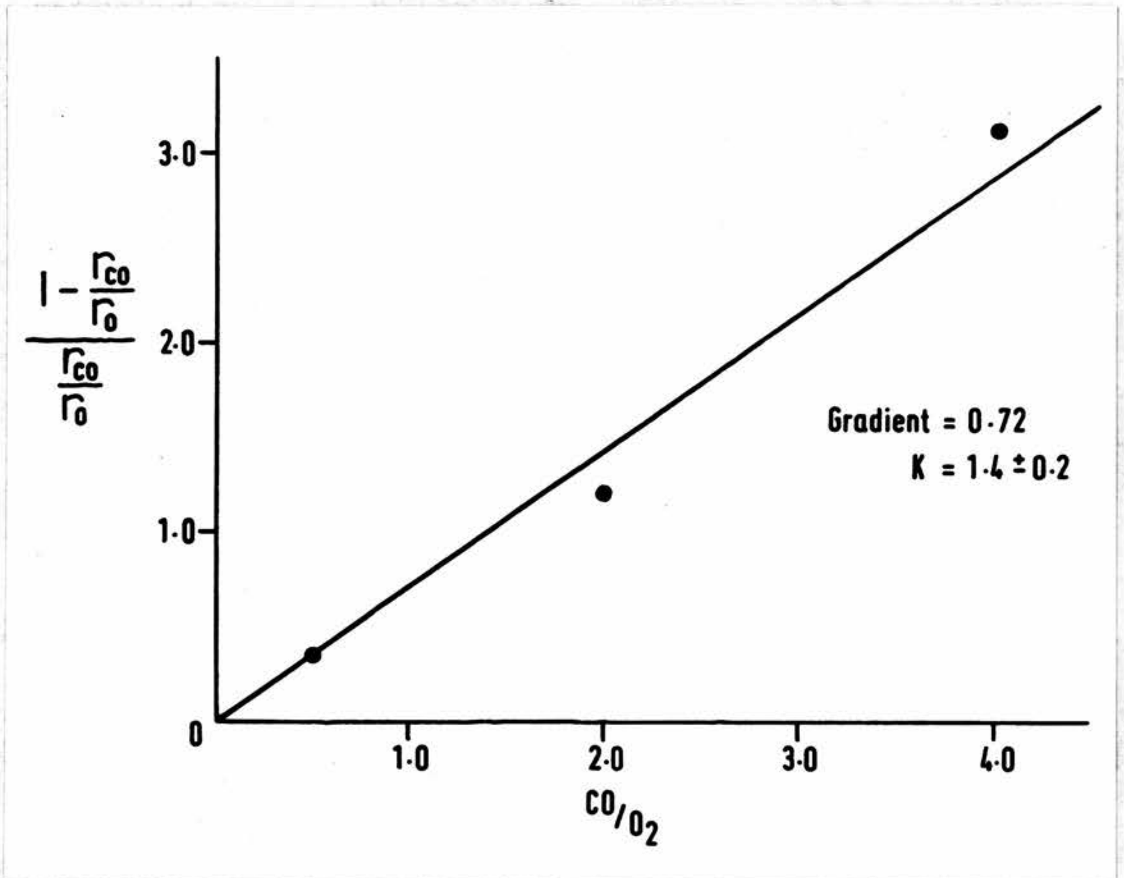
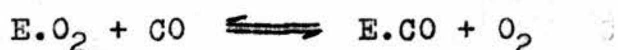


Fig. 5.3. Plot of $\dfrac{1 - \dfrac{r_{co}}{r_o}}{\dfrac{r_{co}}{r_o}}$ versus $\dfrac{CO}{O_2}$ for cholesterol side-chain cleavage reaction. The gradient is 1/K.

cleavage enzyme system, it is assumed that carbon monoxide is competing with oxygen for an enzyme site resulting in inhibition of the enzymic activity.

For the reaction -



$$E.O_2 + CO \rightleftharpoons E.CO + O_2$$

where E is the enzyme binding site, the equilibrium constant K is given by -

$$K = \frac{(E.O_2).(CO)}{(E.CO).(O_2)}$$

Therefore,

$$\frac{CO}{O_2} = K.\ \frac{E.CO}{E.O_2}$$

Hence -

$$\frac{CO}{O_2} = K.\ \frac{1 - \frac{r_{co}}{r_o}}{\frac{r_{co}}{r_o}}$$

where r_{co} is the reaction rate in the presence of carbon monoxide and r_o the reaction rate when no carbon monoxide is present. The equilibrium constant K is known as the partition constant and is a characteristic of the system.

If one plots,

$$\frac{1 - \frac{r_{co}}{r_o}}{\frac{r_{co}}{r_o}} \quad \text{versus} \quad \frac{CO}{O_2}$$

a straight line of gradient 1/K should be obtained.

Figure 5.3 shows the results of plotting the data of Figure 5.2 in this way. It can be seen that the points give a reasonable fit to a straight line. The value of K obtained, calculated on the assumption that line passes

through the origin, is $- K = 1{\cdot}4 \pm 0{\cdot}2$ (s.d.) where N = 4.

Simpson and Boyd (1966) obtained values for the partition constant varying between 0·2 and 1·2 in a study of the bovine adrenocortical mitochondrial cholesterol side-chain cleavage enzyme. The present finding is also in keeping with those of Estabrook et al. (1963) who quote values between 0·6 and 1·8 for the partition constant for the steroid 21-hydroxylase of adrenal cortex microsomes. Simpson and Boyd (1966) also found a correlation between the concentration of carbon monoxide in the gas phase and the size of the cytochrome P-450 chromophore. This enabled the partition constant for cytochrome P-450 to be obtained. However, due to presence of only very small amounts of cytochrome P-450 in the solubilised placental mitochondrial preparations, no such studies were carried out in this investigation.

Thus, in common with several other mixed-function oxidases, and more particularly the steroid 11β- and 21-hydroxylases and the cholesterol side-chain cleavage enzyme of the adrenal cortex, the cholesterol side-chain cleavage enzyme of human placental mitochondria is inhibited by carbon monoxide. This is in agreement, with the results of Meigs and Ryan (1968) on the effect of carbon monoxide on the placental cholesterol side-chain cleavage enzyme. These workers do not quote a partition constant for comparison with the one obtained in this study. Furthermore, the kinetics of this inhibition are consistent with a competition between oxygen and carbon monoxide for a common binding site. Such

competition is a well known feature of several haemoproteins such as haemoglobin and cytochrome oxidase. The partition constant for the placental cholesterol side-chain cleavage enzyme is about unity, and therefore similar to that for other steroid hydroxylases. This value for mixed function oxidases is quite different from the partition constants of haemoglobin and cytochrome oxidase which are $1 \cdot 8 - 8 \cdot 0 \times 10^{-3}$ and 10 - 20 respectively. (Keilin and Wang, 1946). It is, however, very similar to the partition constant for cytochrome P-450, which was calculated for the adrenal cortex mitochondrial cytochrome P-450 (Simpson and Boyd, 1966) to be $1 \cdot 07 \pm 0 \cdot 30$. This suggests the possibility that cytochrome P-450 may be involved as the oxygen-binding site for the cholesterol side-chain cleavage enzyme of human placental mitochondria.

<u>Effect of light on the carbon monoxide inhibition of the placental cholesterol side-chain cleavage reaction</u>

A characteristic of the carbon monoxide complexes of haemoproteins is that the enzyme—CO combination can be dissociated by light of the appropriate wavelength. Estabrook *et al.*, (1963) and Omura *et al.*, (1965) showed that the carbon monoxide inhibition of mixed-function oxidases, and particularly the steroid 21-hydroxylase, was light reversible and furthermore, that the most effective wavelength for reversing the carbon monoxide inhibition in the presence of oxygen was 450 nm. This very much strengthened the evidence for the involvement of cytochrome P-450 as the site of oxygen binding in these mixed-function oxidases. Simpson and Boyd (1967a) examined the light reversibility of the cholesterol side-chain cleavage system of adrenocortical mitochondria. The inhibition was found to be light reversible, the most effective wavelength again being 450 nm. This was convincing evidence for the involvement of cytochrome P-450 in the adrenal cholesterol side-chain cleavage enzyme system. It was decided, therefore, to examine the light reversibility of the carbon monoxide inhibition of the placental cholesterol side-chain cleavage activity.

According to the theory of Warburg (1949), the partition constant in the absence of light is -

$$K_d = \left[\frac{\frac{r_{co}}{r_o}}{1 - \frac{r_{co}}{r_o}} \right]_d \cdot \frac{CO}{O_2}$$

where subscript d refers to reactions in the dark.

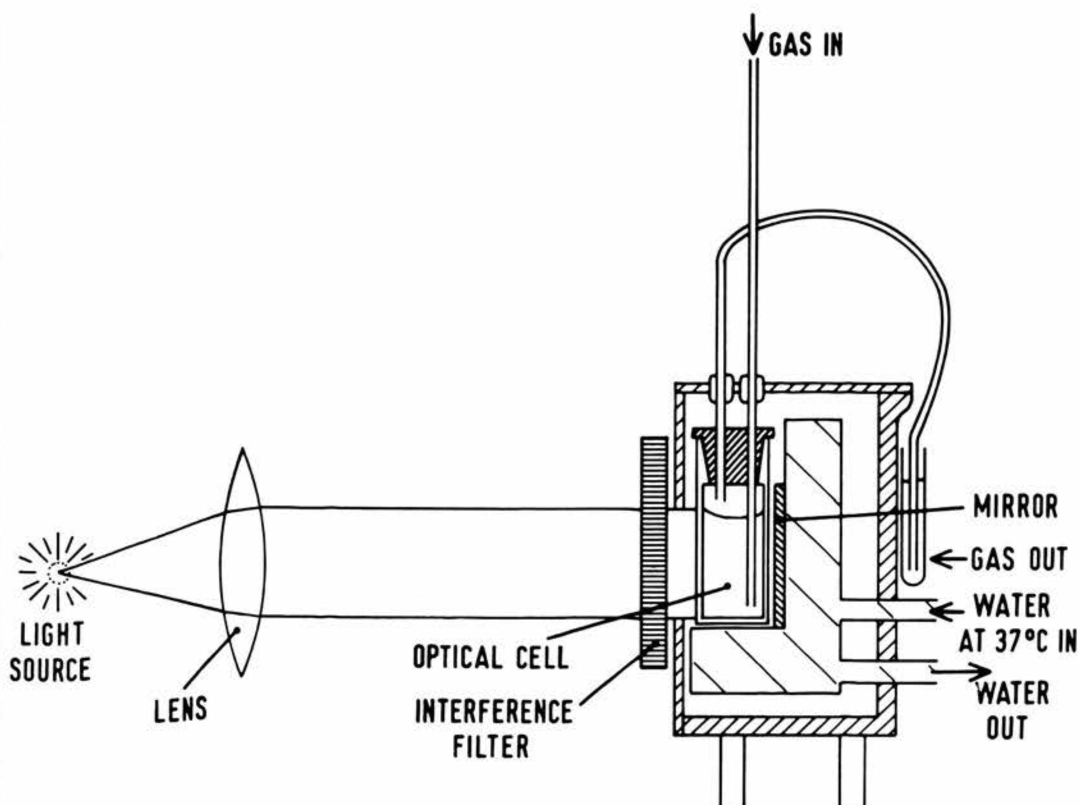


Fig. 5.4. Apparatus for the study of the light reversal of the carbon monoxide inhibition of cholesterol side-chain cleavage activity.

In the presence of light a new constant is obtained -

$$K_1 = \left[\frac{\frac{r_{co}}{r_o}}{1 - \frac{r_{co}}{r_o}}\right]_1 \cdot \frac{CO}{O_2}$$

where subscript 1 refers to reactions in the presence of light.

The percentage displacement of the partition constant -

$$\frac{\Delta K}{K_d} = \frac{K_1 - K_d}{K_d}$$

is proportional within limits to the light intensity, i, for any wavelength.

Thus -

$$\frac{\Delta K}{K_d} = Li$$

where L is a proportionality factor called the "light sensitivity" of which the dimensions are a reciprocal of intensity. Hence plotting L against the wavelength of the incident light gives the photo-chemical action spectrum of the carbon monoxide-sensitive system and reveals the wavelength which is the most effective in reversing the carbon monoxide inhibition in the presence of oxygen.

Experimental procedure. This was essentially as described by Simpson and Boyd (1967a). The light source was a 250 watt high pressure Xenon lamp. Since the degree of reversal of inhibition is proportional to the light intensity, the area of illumination was reduced as much as possible, whilst at the same time ensuring uniform illumination of the entire solution. The experimental arrangement is shown in Fig. 5.4. The optical cuvette provided an incident area of

about 2 cm^2. A mirror was placed behind the cuvette, in order to increase the light flux. The cuvette was kept at 37°C in the Unicam water-jacketed cell holder. The lamp was enclosed in an aluminium shielding blackened on the inside, except for a slit. The lens was so arranged as to cause the light beam from the slit to focus on the cuvette. The filters used were interference filters (Grubb Parsons Ltd., Newcastle-upon-Tyne). The average band width was 8 - 10 nm. The relative intensity of the lamp beam passing through each filter had been previously determined by Dr. E.R. Simpson (1967). This had been determined using a Mullard photo-emissive cell with a type 'A' caesium-antimony photocathode, connected to a Varian 33B-2 electrometer.

The experimental procedure was exactly as described for experiments on the carbon monoxide inhibition. However, the enzyme preparation was the 105,000 g. x 60 min. supernatant of a mitochondrial acetone powder suspension. The standard cholesterol side-chain cleavage assay was used, except that the final volume was halved i.e. 2·5 ml.; thus effectively doubling the concentration of the reactants (see Table 3.1). Light passing through the appropriate filter illuminated the cuvette and its contents during the 5 min. pre-incubation period prior to the addition of the [4-^{14}C] cholesterol. Gassing of the cuvette contents continued throughout the experiment.

An initial experiment was carried out to determine whether light from the lamp, in the absence of any filters, was effective in reversing the carbon monoxide inhibition.

Incubations were initially carried under a gas phase composed of 10% oxygen, 10% carbon monoxide and 80% nitrogen. However, very little carbon monoxide inhibition of cholesterol side-chain cleavage activity was detected. From the previous investigations of the carbon monoxide sensitivity of the enzyme system which gave a value near unity for the Warburg partition constant between carbon monoxide and oxygen, an approximately 50% inhibition of the enzyme activity was expected. The enzyme source in this study was a mitochondrial acetone powder extract instead of the mitochondrial sonicate preparation used in the earlier carbon monoxide inhibition experiments and the acetone powder preparation had a greater activity compared to the sonicate preparation. This allowed a reduction in the assay period from 1 hour to 15 mins. Since assays could only be performed singly, this facilitated more ideal experimental conditions. When the carbon monoxide to oxygen ratio was increased, inhibition of enzymic activity was observed. This problem will be dealt with more fully in the Discussion.

Table 5.1 shows the results obtained in an experiment outlined below.

Incubation(1) was conducted under a gas phase of 10% oxygen, 90% nitrogen; incubations (2) and (3) were carried out under a gas phase composed of 10% oxygen, 40% nitrogen and 50% carbon monoxide. Incubations (1) and (2) were conducted in the dark while incubation (3) was carried out with the cell illuminated with the light beam from the Xenon lamp. The results clearly demonstrate that the carbon monoxide

TABLE 5.1

THE LIGHT REVERSAL OF THE CARBON MONOXIDE INHIBITION OF THE CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY OF A SOLUBILISED PLACENTAL MITOCHONDRIAL PREPARATION

Incubation No.	Conditions	Percentage Conversion
1	Dark	21·1
2	Dark + CO	14·6
3	Light + CO	19·7

inhibition in the presence of oxygen was reversed by light. The light beam caused no heating of the incubation medium so that the observed reversal was not due to thermal effects.

Photochemical action spectrum

In this study three interference filters were used, with transmission maxima at 418, 448 and 470 nm. The enzyme source was the same preparation as for the previous experiment, i.e. a 105,000 g. x 60 min. supernatant of a placental mitochontrial acetone powder suspension (equivalent to 30 mg. mitochondrial powder per ml.). The incubation period was again 15 minutes. One value of the enzymic activity was obtained using each filter. Control incubations were also conducted, namely an incubation in the presence of carbon monoxide but in the absence of light and an incubation in the absence of carbon monoxide. ΔK was calculated by subtracting the value of K_d for the particular preparation from the value of K_l obtained for each filter. The final results are expressed as the ratio -

$$\frac{L}{L_{448}} = \frac{i_{448}}{i} \cdot \frac{\frac{\Delta K}{K_d}}{\frac{\Delta K_{448}}{K_d}}$$

The subscript 448 refers to the value at 448 nm.

The results are set out in Table 5.2.

The relative light intensities determined for this particular apparatus by Simpson (1967) are tabulated in Table 5.3.

It can be seen that of the three wavelengths investigated

TABLE 5.2

THE LIGHT REVERSIBILITY OF THE CARBON MONOXIDE INHIBITION OF THE CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY OF A SOLUBILISED PLACENTAL MITOCHONDRIAL EXTRACT USING LIGHT OF DIFFERENT WAVELENGTHS

λ (nm)	$\frac{r_{co}}{r_o}$	K_1	ΔK	$\frac{L}{L_{448}}$
418	0·74	13·80	2·5	0·20
448	0·87	34·00	22·7	1·00
470	0·77	16·50	5·2	0·16

K_d for each determination = 11·3

TABLE 5.3

RELATIVE LIGHT INTENSITIES OF THE LIGHT FILTERS

Wavelength of maximum transmission of filter (nm)	418	448	470
Relative light intensity (448 nm = 1·0)	0·57	1·00	1·44

light of 448 nm wavelength was the most effective in reversing the carbon monoxide inhibition. The results obtained for the placental cholesterol side-chain cleavage enzyme system are very similar to those obtained for the adrenal enzyme (Simpson and Boyd, 1967a) and other mixed-function oxidases (e.g. Omura _et al_., 1965). This type of result has been taken as strong evidence for the involvement of cytochrome P-450 as the site of oxygen binding in these systems. One might conclude, therefore, that cytochrome P-450 is involved in oxygen activation in the placental cholesterol side-chain cleavage enzyme system also.

Studies on the resolution of the components of the cholesterol side-chain cleavage system of human placenta

In 1957, Ryan and Engel observed that carbon monoxide inhibited steroid C-21 hydroxylation in adrenocortical microsomes. Garfinkel (1958) and Klingenberg (1958), working independently, discovered a carbon monoxide combining substance in liver microsomal preparations. This was later shown to have cytochrome-like properties and was subsequently termed cytochrome P-450 (Omura and Sato, 1964a, b). Early attempts to isolate components of steroid hydroxylating systems showed the existence of at least three proteins and an unknown heat stable factor (Tomkins et al., 1958).

Studies on the steroid 11β-hydroxylase of adrenal cortex mitochondria (Omura et al., 1965; Omura et al., 1966; Kimura and Suzuki, 1967) have shown that this system consists of at least three protein fractions - a fraction containing cytochrome P-450, a flavoprotein dehydrogenase and a non-haem iron protein, which upon recombination restored the 11β-hydroxylase activity. Billiar and Little (1966, 1969) observed that placental mitochondria contained a factor which in the presence of specific adrenal mitochondrial fractions could stimulate steroid 11β-hydroxylation. The factor corresponded to the adrenal non-haem iron protein, adrenodoxin, but the factor appeared different to adrenodoxin, although isolated by a similar method.

Constantopoulos and Tchen (1961) and Bryson and Kaiser

(1962) resolved the cholesterol side-chain cleavage enzyme into at least two protein fractions on the basis of ammonium sulphate fractionation studies. Simpson and Boyd (1967b) having earlier shown that the adrenal cholesterol side-chain cleavage enzyme was similar to the steroid 11β-hydroxylase, displaying carbon monoxide sensitivity and the subsequent reversal of the inhibition by light (Simpson and Boyd, 1966), presented evidence for the existence of three protein components in the adrenal enzyme. These were, namely, a fraction containing cytochrome P-450, a flavoprotein fraction together with a non-haem iron protein (adrenodoxin). This latter study emphasised the similarity between the steroid 11β-hydroxylase and the cholesterol side-chain cleavage enzyme.

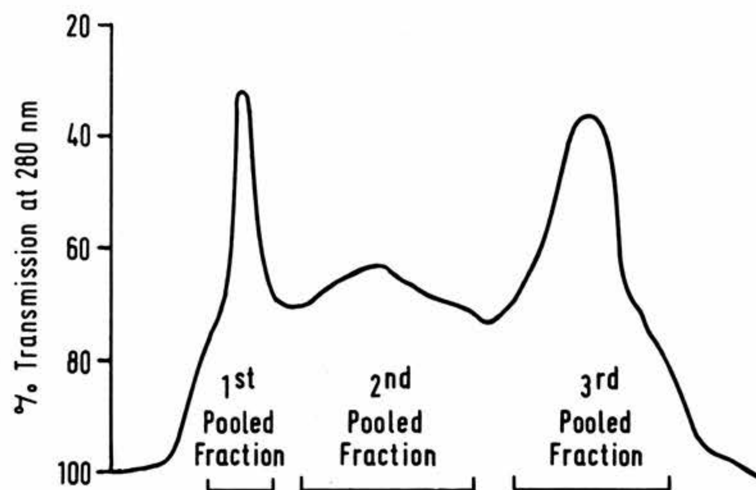
Billiar and Little (1966, 1969) have shown that placental mitochondria contain a factor which in the presence of specific adrenal mitochondrial fractions can stimulate 11β-hydroxylation although the placental mitochondria cannot themselves catalyse 11β-hydroxylation. Similar observations have been made with liver (Nakamura _et al_., 1965, 1966), testis (Ohno _et al_., 1967; Kimura and Ohno, 1968) and ovarian factors (Kimura and Ohno, 1968). The testicular protein which stimulates 11β-hydroxylation is similar to adrenodoxin (Kimura and Ohno,(1968)). Adrenodoxin is a low molecular weight protein which contains non-haem iron and acid-labile sulphur and participates in the electron transfer from NADPH and a flavoprotein (adrenodoxin reductase) to cytochrome P-450 for steroid hydroxylation (Omura _et al_., 1965). However, Billiar and Little (1969) found that the placental factor which stimulates 11β-hydroxylation was different to

adrenodoxin although isolated by similar methods. Morrison et al. (1965) attempted to resolve the cholesterol side-chain cleavage enzyme present in a placental mitochondrial acetone powder extract using Sephadex gel chromatography and ammonium sulphate fractionation. However, loss of enzymic activity after such procedures did not allow any conclusions to be made about the enzymic system.

An attempt was therefore made to characterise the protein components of the human placental cholesterol side-chain cleavage enzyme system.

Fractionation of the 'solubilised' enzyme using Sephadex G-200

The human placental cholesterol side-chain cleavage enzyme was solubilised as previously described, i.e. 900 mg. lyophilized mitochondria were suspended in 30 ml. 0·1 M phosphate buffer (pH 7·4) and centrifuged at 105,000 g. for 60 minutes. The supernatant which contained the enzymic activity was concentrated using Carbowax 20 W (Union Carbide Co.). The concentrated sample was applied on to a Sephadex G-200 column which had been prepared as follows: Sephadex G-200 was allowed to swell for 7 days in 0·1 M phosphate buffer, pH 7·4, before use. A glass column (35 cm. x 3 cm.) was filled with the Sephadex G-200 and allowed to run overnight to enable settling and equilibration of the gel. Elution was carried out using 0·1 M phosphate, pH 7·4. The eluate was monitored at 280 nm by means of an L.K.B. 8300 A 'Uvicord II' absorptiometer and a 6520 H recorder. The fraction collector was an L.K.B. 7000 A "Ultra Rac". A typical elution pattern is shown in Fig. 5.5.



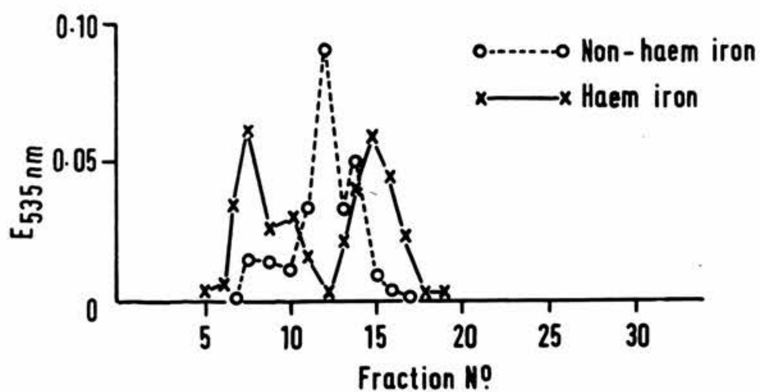


Fig. 5.5 Elution pattern of a lyophilized placental mitochondrial extract chromatographed on Sephadex G-200, showing the distribution of haem and non-haem iron.

The first peak was shown to contain haem iron, the maximum of which corresponded to the protein concentration maximum (Fig. 5.5). In the Sephadex G-200 chromatography of the solubilised adrenal and ovarian enzymes (Simpson and Boyd (1967); Sulimovici and Boyd, 1968) this first peak was found to contain the cytochrome P-450. In the present experiment, although the fraction contained haem iron, no cytochrome P-450 was detected. The carbon monoxide reduced difference spectrum of the fraction did, however, show an absorption at 420 nm, presumably cytochrome P-420. The second protein peak contained the relatively large amount of haemoglobin which contaminated the solubilised enzyme and in fact, the 280 nm trace of this fraction appeared to reflect the haemoglobin content of the preparation. The pooled fractions I, II and III (see Fig. 5.5) were separately concentrated using either "Carbowax" or lyophilization to reduce the volume. The fractions were then incubated in the presence of an NADPH generating system with [4-^{14}C] cholesterol using the normal assay procedure. The protein concentration of each fraction corresponded to approximately twice the protein concentration used in the standard assay. It was seen (Table 5.4) that optimal activity was obtained when fraction I and II were present. Addition of fraction III appeared to produce some inhibition of the cholesterol side-chain cleavage activity. It was found, later that when the enzyme preparation was dialysed prior to application on to the column, this third peak was no longer present. This indicated that it consisted of low molecular weight

TABLE 5.4.

RECONSTITUTION OF CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY AFTER GEL CHROMATOGRAPHY OF THE SOLUBILISED LYOPHILIZED MITOCHONDRIAL PREPARATION ON SEPHADEX G-200

Pooled fractions	Percentage Conversion
I	1·2
II	4·9
III	0·5
I + II	19·4
I + II + III	5·7

Each pooled fraction was present in an amount equivalent to 60 mg. lyophilized mitochondria.

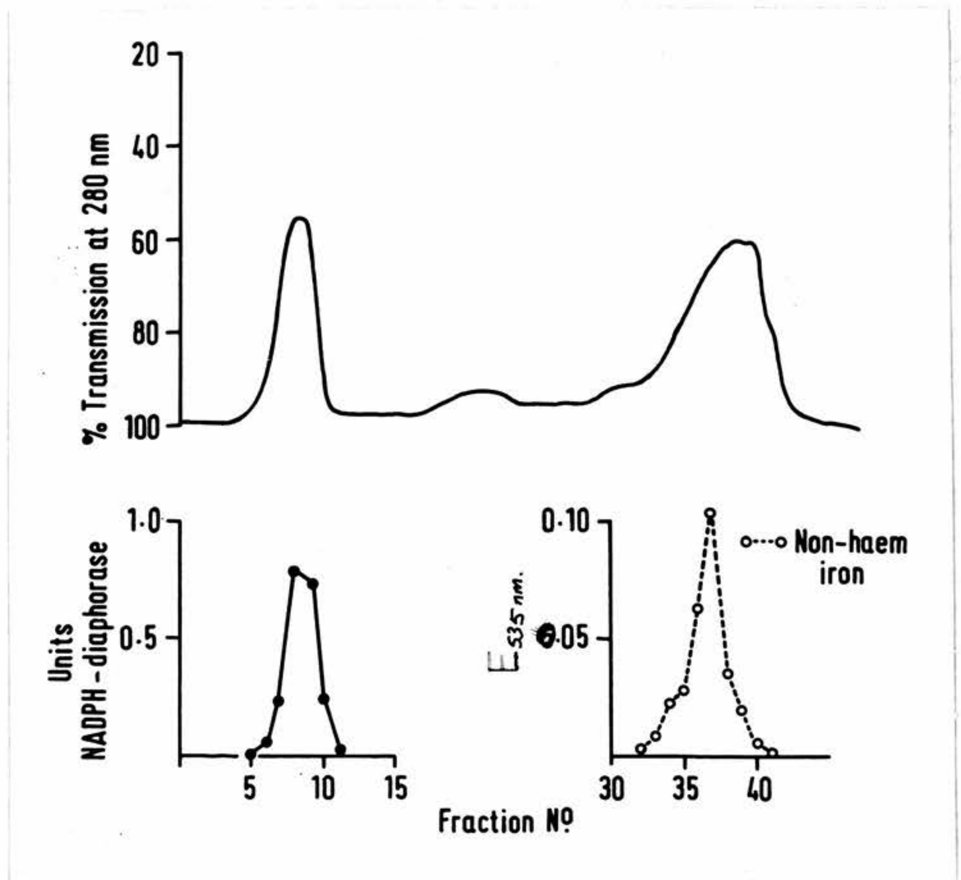


Fig. 5.6 Elution pattern of 2nd pooled fraction (see Fig. 5.5) on DEAE-Sephadex (A-25), showing the distribution of NADPH diaphorase and non-haem iron.

material, possibly polynucleotide phosphates.

Separation on DEAE-Sephadex.

The pooled fraction II after Sephadex G-200 chromatography was applied on to a DEAE-Sephadex column (25 cm. x 2·5 cm.). The column was equilibrated with 0·04 M phosphate buffer pH 7·4 and elution was carried out utilizing a salt (sodium chloride) gradient up to 0·7 M. using a flow rate of 40 ml. per hour. Figure 5.6 shows a typical 280 nm. elution trace after such chromatography. The initial fraction, which was eluted with 0·04 M phosphate buffer, exhibited NADPH-diaphorase activity - indicative of the presence of a flavoprotein. However, the major component of the fraction was haemoglobin. The second major fraction which was eluted at high salt concentration (0·7 M NaCl) contained non-haem iron. This fraction was not apparently eluted as a single component. Comparison of the E_{280} trace and the non-haem iron estimations revealed that the non-haem iron containing component was eluted in the front portion of the fraction.

Non-haem and haem iron estimations were carried out as described in Chapter 2.

NADPH diaphorase activity was estimated by a modification of the procedure of Omura et al. (1966). To 1·5 ml. aliquots were added 0·2 ml. NADPH generator system (3·7 mg. $NADP^+$, 12·5 mg. glucose-6-phosphate and 1 i.u. glucose-6-phosphate dehydrogenase per ml. water). After 2 minutes 0·2 ml. of an aqueous solution (0·3 mg./ml) of 2:6 dichlorophenol indophenol (DCPIP) was added. The decrease in absorbancy at 590 nm. was followed.

The elution patterns obtained on both Sephadex G-200 and DEAE-Sephadex chromatography were very similar to those obtained by Omura _et al_. (1966) and by Simpson and Boyd (1967) after similar chromatography of adrenal mitochondrial acetone powder extracts. Sulimovici and Boyd (1968) also achieved a similar fractionation of a rat ovarian mitochondrial extract.

The NADPH-diaphorase (flavoprotein) fraction and the non-haem iron protein fraction were dialysed against 0·04 M phosphate for 3 hours (3 changes of the dialysing buffer) and then concentrated using "Carbowax". The three fractions, i.e. the haem-containing fraction, the flavoprotein and the non-haem iron protein, were incubated individually and collectively with [4-^{14}C] cholesterol employing the normal assay procedure. Cholesterol side-chain cleavage activity was only observed when all three components were present. However, the activity observed compared to the unfractionated enzyme was minimal. The results are presented in Table 5.5. It appeared that DEAE-Sephadex Chromatography was harmful to one or both of the protein components. Cooper _et al_. (1968) have also observed that for maximal activity after fractionation of the steroid 11β-hydroxylase, relatively larger molar amounts of non-haem iron protein compared to the other two components were required. It is, therefore, possible that limiting amounts of placental non-haem iron protein were present in the reconstituted system.

TABLE 5.5

RECONSTITUTION OF CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY OF THE SOLUBILISED ENZYME AFTER CHROMATOGRAPHY ON SEPHADEX G-200 AND DEAE-SEPHADEX

Fraction	Percentage Conversion
I	0·2
NADPH diaphorase (F_p)	0·2
Non-haem iron protein (NHIP)	0·3
I + F_p	0·2
I + NHIP	2·3
F_p + NHIP	0·3
I + F_p + NHIP	3·5

Each fraction was present in an amount equivalent to 60 mg. lyophilized mitochondria.

Characterisation of the protein components

Cytochrome P-450. Fraction I after Sephadex G-200 chromatography contained a haemoprotein. The carbon monoxide reduced difference spectrum - the common method of showing the presence of cytochrome P-450, revealed an absorption band at 420 nm. This absorption was due either to the presence of cytochrome P-420 - the inactive form of cytochrome P-450 - or to the presence of contaminating haemoglobin, the bulk of this major contaminant of the enzyme preparation being found in fraction II. Due to the small size of this absorption band, one could not distinguish between the two possibilities using the usual method i.e. formation of the cyanide complex. Thus cytochrome P-450 could not be identified in the high molecular weight fraction i.e. fraction I, where, by analogy with the adrenal and ovarian enzymes, it should be located. However, it was shown earlier in this chapter that the enzyme preparation prior to fractionation contained very low amounts of cytochrome P-450. A small conversion of this cytochrome P-450 to cytochrome P-420 during chromatography could reduce the cytochrome P-450 content to an undetectable level.

Flavoprotein. After DEAE-Sephadex chromatography, a fraction was obtained of which the major component was haemoglobin. The fraction, however, exhibited NADPH-DCPIP reductase activity - characteristic of a flavoprotein. It was not possible to separate the flavoprotein activity from haemoglobin. The cholesterol side-chain cleavage activity had an absolute requirement for this fraction.

Non-haem iron protein. Kimura and Suzuki (1965) and Suzuki and Kimura (1965) in studies on pig adrenal steroid hydroxylases identified a non-haem iron protein as one of the components involved in the electron transfer system associated with the steroid 11β- hydroxylase activity of the mitochondria. This fraction was named 'adrenodoxin'. It was found to possess similar physical and chemical properties to the plant ferredoxins, i.e. it contained non-haem iron and labile sulphur-containing groups. The redox potential of adrenodoxin was, however, higher than that of, say, spinach ferredoxin (approximately zero compared to -0·43 volt). Simpson and Boyd (1967b) demonstrated that adrenodoxin was also involved in the cholesterol side-chain cleavage reaction occurring in adrenal mitochondria. Ohno, Suzuki and Kimura (1967) have also isolated and identified a non-haem iron protein from pig testis similar to adrenodoxin and proposed the name of testodoxin.

In the present study it has been possible to show the presence of a non-haem iron protein in human placental mitochondria. The protein has similar chromatographic properties to adrenodoxin and appears to be involved in the cholesterol side-chain cleavage enzyme system. Billiar and Little (1969) have recently isolated a non-haem iron protein from placental mitochondria, which stimulated adrenal steroid 11β-hydroxylase activity. These workers, however, reported that the placental non-haem iron protein differed from adrenodoxin and testodoxin in that the labile inorganic sulphide content was very low compared to the non-haem iron content. The placental

protein, they suggest, is more similar to the liver factor that can stimulate adrenal steroid 11β-hydroxylase (Nakamura _et al_., 1966). Liver does not appear to contain a protein similar to adrenodoxin (Kimura and Ohno, 1968). Kimura and Ohno (1968) have suggested that tissue extracts with low ratios of acid-labile sulphur to non-haem iron do not contain proteins similar to adrenodoxin.

In the present study, the non-haem iron and acid-labile sulphur contents of the placental non-haem iron protein fraction eluted from the DEAE-Sephadex column were estimated as described in Chapter 2. Protein determinations were carried out according to Lowry _et al_. (1951). The non-haem iron content was estimated to be 80 n atoms per mg. protein while the labile sulphur content was 40 nmoles per mg. protein. Kimura (1968) has reported that 'adrenodoxin' contains 109 n atoms of iron per mg. protein and 100 nmoles labile sulphur per mg. protein, i.e. a non-haem iron to labile sulphur ratio of approximately unity. The placental non-haem iron protein has a non-haem iron to labile sulphur ratio of 2. The lower non-haem iron content of the placental protein is probably due to the fact that Kimura's adrenodoxin preparation had undergone further purification procedures. However, the much lower labile sulphur content is either a characteristic of the placental protein or that some labile sulphur has been shed during the fractionation procedures.

The purity of the placental non-haem iron protein was examined using acrylamide gel disc electrophoresis, but due to the small amount of a rather dilute sample being available, the results were not conclusive.

<u>Distribution of the 3β-hydroxysteroid dehydrogenase-isomerase system after Sephadex G-200 chromatography.</u>

The 105,000 g. - 60 min. supernatant extracts of lyophilized placental mitochondria contained appreciable 3β-hydroxysteroid dehydrogenase-isomerase activity. The various fractions obtained after Sephadex G-200 chromatography of such extracts were examined in order to determine the location of the dehydrogenase enzyme system. The assay consisted of the incubation of various fractions with [4-^{14}C] pregnenolone in the presence of NAD^+ (0·2 mg./5 ml. incubation). The incubation period was 15 minutes. The remainder of the assay procedure was as described in Chapter 2. The results are presented in Table 5.6. The highest dehydrogenase activity was located in fraction I. Thus the dehydrogenase-isomerase system appeared to be associated with the high molecular weight material. This finding implies that the dehydrogenase system is closely associated with the cholesterol side-chain cleavage enzyme system, i.e. the possible existence of a mitochondrial 3β-hydroxysteroid dehydrogenase-isomerase enzyme system. The enzyme is usually said to be located only in the microsomal fraction. However, Koide and Torres (1965) have presented evidence for the presence of separate mitochondrial and microsomal 3β-hydroxysteroid dehydrogenase-isomerase systems in adrenal tissue. Thus placental mitochondria are possibly capable of converting cholesterol to progesterone before release of the latter from the mitochondria.

TABLE 5.6

DISTRIBUTION OF 3β-HYDROXYSTEROID DEHYDROGENASE-ISOMERASE ACTIVITY AFTER CHROMATOGRAPHY OF THE SOLUBILISED MITOCHONDRIAL PREPARATION ON SEPHADEX G-200

Fraction	% Conversion of [4-^{14}C] Pregnenolone to ^{14}C-Progesterone
I	9·7
II	5·2
III	0·7

Each fraction was present in an amount equivalent to 60 mg. lyophilized mitochondria.

CHAPTER 6

D I S C U S S I O N

DISCUSSION

The particular mammalian sterol mono-oxygenase investigated in this study has been the cholesterol side-chain cleavage enzyme system of human term placenta. The work of Halkerston _et al_. (1961) and later Shimizu (1968) has shown that the adrenal enzyme has a requirement for molecular oxygen and NADPH. It thus satisfies the criterion to be classified as a mixed-function oxidase, as defined by Mason (1957).

The side-chain cleavage of cholesterol is the first step in the degradation of this substance to steroid hormones by endocrine tissue. There is evidence that the initial 20α-hydroxylation of cholesterol is the first step in the reaction and it is thought to be the rate limiting step in steroid hormone biosynthesis, possibly controlled by trophic hormones (Stone and Hechter, 1954). The cholesterol side-chain cleavage enzyme system has been most extensively studied in adrenocortical tissue and to a lesser extent in ovarian and testicular tissue (see Sulimovici and Boyd, 1969b, for a review of earlier studies).

Earlier studies on the cholesterol side-chain cleavage in other tissues were hampered by the lack of a suitable assay. Two methods have been used, in general - estimation of the C_6 fragment, isocaproic acid (Ichii _et al_., 1963) or measurement of pregnenolone, the first C_{21} steroid product of the cleavage reaction (Hall and Koritz, 1964). The first method had the disadvantage that it yielded no information on the nature of the steroid produced in the reaction,

while the second assumed that pregnenolone was the sole product of the reaction, which is not always the case. These problems were largely overcome by the use of [4-^{14}C] labelled cholesterol as substrate coupled with the use of thin-layer chromatography for the separation of the substrate and products. Radio-chromatogram scanning and liquid scintillation spectrometry were the procedures used to detect and quantitate the products. Such an assay, which had been developed in this laboratory (Simpson, 1967) was found to be rapid, reproducible and sensitive allowing the detection of metabolites which might occur in small amounts.

Using such an assay technique, when mitochondrial and microsomal preparations obtained from placental homogenates were incubated with [4-^{14}C] cholesterol as substrate in the presence of an NADPH generating system, cholesterol side-chain cleavage activity was observed only in the mitochondrial fraction (Table 3.2). This observation was in agreement with other studies of the cholesterol side-chain cleavage reaction in other endocrine tissues (Sulimovici and Boyd, 1969b).

Using the "normal" assay concentration of the NADPH generating system (1 mM), which gave maximal metabolite production, the only product detected was [^{14}C] progesterone. The identity of this steroid product was confirmed by thin-layer chromatography in other solvent systems and radio-GLC chromatography. In all instances the radioactive material chromatographed with progesterone. The percentage conversion of the tracer cholesterol to progesterone was between

4 and 10% in a one hour incubation. The activity varied from one preparation to the next (Table 3.2). These activities were therefore about ten-fold higher than those previously observed by Ryan *et al.* (1966). These workers observed a 0·6% conversion in one hour, but used a much lower concentration of the NADPH generating system (5 μM). That progesterone was the major metabolite of the cholesterol side-chain cleavage reaction was in some ways surprising. While it is well known that the placenta contains a very active 3β-hydroxysteroid dehydrogenase-Δ^{4-5} isomerase enzyme system capable of converting pregnenolone to progesterone, it is usually assumed to be a microsomal enzyme. However, repeated washing of the mitochondrial fraction did not influence the nature of the reaction product. This observation is therefore in agreement with that of Koide and Torres(1965) who reported that the dehydrogenase-isomerase enzyme appeared to be associated with both the mitochondrial and microsomal material. Sulimovici and Boyd (1969a) have reported the existence of distinct dehydrogenase-isomerase enzymes in immature rat ovarian mitochondria and microsomes. However, it was possible that in this study, the mitochondrial fraction was contaminated with microsomes, even though the mitochondria were subjected to repeated washing. Electron-microscopic examination of the mitochondrial fraction revealed that the preparation was essentially mitochondrial, although some endoplasmic reticulum was present (Fig. 3.1). Most of the mitochondria appeared damaged, presumably caused by the homogenisation procedure.

Despite the fact that intact mitochondria are not permeable to pyridine nucleotides, it was observed that the placental mitochondrial cholesterol side-chain cleavage enzyme system required NADPH supplied by the NADPH generating system. In these studies, the NADPH was, of course, generated extramitochondrially. Yago and Ichii (1969) have observed that the cholesterol side-chain cleavage enzyme of adrenocortical mitochondria is located in the inner mitochondrial membrane. Thus, presumably NADPH has to permeate to the inner membrane in order that it might be utilized. The results obtained demonstrate that the placental mitochondrial preparation did not consist of intact mitochondria, and were in fact 'leaky' mitochondria. The preparation of 'good' mitochondria is a problem when dealing with placental tissue. The presence of large amounts of vascular and connective tissue does not permit the use of gentle homogenisation techniques that are used, for example, in the preparation of liver mitochondria.

Since glucose-6-phosphate dehydrogenase is not a mitochondrial enzyme, the physiological supply of NADPH must arise from some other source. Using bovine adrenocortical mitochondrial preparations, Simpson and Estabrook (1969) have shown that the NADPH required for steroid 11β-hydroxylation arises from malate via mitochondrial "malic enzyme". These workers have found a quantitative relationship between pyruvate production from malate and corticosterone output by adrenal cortex mitochondria. When placental mitochondria were incubated with 10 mM malate and $NADP^+$ no cholesterol

side-chain cleavage activity was observed (Table 3.3). Thus placental mitochondria do not appear to possess a mitochondrial malic enzyme, or at least if it is present, it does not supply the NADPH for the cholesterol side-chain cleavage reaction. Simpson (personal communication) has observed virtually no malic enzyme activity in human placental mitochondria. The other favoured mechanism for the supply of intramitochondrial NADPH involves the pyridine nucleotide transhydrogenase, an energy requiring process. Peron and coworkers (see Peron _et al._,1966) favour this process as the important source of NADPH in rat adrenal mitochondria. When placental mitochondria were incubated with succinate, malate and fumarate in separate incubations, cholesterol side-chain cleavage activity was only observed using succinate as the oxidisable substrate (Table 3.3). It was also observed that the product obtained when the reducing equivalents were supplied from succinate was pregnenolone, and not progesterone. It is possible that the oxidation of succinate resulted in the reduction of the endogenous NAD^+ within the mitochondria using reversed electron flow, and so removed the cofactor for the dehydrogenase reaction. Transhydrogenation from NADH to NADPH could then occur and thus supply the NADPH required for the hydroxylation reaction. The scheme is set out below:-

RH → ROH

NADPH → f_p → NHIP → cyt.P-450

O_2 → H_2O

↑

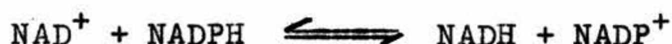
NADH ↔ f_p ⇄ Ubiquinone cyt.b → cyt. C_1 → cyt. C → cyt. a → cyt. a_3 → O_2

Succinate → f_s ↗

Malate could similarly be expected to supply NADH via malic dehydrogenase, but the results do not show this. One must remember, however, that the placental mitochondria are probably not intact i.e. are "leaky", with the possible resultant loss of mitochondrial matrix enzymes as well as substrates and cofactors. Succinic dehydrogenase is a membrane-bound enzyme whereas the other Krebs' tricarboxylic acid cycle enaymes are more soluble.

A further possible source of intramitochondrial NADPH investigated was the action of mitochondrial $NADP^+$-linked isocitrate dehydrogenase. When cholesterol side-chain cleavage assays were carried out using placental mitochondria and isocitrate (5 mM), cholesterol side-chain cleavage activity was observed (Table 3.3). However, isocitrate was not as good an electron donor as was succinate. But, the mitochondrial $NADP^+$-linked isocitrate dehydrogenase-catalysed reaction could act as a source of reducing equivalents in human placental mitochondria. Schreiner and Villee (1965) observed that mitochondria from full term placentae were more uncoupled than mitochondria from 3 month placentae, i.e. had a much higher O_2 consumption. Since the progesterone content of the full term placentae was approximately twice that of the 3 month ones, a relationship between the degree of uncoupling and the progesterone content was suggested. It is possible that during maturation of the placenta, reducing power from oxidisable substrates is redirected from the normal respiratory electron transport system to the electron transport system involved in hydroxylation reactions. A further result that suggested the

existence of a system in placental mitochondria capable of transferring reducing equivalents between NAD and NADP, i.e.



$$NAD^{+} + NADPH \rightleftharpoons NADH + NADP^{+}$$

- was that further increase of the NADPH level resulted in an accumulation of pregnenolone, although the overall cholesterol side-chain cleavage activity was not significantly affected (Table 3.4). This can be explained if the excess NADPH could reduce the endogenous NAD^{+} required for the dehydrogenation of pregnenolone. The results obtained in this study favour a transhydrogenase system as the source of the mitochondrial NADPH required for mixed-function oxygenation reactions rather than a mechanism involving malic enzyme. However, it must be mentioned that isocitrate, coupled presumably with endogenous isocitrate dehydrogenase, was also capable of supplying NADPH for the placental cholesterol side-chain cleavage enzyme. Therefore it is equally possible that mechanisms not involving a transhydrogenase are important in placental mitochondrial NADPH generation. No direct evidence for a placental mitochondrial pyridine nucleotide transhydrogenase exists, although the soluble placental transhydrogenase, involving catalytic amounts of oestradiol and oestrone, has been well documented (see Jarabak, 1969).

Yago, Dorfman and Forchielli (1967) demonstrated that exogenously added NADPH was not oxidised by a "heavy mitochondrial" preparation of bovine corpora lutea. Extramitochondrial NADPH was also found to be a poor electron donor

for the mitochondrial 11β-hydroxylase of rat adrenal (Harding et al., 1965; Peron et al., 1966). Earlier studies on liver and heart mitochondrial respiration showed that external nicotinamide nucleotides were not utilised at appreciable rates (Lehninger, 1951; Chance and Williams, 1955; Lehninger et al., 1960). Koritz (1968) observed that addition of Ca^{++} ions, a known mitochondrial swelling agent, greatly enhanced the cholesterol side-chain cleavage activity of rat adrenal mitochondria in the presence of exogenous NADPH. The stimulated activity was attributed to the easier penetration of the NADPH into the swollen mitochondria. The accessibility of NADPH to the cholesterol side-chain cleavage enzyme could thus be an important controlling factor, especially since the results of Yago and Ichii (1969) indicate that in bovine adrenocortical mitochondria, the enzyme is located in the inner mitochondrial membrane.

When agents such as glutathione (GSH), cysteine known to produce mitochondrial swelling were added to placental mitochondrial preparations, no marked stimulation of cholesterol side-chain cleavage activity was observed. Glutathione, in small amounts, produced some activation of the enzymic activity, whereas higher concentrations of the reducing agent inhibited the enzyme system (Fig. 4.7). Cysteine did not produce any observable effect on the enzymic activity, whereas β-mercapto-ethylamine inhibited the enzyme (Figs. 4.5 and 4.6). It must, however, be remembered that the placental cholesterol side-chain cleavage enzyme responded

to extramitochondrial NADPH in the absence of a known swelling agent. Thus it is likely that the mitochondria were in a swollen state prior to addition of the swelling agents.

In order to study the components of the cholesterol side-chain cleavage enzyme system present in human placental mitochondria, it was desirable to 'solubilise' the enzyme system. 'Solubilisation' was loosely defined as the presence of enzymic activity in the supernatant after high-speed centrifugation at 105,000 g. for 30 minutes in a medium of 0·1 M KCl or 0·1 M phosphate buffer. It was purely an operational definition, implying that the enzyme was in a form amenable to some preliminary fractionation studies.

Three techniques which had previously been used to solubilise the enzyme system in other tissues were all successfully used. Ultrasonication (Simpson and Boyd, 1966) of a placental mitochondrial suspension in water followed by centrifugation at 105,000 g. for one hour resulted in enzymic activity being located in the high-speed supernatant (Table 3.5). Similarly, after lyophilization of the mitochondrial sonicate, the lyophilized mitochondria could be suspended in 0·1 M KCl or 0·1 M phosphate buffer, pH 7·4, to yield a supernatant after high-speed centrifugation which had cholesterol side-chain cleavage activity (Table 3.6). Mitochondrial acetone powder preparations also yielded active 'soluble' enzyme preparations (Table 3.7), in agreement with the previous report of Morrison et al. (1965). The major product detected when these solubilised preparations were

incubated with [4-^{14}C] cholesterol was pregnenolone, with smaller amounts of progesterone being found. It appeared that most of the 3β-hydroxysteroid dehydrogenase remained associated with the particulate material (Table 3.10).

During the procedure of preparing the solubilised enzyme system it was found that whereas the native mitochondrial preparations had demonstrable cholesterol side-chain cleavage activity, the same preparations after ultrasonication or lyophilization appeared to have lost this enzymic activity (Tables 3.5, 3.6 and 3.7). However, after high speed centrifugation of the sonicated or lyophilized preparation enhanced enzymic activity was found in the supernatant. Addition of the high speed (105,000 g. x 60 min.) lyophilized mitochondrial pellet to the supernatant completely inhibited the enzymic activity (Table 3.6). Thus the particulate material appeared to contain a substance (or substances) inhibitory to the cholesterol side-chain cleavage activity. This finding was contrary to that observed during the solubilisation of the enzyme from immature rat ovarian and bovine adrenocortical mitochondria (Sulimovici and Boyd, 1969b; Simpson, 1967). These workers found that disruption of the mitochondria by such procedures as ultrasonication and lyophilization resulted in enhanced cholesterol side-chain cleavage activity. It was thought initially that the inhibitory substance could be endogenous cholesterol. In the native placental mitochondrial preparations it might be proposed that the added [4-^{14}C] cholesterol does not equili-

brate with the total mitochondrial cholesterol and preferentially equilibrates with the "steroidogenic cholesterol pool". One must assume that the mitochondrial cholesterol available for steroidogenesis is a small part of the total cholesterol of the mitochondria. Evidence for this assumption was obtained by Armstrong et al. (1964) from experiments in which acetate or mevalonate were found to be better precursors (on the basis of specific activity determinations) for steroid biosynthesis than was cholesterol. However, cholesterol was found to be intimately associated with steroidogenesis and hence the postulation of a "steroidogenic pool" of cholesterol. Upon disruption of the mitochondria by ultrasonication or lyophilization, it might then be assumed that the tracer cholesterol could equilibrate with a much larger pool of cholesterol. This would result in a very effective dilution of the tracer cholesterol and an apparent inhibition of the cholesterol side-chain cleavage activity as assayed in this study.

However, when experiments were carried out to study the effect of amounts of cholesterol normally expected to be found in mitochondrial preparations (approximately 10 μg. per mg. lyophilized mitochondria) on the apparent cholesterol side-chain cleavage activity, considerable enzymic activity was observed at exogenous cholesterol levels of 400 μg. per incubation (Table 3.12). The total cholesterol content in lyophilized placental mitochondria is certainly less than 400 μg. This experiment can be criticised because the exogenous cholesterol was added in the free form in acetone, whereas

the endogenous cholesterol is undoubtedly bound in some form of lipoprotein structure.

The experiment also permitted an estimate of the apparent K_m of the enzyme for the cholesterol substrate (Fig. 3.7). The apparent K_m varied between 50 and 200 μM depending on the particular solubilised mitochondrial preparation. There are obviously non-specific cholesterol binding sites as well as the binding site on the enzyme. The number of these non-specific sites presumably varies from one solubilised preparation to the next. Raggatt and Whitehouse (1966) obtained an apparent K_m for the adrenocortical enzyme of about 50 μM but these workers also observed considerable variations in the K_m value obtained. Young and Hall (1969) have recently obtained an apparent K_m of 40-50 μM for the adrenal enzyme.

Evidence was obtained for the lipid character of the inhibitory substance. It was heat stable (Table 3.8) and could be extracted from the particulate material using the general lipid solvent, chloroform-methanol (2:1, v/v) (Fig. 3.5) while it was not extractable using a hot aqueous solution (Table 3.8). Further evidence for the lipid nature of the inhibitor was from observations on the preparation of placental mitochondrial acetone powders. It was found that a long exposure (10-15 minutes) of the mitochondria to acetone resulted in the removal of the inhibitory material (Table 3.10). This observation was indicative of the removal of lipid(s) from the mitochondria.

Examination of the chloroform-methanol extracts of the 105,000 g. x 60 min. pellet from lyophilized mitochondrial suspensions using thin-layer chromatography revealed that the main components present were phospholipids and cholesterol and the most inhibitory fraction was the phospholipid (Table 3.9). The phospholipid fraction was further resolved into phosphatidyl choline (lecithin), phosphatidyl ethanolamine and probably cardiolipin. The lecithin fraction was the most inhibitory (Fig. 3.6.).

The acetone wash after the preparation of mitochondrial acetone powders was also found to be inhibitory (Table 3.11). TLC examination of the acetone wash revealed that the components present were phosphatidyl ethanolamine, lecithin, cardiolipin and cholesterol.

It appeared, therefore, that the observed inhibition in sonicated and lyophilized mitochondrial suspensions was due to the mitochondrial phospholipid, especially lecithin.

In order to study the effect of phospholipids more clearly, cholesterol side-chain cleavage assays (using a 105,000 g. x 60 min. mitochondrial acetone powder supernatant) were performed in the presence of varying amounts of commercially available highly purified lecithin and phosphatidyl ethanolamine (Fig. 3.8). Lecithin was seen to be a strong inhibitor of the cholesterol side-chain cleavage enzyme at a concentration close to the phospholipid content of mitochondria, i.e. about 30% of the dry mitochondrial weight (Rouser _et al_., 1968). Phosphatidyl ethanolamine was a less potent inhibitor of the cholesterol side-chain cleavage

activity, and at low concentrations (0·1 - 0·5 mM) stimulated the enzymic activity.

Comparison of Figs. 3.8 and 3.9b showed that the inhibitory substance of the lyophilized mitochondrial 105,000 g. pellet and lecithin had similar inhibitory effects on the enzymic activity. The inhibitor characteristic, K_i, for the inhibitor from lyophilized mitochondria was not determined due to the difficulty of estimating the amount of endogenous "steroidogenic" cholesterol present in the 105,000 g. mitochondrial pellet.

It was thought possible that the mitochondrial phospholipid might be exerting its inhibitory effect by sequestering the added cholesterol into a form which could not be metabolised by the enzyme. However, a plot of I versus $\frac{i}{1 - i}$ where I was the inhibitor concentration and i was the fractional inhibition $(1 - \frac{\text{inhibited rate}}{\text{uninhibited rate}})$, was found to be a straight line (Fig. 3.11). This result, according to Reiner (1969), is indicative of an involvement of the enzyme in the inhibition to form an inhibitor-substrate-enzyme inactive complex and not simply an inhibitor-substrate inactive complex. The straight line plot was obtained with both lyophilized mitochondrial pellet and pure lecithin as the inhibitor. It is possible that mitochondrial phospholipid, and cholesterol could form a "lipoprotein" state together with the enzyme, but the cholesterol is in a form that is not metabolised.

The formation of a lipoprotein form of complex would also provide an explanation of the differences observed

between the placental and adrenocortical enzymes when the mitochondria are disrupted and subjected to ultrasonication or lyophilization. In the adrenal mitochondria, disruption results in an enhanced cholesterol side-chain cleavage activity (Simpson, 1967) while the present study has shown disruption of placental mitochondria results in an apparent inhibition of the enzymic activity. The formation of a "lipoprotein complex" requires the presence of critical concentrations of the complex components. If any component is present in excess of the critical complex concentration, then the component will not be found in a complexed form. One major difference between the adrenal and placental mitochondria is likely to be their respective cholesterol side-chain cleavage enzyme contents, since adrenocortical mitochondria contain much more of the enzyme based on the mitochondrial cytochrome P-450 content. This present study (Fig. 5.1), as well as that of Meigs and Ryan (1968), has shown that the absolute amounts of cytochrome P-450 in placental mitochondria are approximately one tenth those calculated from data on adrenal mitochondria (Harding and Nelson, 1966). Accepting that cytochrome P-450 is involved in the cholesterol side-chain cleavage enzyme, the cytochrome P-450 content might be taken as an approximate index of the enzyme content. It might then be argued that the placental enzyme concentration is close to the critical complex concentration while the adrenal enzyme concentration is much greater (ten times) the critical concentration.

Evidence for this concept was obtained in two experiments, both employing lyophilized adrenocortical mitochondria. The 105,000 g. x 30 min. supernatant and pellet of the lyophilized adrenal mitochondria were obtained in the normal manner. After inactivation by heating of the residual activity of the pellet, the effect of varying amounts of the pellet on the cholesterol side-chain cleavage activity of the supernatant was studied. The latter contains only a small proportion of the total mitochondrial cholesterol side-chain cleavage activity (Simpson, 1967). The pellet material was found to be inhibitory (Table 3.14). The second experiment was to investigate the effect of pure lecithin on the cholesterol side-chain cleavage activity of the adrenal mitochondrial 105,000 g. supernatant (Fig. 3.10). Lecithin was found to inhibit the solubilised adrenal enzyme, just as it did the solubilised placental enzyme. It therefore seems likely the ratio of phospholipid to the enzyme is critical for the observation of the enzymic inhibition. One of the simplest explanations would be the formation of an enzymatically inactive complex composed of enzyme, phospholipid and cholesterol, with only a limited amount of enzyme being taken into the micellar phase.

It has been established that mitochondrial phospholipid, especially lecithin, can inhibit the solubilised cholesterol side-chain cleavage activity of placental mitochondria at concentrations similar to those present in the native mitochondria. However, this inhibition is very unlikely to be of physiological significance since intact

mitochondria have a very organised structure, giving an environment completely unlike that in the enzymic assay. This is emphasised by the observation that native mitochondria display cholesterol side-chain cleavage activity, which is apparently inhibited when the mitochondrial structure is disrupted by such processes as ultrasonication or lyophilization.

A similar inhibitory effect was observed by Sharma et al., (1962) during attempts to solubilise the steroid 11β-hydroxylase of adrenocortical mitochondria. After ultrasonication of the mitochondria, steroid 11β-hydroxylase activity could not be demonstrated until high speed centrifugation of the sonicate had been performed. The resulting particulate material was found to be inhibitory. This suggested the presence of an 11β-hydroxylase inhibitor associated with the insoluble particles. However, this observation was not further investigated.

In summary, it appears that after disruption of the placental mitochondrial structure, the endogenous phospholipid, especially lecithin, inhibits the cholesterol side-chain cleavage activity. It is of interest since phospholipid appears to be important for the activity of other steroid and fatty acid hydroxylases (Williamson and O'Donnell, 1969; Lu et al., 1969). The phospholipid could be important in the interaction between the lipophilic substrates and the enzyme protein. In the disrupted mitochondrial system, the mitochondrial phospholipid bulk could very feasibly interfere

in the interaction between the enzyme and the substrate, especially when the enzyme content is very low.

Chapters 4 and 5 are concerned with studies performed on the solubilised cholesterol side-chain cleavage enzyme of human placental mitochondria with regard to its properties and characteristics. In all the studies discussed, the solubilised enzyme system was obtained by ultracentrifugation at 105,000 g. for 60 min. of ultrasonicated, lyophilized or acetone powder suspensions (in 0·1 M KCl or 0·1 M phosphate buffer pH 7·4) of placental mitochondria. The acetone powder preparations generally gave the most active systems, presumably due to removal of endogenous cholesterol.

Mono-oxygenases require a source of reducing equivalents, usually either NADPH or NADH (Mason, 1965). The placental mitochondrial cholesterol side-chain cleavage enzyme, in common with other mammalian steroid mono-oxygenases, was found to have a specific requirements for NADPH. The K_m of the enzyme for NADPH was found to be about 1μM (Fig. 4.2). Concentrations of NADPH from 2-500 μM produced very little change in the enzymic activity. NADH (1 mM) did not support any detectable cholesterol side-chain cleavage activity. Satoh et al. (1966) found the optimal concentration of NADPH for the adrenal cholesterol side-chain cleavage enzyme to be about 50 μM. Boyd (1970) reported the K_m of NADPH for the liver microsomal cholesterol 7α-hydroxylase to be 300 μM. However this was in a crude microsomal preparation where there are undoubtedly other NADPH oxidising systems.

It might be noted that recently Sih (1969) has produced

evidence to support the concept that NADH can replace NADPH as the reductant for the flavoprotein-adrenodoxin reductase but much higher concentrations of NADH are required; the K_m being much larger. However, his postulated mechanism for steroid 11β-hydroxylation still has an absolute requirement for NADPH to provide reducing equivalents to keep the autoxidisable cytochrome P-450-Fe(II) in the ferrous state of oxidation, a course which does not enter into the stoichiometry of the hydroxylation reaction. In the present study no evidence was obtained to support the type of mechanism suggested by Sih for enzymic hydroxylation reactions.

The pH-activity profile showed a broad maximal activity in the pH region 6·8-7·6 (Fig. 4.1). The profile was very similar to those obtained for the cholesterol side-chain cleavage enzyme of rat ovarian mitochondria (Sulimovici, 1968) and the liver microsomal cholesterol 7α-hydroxylase (Scholan, 1969).

Increasing buffer concentration was found to have a marked inhibitory effect on the cholesterol side-chain cleavage activity (Fig. 4.3). Phosphate at a concentration of 200 mM completely abolished the enzymic activity. Satoh <u>et al</u>. (1966) had previously observed that the conversion of cholesterol to pregnenolone in bovine adrenal cortex mitochondria was greatly stimulated by inorganic phosphate; the optimal concentration was about 10 mM. Higher concentrations of inorganic phosphate were found by these workers to be inhibitory and 100 mM phosphate produced essentially complete inhibition. The placental enzyme, therefore, appeared to

be similarly affected by the presence of phosphate as was the adrenal enzyme. However, the very low concentration effect of phosphate was not studied due to problems of the buffering power. The inhibitory effect of phosphate was found not to be specific since other buffer salts (Tris-HCl, citrate, glycylglycine and triethanolamine-HCl) at high concentrations inhibited the solubilised placental enzyme (Fig. 4.4.). An increase in the ionic strength of the incubation medium by the addition of KCl also produced a similar inhibitory pattern (Fig. 4.4.). Thus the effect of phosphate was not a specific one, say by competing with the phosphate groups of NADPH (Table 4.1) but was part of a general effect of salt concentration of the medium. The inhibitory effect was found to be reversible since after dialysis of the enzyme which had been subjected to a high salt concentration, enzymic activity was restored (Table 4.2). An increase in ionic strength produces an increase in hydrophobic bonding. The degree of such bonding is likely to be very important for the activity of solubilised lipophilic enzyme systems. This effect of salt concentration is not of physiological importance since the enzymic activity of native placental mitochondria is not affected by increases in the phosphate concentration (Table 4.3). Scholan (1969) observed that the liver microsomal cholesterol 7α-hydroxylase was insensitive to increases in the phosphate concentration. Thus membrane-bound sterol hydroxylases appear to be protected from the inhibitory effect of high salt concentration, presumably by their membrane environment.

Simpson and Boyd (1966) have shown that the cholesterol side-chain cleavage enzyme present in bovine adrenocortical mitochondria is inhibited by carbon monoxide. Carbon monoxide inhibition and its reversal by light implicates an iron-containing catalyst (Warburg, 1949) in the cholesterol side-chain cleavage reaction. Cytochrome P-450, some other haemoprotein or even a non-haem iron component could be the site of the CO inhibition. Simpson and Boyd (1967a) showed from an action spectrum of the light reversal that cytochrome P-450 was indeed the CO-sensitive component of the cholesterol side-chain cleavage system of the adrenal mitochondrial preparations. The steroid 11β- and 18-hydroxylases of adrenal mitochondria (Harding et al., 1965; Greengard et al., 1967) as well as the steroid 21-hydroxylase of adrenal microsomes (Estabrook et al., 1963) had displayed a light reversible carbon monoxide sensitivity. Photochemical action spectra confirmed the involvement of cytochrome P-450. Thus the adrenal cholesterol side-chain cleavage enzyme appeared to be very similar to the other adrenal steroid hydroxylases. The present studies show the placental mitochondrial cholesterol side-chain cleavage enzyme is inhibited by carbon monoxide. It was found that using an atmosphere containing carbon monoxide and oxygen in the ratio 1·4:1 produced a 50% inhibition of the cholesterol side-chain cleavage activity of an extract (105,000 g. x 60 min.) of sonicated placental mitochondria (Fig. 5.2). The Warburg partition constant, K, for the placental cholesterol side-chain cleavage system of 1·4 (Fig. 5.3) is very similar

to the K values reported for the adrenal cholesterol side-chain cleavage system by Simpson and Boyd (1966) and by Harding et al. (1969) when K values between 0·8 and 2·0 were reported. The K value for cytochrome P-450 is very different to those of haemoglobin and cytochrome oxidase which are 1·8-8·0 x 10^{-3} and 10-20 respectively (Keilin and Wang, 1946). Light reversal studies showed that the carbon monoxide inhibition was light reversible and that the most effective light wave-length was around 450 nm (Tables 5.1 and 5.2). Thus cytochrome P-450 appeared to be involved in the placental cholesterol side-chain cleavage reaction, and thus must have been present in the enzyme preparations.

When the solubilised enzyme preparations were examined for the presence of cytochrome P-450 using the carbon monoxide-reduced difference spectrum technique developed by Omura and Sato (1964a), virtually no cytochrome P-450 was detectable. The spectral determinations were complicated throughout this study by the presence of haemoglobin which reacts with carbon monoxide yielding a spectral absorption maximum at 420 nm, the same as that of cytochrome P-420, the inactive form of cytochrome P-450. Human placenta is a very vascular tissue and removal of the last traces of blood is very difficult. Though perfusion of the foetal side via the umbilical cord is possible, perfusion of the maternal side is not possible once the tissue is detached from the uterine wall. Figure 5.1(a) shows a typical carbon monoxide-reduced difference spectrum of a solubilised enzyme preparation. Large amounts of haemoglobin relative to the cytochrome P-450

produces a spectrum which could possibly mask the cytochrome P-450 absorption. However, examination of the particulate material which remained after the extraction of the lyophilised placental mitochondria showed that this fraction contained cytochrome P-450 as well as haemoglobin and possibly cytochrome P-420 (Fig. 5.1(b)). Thus the bulk of the placental mitochondrial cytochrome P-450 was firmly associated with the particulate phase. It appeared that the cholesterol side-chain cleavage activity was distinct from the bulk of the cytochrome P-450. Simpson (1967) observed a similar partition in studies on the solubilised cholesterol side-chain cleavage enzyme from adrenocortical mitochondrial sonicates. In that work the anomaly was resolved when it was found that although part of the enzyme had been 'solubilised', the bulk of the enzyme was still in the particulate phase. In the present study, a similar argument probably applies but could not be tested due to the presence of the endogenous inhibitor associated with the particulate material.

Although Juchau et al., (1968) failed to detect cytochrome P-450 in 3-month placental tissue and suggested some other component to be involved in placental drug metabolism, Meigs and Ryan (1968) detected cytochrome P-450 in both placental mitochondria and microsomes. These workers observed that cholesterol side-chain cleavage in this tissue was inhibited by carbon monoxide in the dark and that light partially reversed this inhibition. This present study has confirmed these observations and further shown almost certainly that cytochrome P-450 is the CO-sensitive component.

This present study reported a Warburg partition constant, K, of close to unity for the carbon monoxide inhibition of the placental mitochondrial cholesterol side-chain cleavage reaction. The enzyme source was a sonicated placental mitochondrial extract. The light reversal studies were carried out using a mitochondrial acetone powder extract since their greater enzymic activity allowed shorter incubation periods. However, using the acetone powder extracts, a carbon monoxide to oxygen ratio of 5:1 was required to produce an approximately 30% inhibition of the enzymic activity (Table 5.1). Meigs and Ryan (1968) using a placental mitochondrial acetone powder extract observed an approximately 60% inhibition of cholesterol side-chain cleavage activity using a carbon monoxide to oxygen ratio of 10:1. A similar situation has been reported for the adrenal steroid 11β-hydroxylase. Cammer _et al._, (1968) found a K value of approximately unity whereas Harding _et al._ (1969) obtained K values in the region of 5. Estabrook _et al._ (1968) also observed a significant variation of the partition constant, K, for the carbon monoxide inhibition of aminopyrine demethylation by liver microsomes. Estabrook and coworkers found that the microsomal demethylation of aminopyrine was less inhibited by carbon monoxide as the rate of electron flux was decreased. The rate of electron flux was varied by modifying the rate of NADPH generation. They also noted that Warburg and Kubowitz (1929) in describing the requirements for demonstrating the CO inhibition of an oxidase reaction, had discussed the influence of the rate of electron

flux through the electron transport system on the extent of the inhibition observed with carbon monoxide. Cooper et al., (1968) also required saturating levels of the reducing system to establish the CO-inhibition of reconstituted steroid 11β-hydroxylase preparations. Saturating levels of the reducing system required molar ratios of non-haem iron protein to flavoprotein to cytochrome P-450 of 50:1:1, i.e. a large excess of the non-haem iron protein. A dependence of the system on the rate of electron flux could therefore explain the variations in the value of the partition constant for a particular hydroxylase.

The cytochrome P-450 content of lyophilized placental mitochondria was found to be about 0·05 nmole per mg. mitochondrial protein. The cytochrome P-420 content was found to be approximately 0·07 nmole per mg. mitochondrial protein. The cytochrome P-420 content was estimated by subtracting the absorption of the oxidised haemoprotein-CO complex (at 420 nm) from that of the reduced haemoprotein-CO complex (at 420 nm) since only the reduced form of cytochrome P-420 reacts with CO. This is a very approximate method of estimating cytochrome P-420. Meigs and Ryan (1968) found that the cytochrome P-450 content of native placental mitochondria was 0·12 nmoles per mg. mitochondrial protein and the cytochrome P-420 content, 0·06 nmoles per mg. mitochondrial protein. Thus the present estimations are slightly lower than those of Meigs and Ryan and apparently some of the cytochrome P-450 has undergone degradation to cytochrome P-420 during the lyophilization procedure. In the present study, cytochrome P-450 could not be detected in native

placental mitochondria due to large amounts of haemoglobin. Meigs and Ryan (1968) partially overcame this difficulty by perfusing the tissue prior to homogenisation. Such a perfusion was not very successful in the present studies.

The effect of steroid substrates such as 20α-hydroxycholesterol and 11-deoxycorticosterone on the placental mitochondrial P-450 was investigated. However, the low cytochrome P-450 content together with turbidity problems did not permit any conclusions to be drawn, i.e. whether the cytochrome P-450 was in the high-spin or low-spin state.

After the solubilisation of the placental mitochondrial cholesterol side-chain cleavage enzyme system, attempts at the separation and the identification of the protein components of the soluble enzyme system were carried out. Morrison _et al_. (1965) had attempted to separate the components of the placental cholesterol side-chain cleavage enzyme system using ammonium sulphate fractionation and Sephadex G-200 gel chromatography but with negative results.

The soluble mitochondrial preparations were chromatographed on Sephadex G-200, yielding three fractions (Fig. 5.5). Fractions I and II were both required for maximum activity (Table 5.4). Fraction I contained a haem iron protein (Fig. 5.5) and fraction II was heavily contaminated with haemoglobin. Fraction III was not required for enzymic activity and was inhibitory towards the enzymic activity. Fraction II was rechromatographed on DEAE-Sepahdex (A-25) using a salt gradient (Fig. 5.6). Two further fractions were identified, one displaying NADPH diaphorase activity

(flavoprotein) and the other containing a non-haem iron protein (Fig. 5.6). Fraction I, the flavoprotein fraction and the non-haem iron protein were all required for enzymic activity (Table 5.5), although the recovered activity was low. The combination of fraction I and the non-haem iron protein fraction also had a small amount of cholesterol side-chain cleavage activity, probably due to the contamination of fraction I with flavoprotein.

Omura et al. (1966) isolated three protein components from the steroid 11β-hydroxylase of adrenocortical mitochondria. These were identified as cytochrome P-450, flavoprotein and a non-haem iron protein (adrenodoxin) and all were required for the 11β-hydroxylase activity. They proposed that in this hydroxylation the electron flow was NADPH ⟶ flavoprotein ⟶ non-haem iron protein ⟶ cytochrome P-450. Simpson and Boyd (1967b) isolated three similar protein components required for the cholesterol side-chain cleavage enzyme system of bovine adrenocortical mitochondria.

In the present studies the haem protein component of fraction I is at least in part cytochrome P-450, although the cytochrome P-450 content of this fraction must be very low since it could not be detected using the reduced carbon monoxide difference spectrum technique (Omura and Sato, 1964a). The failure to detect the presence of cytochrome P-450 was not unexpected since the original solubilised extract contained no significant amount of this cytochrome (Fig. 5.1a). However, the light reversal studies of the carbon monoxide

inhibition of the enzymic activity (Table 5.2) indicate cytochrome P-450 to be a component of the enzyme system. By comparison with the studies of Simpson and Boyd (1967b), cytochrome P-450 should be located in fraction I. Fraction II contained both the flavoprotein and the non-haem iron components. Thus the placental enzyme appeared to be very similar to the adrenal cortex enzyme studied by Simpson and Boyd (1967b).

The flavoprotein was also present in small amounts in the solubilised enzyme (Fig. 5.5). The flavoprotein chromatographed on DEAE-Sephadex together with haemoglobin and so was very contaminated.

The non-haem iron containing fraction was also present in relatively small amounts and spectral observations on the fraction were not informative due to the small amount of material available. Billiar and Little (1969) have recently isolated placental and liver mitochondrial factors which supported steroid 11β-hydroxylation in the presence of bovine adrenal mitochondrial particles. The non-haem iron proteins of testis and ovary, which are known to be similar to adrenodoxin, can similarly support steroid 11β-hydroxylation (Kimura and Ohno, 1968). Billiar and Little (1969), however, found that although the placental and liver factors had similar chromatographic properties on DEAE-cellulose to adrenodoxin, they did not contain non-haem iron or acid-labile sulphur. The present study has shown that placental mitochondria do contain a protein which has non-haem iron and acid-labile sulphur. The placental protein, as prepared

in this investigation, contained approximately 2 parts non-haem iron to 1 part labile sulphur compared to adrenodoxin which contains approximately equal parts of non-haem iron and labile sulphur (Kimura, 1968). Possibly, part of the labile sulphur has been shed during the purification procedure.

It is apparent that placental mitochondria do contain protein components similar to those involved in the electron transport system associated with adrenal steroid hydroxylation. Thus the placental cholesterol side-chain cleavage enzyme system appears to be very similar to the adrenal and ovarian enzymes. (Simpson and Boyd, 1967b; Sulimovici and Boyd, 1968b). The placental components, however, appear to be present in much smaller amounts than in the adrenal cortex. This is interesting because <u>in vivo</u> studies suggest that the placenta at full term may be degrading about 300 mg. cholesterol to progesterone per 24 hours (Pearlman, 1957).

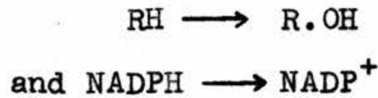
Recovery of cholesterol side-chain cleavage activity after DEAE-Sephadex chromatography was low (Table 5.5). Sulimovici (1968) observed a similar loss of enzymic activity after fractionation of the ovarian enzyme. DEAE-Sephadex chromatography, therefore, is apparently harmful to one (or more) of the protein components. An alternative explanation can be attributed to a diluted reconstituted enzyme system since a two-fold dilution of each component of a three component system should produce an overall eight-fold decrease of enzymic activity. Cooper <u>et al</u>. (1968) found that the activity of a reconstituted adrenal steroid 11β-hydroxylase system was fully restored only when the molar ratios of non-haem iron protein to flavoprotein to cytochrome P-450 were

about 50:1:1. They suggested that the large excess of non-haem iron protein required might possibly be related to the low redox potential of cytochrome P-450 (about -0·4 V.). Since the redox potential of the NADPH/$NADP^+$ system is about -0·3 V and that of the reduced/oxidised adrenodoxin is about zero (Kimura, 1968), the overall redox system could possibly be rather sluggish. Hence, there could be a requirement for a large excess of the non-haem iron protein. Such a large excess of placental non-haem iron protein was not used in this investigation due to the small amount of the protein present in placental mitochondria.

The solubilised enzyme preparations, especially those from lyophilized placental mitochondria, contained considerable 3β-ol dehydrogenase and Δ^5-3-ketosteroid isomerase activity (Table 3.13). After Sephadex G-200 chromatography, the bulk of this dehydrogenase activity was associated with the first fraction eluted, i.e. the high molecular weight material eluted in the void volume (Table 5.6). Thus the dehydrogenase-isomerase was firmly associated with very high molecular weight components. This was evidence for the mitochondrial location of these enzymes normally assumed to be of microsomal origin. Koide and Torres (1965) have suggested a possible bimodal distribution of these enzymes.

Mixed-function oxidases possess both interesting and unusual properties. The hydroxylation reaction is virtually irreversible and thus the enzymes involved are of considerable significance as control steps of metabolism. Hydroxylation reactions are 'energetically expensive' since NADPH is consumed.

Thus steroid hydroxylations involve the coupling of two exergonic reactions



$$RH \longrightarrow R.OH$$

$$\text{and } NADPH \longrightarrow NADP^+$$

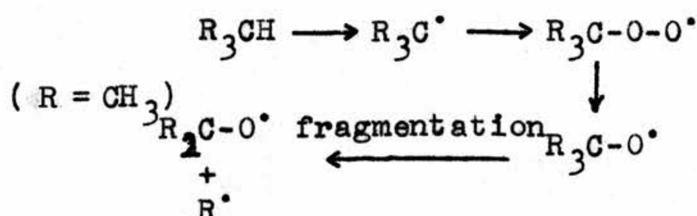
This implies that enzymic hydroxylation reactions have a rather high activation energy. The activation energy for the placental cholesterol side-chain cleavage enzyme was found to be 18·4 ± 2·5 kcal.mole^{-1}. This is of the same order as that of the liver microsomal cholesterol 7α-hydroxylase which was found to be 22 ± 4 kcal mole^{-1} (Boyd, 1970). It seems probable, therefore, that the activation process is similar for each of these hydroxylases. Molecular oxygen is a rather inert molecule. The oxygen molecule has to accept two electrons in the 'activation' process. If, as seems likely, this process occurs stepwise, $O_2^{\cdot -}$ will possibly be an intermediate (see Bray, 1970). However Sih (1969) has objected to the postulation that the perferryl ion FE(II)-O_2 is the so-called "active oxygen", since it is known that, in general, oxygen complexes of d_6-transition metals are kinetically stable toward oxidation of unactivated carbon-hydrogen bonds. This is because in Fe(II)-O_2, both (σ) and (π) bonding occur. This strengthens the metal-ligand bond and contributes to the unusual stability of the Fe(II)-O_2 complex. Sih has suggested that a much more reactive species would be the hydroperoxo complex of ferrous ion $[Fe(II)\text{-}O\text{-}OH]^-$. Within this complex, all the p orbitals of oxygen are occupied and the metal-ligand interaction is weaker. This facilitates the rupture of the oxygen-oxygen bond and

generates the ferryl ion complex Fe(II)-Ö: which is a highly reactive species (Hamilton et al., 1966).

The direct involvement of NADPH is the formation of $[Fe(II)-O-OH]^-$ in steroid hydroxylation had been proposed earlier by Hayano (1962). NADPH has a dual function in this mechanism. Reduced NADP serves in an accessory capacity by keeping the autoxidisable P-450 in the reduced state by means of the NADPH-cytochrome P-450 reductase system, a sequence which is steroid independent. Secondly, NADPH is directly involved in the steroid hydroxylation reaction to generate the hydroperoxo complex $[Fe(II)-O-OH]^-$. Experimental evidence in favour of the dual role of NADPH in the 11β-hydroxylation of deoxycorticosterone has been obtained (Sih et al., 1968). The existence of the transient $[Fe(II)-O-OH]^-$ species still remains to be demonstrated. Further developments in the purification and examination of cytochrome P-450 are required for the elucidation of the mechanism of steroid hydroxylation.

The biosynthetic pathway by which pregnenolone is produced from cholesterol reportedly involves two hydroxylated sterol intermediates, 20α-hydroxycholesterol and 20α, 22R-dihydroxycholesterol. A search was carried out in the present study of the placental cholesterol side-chain cleavage reaction for the presence of possible intermediates between cholesterol and pregnenolone. However, none were ever detected. The evidence for the existence of these compounds is based mainly on incubation studies in which

synthetic samples of the two proposed intermediates were converted more efficiently than cholesterol into pregnenolone. (Shimizu *et al.*, 1961; 1962). Neither of these compounds has been isolated from natural sources in adequately characterised form. Chaudhuri *et al.* (1962) also proved that 22R-hydroxycholesterol could be converted into pregnenolone by a bovine adrenal preparation. This finding complicated the scheme since it made it necessary to postulate more than one pathway between cholesterol and pregnenolone, and this, as has been pointed out by Simpson and Boyd (1967a) is unlikely. Recently, Burstein and Gut (1969) have shown that the pathway involving 22R-hydroxycholesterol is probably much more significant than that involving 20α-hydroxycholesterol. Although using adrenal mitochondrial preparations which converted 20α-hydroxycholesterol to pregnenolone in good yield, many workers (Koritz and Hall, 1964; Hall and Koritz, 1964; Simpson and Boyd, 1967a; Lieberman *et al.*, 1969) have been unable to "trap" radioactivity in this hydroxysteroid when labelled cholesterol is used as substrate for this conversion. Simpson and Boyd (1967a) suggested that the intermediates remained enzyme-bound. Lieberman *et al.* (1969) have considered the possibility that the intermediates might resemble some of the transitory free radicals involved in the autoxidation of isobutane (Mayo, 1968). The intermediates in this reaction are postulated to be:



In this scheme, 20α-hydroxycholesterol will not be an intermediate even though, by its ready conversion into a product which behaves like an alkoxy radical, it may be efficiently converted into pregnenolone. Lieberman et al. (1969) support this postulation by demonstrating that the treatment of 20α-hydroxycholesterol-3-acetate with lead tetra-acetate (a reagent which abstracts $H^{\cdot}$ from hydroxyl groups) results in the formation of pregnenolone acetate which undoubtedly arose by fragmentation of the alkoxy radical. Van Lier and Smith (1968) have reported that autoxidation of cholesterol (most likely mediated by free radical intermediates) led to the formation of several products among which were cholesterol-20α-hydroperoxide, cholesterol-25-hydroperoxide and pregnenolone. The possibility exists that the mechanisms involved in this process might be similar to those involved in steroid hormone production from cholesterol. It is well accepted that oxygen radicals are involved in some biological oxidations and hydroxylations (Staudinger, 1966).

Shimizu (1968) has reported that the side-chain cleavage of 20α-22R-dihydroxycholesterol still retains a requirement for NADPH and molecular oxygen. Thus the metabolism of 20α-hydroxycholesterol, 22R-hydroxycholesterol and 20α,22R-dihydroxycholesterol, might reflect not the intermediacy of these compounds but lack of specificity of the enzyme.

The intermediate problem will not be resolved until the intermediates are formed from the natural precursor by a single purified enzyme. Details of the stoichiometry of the

reaction would be of considerable value to the solution of this problem.

This investigation has been concerned with a study of the human placental cholesterol side-chain cleavage enzyme. The enzyme was found to have the characteristics of a mono-oxygenase requiring NADPH and molecular oxygen. The enzyme was located in the mitochondrial fraction. The intra-mitochondrial reducing equivalents source for NADPH generation appeared to be succinate mediated possibly by an energy-linked trans-hydrogenase coupled with reversed electron flow. Solubilisation of the enzyme system was achieved. These studies were complicated by the activation of an endogenous inhibitor of the enzyme, which appeared to be mitochondrial phospholipid, when the placental mitochondria were disrupted. The solubilised enzyme system was fractionated into three components, cytochrome P-450, a flavoprotein and a non-haem iron protein. The presence of all the three components was essential for enzymic activity. Thus the placental enzyme appeared very similar to the adrenocortical enzyme, except that the concentrations of the enzyme components in placental mitochondria were much less than those in adrenal mitochondria.

APPENDIX

The following chemicals were purchased from the Sigma Chemical Co. - $NADP^+$ monosodium salt, G6P disodium salt, G6P dehydrogenase, NADH, NAD^+, 2,6-dichlorophenol indophenol, ATP, bathophenanthroline sulphonate (sodium salt), β-mercapto-ethylamine.

Silica gel H was purchased from Merck.

[4-^{14}C] Cholesterol and [4-^{14}C] pregnenolone were purchased from the Radiochemical Centre, Amersham.

The purified lecithin and phosphatidyl ethanolamine were supplied by Lipid Products, Epsom.

PPO scintillation grade and POPOP scintillation grade were from the Packard Instrument Co. Inc.

Sephadex G-200 and DEAE-Sephadex (A-25) were purchased from Pharmacia, Uppsala.

Polyethylene glycol (Carbowax 20M) was obtained from the Union Carbide Chemical Co.

Standard steroids were supplied by Organon.

All other reagents and solvents were analar reagent grade, with the exception of toluene and acetone. Toluene was washed with conc. sulphuric acid and water, and dried over sodium sulphate. Acetone was distilled over potassium permanganate.

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The Presence of an Endogenous Inhibitor of the Cholesterol Side-Chain-Cleavage System of Human Placenta

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The presence of an active cholesterol side-chain-cleavage enzyme system in human placental mitochondria was reported by Morrison, Meigs & Ryan (1965). Solubilization of the enzyme was achieved from an acetone-dried powder preparation. These workers noted that thorough treatment of the powder with ether was necessary to obtain active preparations.

The adrenal cholesterol side-chain-cleavage enzyme and the steroid 11β-hydroxylase systems possess similar characteristics (see Omura, Sanders, Estabrook, Cooper & Rosenthal, 1966; Simpson & Boyd, 1967*a*). Sharma, Forchielli & Dorfman (1962) reported the preparation of a soluble steroid 11β-hydroxylase from bovine adrenocortical mitochondria. They observed that ultrasonic treatment followed by high-speed centrifugation resulted in increased enzymic activity as compared with the initial ultrasonically treated preparation. This suggested the presence of an 11β-hydroxylase inhibitor associated with the insoluble particles.

We have investigated various methods for the solubilization of the cholesterol side-chain-cleavage enzyme system present in human term-placental mitochondria. The assay was performed as described by Simpson & Boyd (1967*b*). Native placental mitochondria converted [4-^{14}C]cholesterol into progesterone with a 4–8% conversion in 1h. Ultrasonic disruption of the mitochondria produced a decrease in enzymic activity. However, after ultracentrifugation (105000*g* for 1h) of the sonicated preparation, increased enzymic activity was shown in the supernatant. Similarly, freeze-drying of the sonicated mitochondria resulted in a preparation that did not show maximal activity until ultracentrifugation at 105000*g* for at least 90min. A mitochondrial acetone-dried-powder preparation exhibited a similar phenomenon.

The results suggested the presence of an enzyme inhibitor associated with the insoluble material. It was heat-stable and firmly bound to the insoluble particles, but could be extracted with chloroform–methanol (2:1, v/v). Prolonged acetone treatment of the mitochondria also resulted in removal of the inhibitor. The main components present in the acetone wash were phospholipid (mainly phosphatidylcholine and phosphatidylethanolamine) and cholesterol. The phospholipid fraction was found to be inhibitory.

The effect of authentic phosphatidylcholine and phosphatidylethanolamine on the enzymic activity was investigated. The former was found to be the more potent inhibitor of the enzymic activity. Mitochondrial phospholipid, together with other factor(s) present in the placental mitochondria, inhibited cholesterol side-chain-cleavage activity. The mechanism may involve sequestration of the cholesterol substrate in some micellar or lipoprotein form.

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