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STEROIDOGENESIS IN RAT TESTIS

By

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C O N T E N T S

Steroidogenesis in Rat Testis

ABSTRACT OF THESIS

The first and rate limiting step in steroid biosynthesis from cholesterol, namely the side-chain cleavage of cholesterol, was investigated in rat testes.

1. A sensitive and quantitative method for the assay of cholesterol side-chain cleavage is described. The procedures developed for preparing subcellular fractions free of sperms; fractionation of the heavy mitochondria and separation of the steroid products formed from cholesterol on a single thin-layer chromatogram plate are also presented.

2. The subcellular distribution of the cholesterol side-chain cleavage enzyme system was investigated. This enzyme was localized exclusively in the heavy mitochondrial fraction.

3. The phosphorylative and respiratory control capacities of these intact testicular heavy mitochondria were measured. Respiratory control ratios could be demonstrated only when potassium phosphate, ethylenediamine tetraacetic acid and bovine serum albumin (free of fatty acid) were present together. The optimal requirements for these factors were investigated and the results discussed.

4. The effect of various extra and intra-mitochondrial sources of reducing equivalents on the sterol hydroxylase enzymic activity were examined. The cholesterol side-chain cleavage enzyme had an absolute requirement for NADPH. Certain Krebs cycle intermediates were found to be effective electron donors for the enzyme activity.

5. The optimum conditions for cholesterol side-chain cleavage activity were investigated. These included the effect of varying substrate concentrations (cholesterol and NADPH); different buffer systems, varying the pH and increasing amounts of mitochondrial protein.

6. The rate, total yield and nature of the steroid products formed from [4-^{14}C] cholesterol by testicular heavy mitochondria were found to be greatly influenced by the type of electron donor used (NADPH or isocitrate), as well as by various anions, cations, nucleotides and bovine serum albumin.

7. The influence of inhibitors on the <u>in vitro</u> biosynthesis of steroids in mitochondria were tested. Cyanoketone at low concentrations (6 μM) did not inhibit the cholesterol side-chain cleavage activity but blocked the further metabolism of pregnenolone to progesterone. Aminoglutethimide at 400 μM completely inhibited the conversion of cholesterol to pregnenolone. These effects are discussed.

8. By difference spectroscopy it was demonstrated that rat testes heavy mitochondria contain a complement of cytochromes of the normal respiratory chain but the oxygen activating and substrate binding component of steroid hydroxylation reactions - cytochrome P450 - could not be demonstrated as the CO-binding pigment. Using ficoll fractionated heavy mitochondria and certain specific lipophilic amines a method was developed for the detection and measurement of testicular mitochondrial cytochrome P450. Aminoglutethimide was bound only to mitochondrial cytochrome P450, whereas metyrapone interacted with both the mitochondrial and microsomal cytochrome P450. This fact suggested

that the mitochondrial cytochrome P450 is quite different from the cytochrome P450 found in the microsomes. The amount of high and low spin cytochrome P450 was estimated and the effect of changes of pH and the effect of steroids on cytochrome P450 binding was studied.

9. Treatment of intact rats with cycloheximide resulted in a significant decrease in the conversion of cholesterol to pregnenolone as well as the amount of cholesterol bound to the mitochondrial cytochrome P450. The significance of these findings are discussed.

10. Whether the seminiferous tubules were capable of de novo steroid biosynthesis from cholesterol was studied using subcellular fractions from interstitial tissue and seminiferous tubules dissected out from rat testes. On the basis of cholesterol side-chain cleavage activity and cytochrome P450 spectral evidence it was established that the interstitium is the only testicular tissue capable of de novo steroid synthesis from cholesterol. The seminiferous tubules appear to lack the necessary oxygenase enzyme systems.

11. Preliminary observations are presented which suggest the existence of an alternative route of steroid biosynthesis from cholesterol. The possible significance of these observations are discussed.

ABBREVIATIONS USED IN THE TEXT

ACTH	-	Adrenocorticotrophic hormone
ADP	-	Adenosine 5'-diphosphate
AMP	-	Adenosine 5'-monophosphate
ATP	-	Adenosine 5'-triphosphate
BSA	-	Bovine serum albumin
Cyclic AMP	-	Adenosine 3',5'-cyclic monophosphate
CSCC	-	Cholesterol side-chain cleavage
cpm	-	Counts per minute
DHA	-	Dehydroepiandrosterone
DOC	-	Deoxycorticosterone
EDTA	-	Ethylenediamine tetra-acetic acid
EGTA	-	Ethyleneglycol-bis(β-amino-ethyl ether) N_1N'-tetra acetic acid
EPR	-	Electron paramagnetic resonance
FAD	-	Flavin adenine dinucleotide
FSH	-	Follicle-stimulating hormone
G-6-P	-	D-Glucose-6-phosphate
GLC	-	Gas-liquid chromatography
GSH	-	Reduced glutathione
HCG	-	Human chorionic gonadotrophin
ISP	-	Iron sulphur protein
LH	-	Luteinizing hormone
MAD	-	17α-Methyl androst-5-ene-3β,17β-diol
NAD(H)	-	Nicotinamide adenine dinucleotide (reduced)
NADP(H)	-	Nicotinamide adenine dinucleotide phosphate (reduced)
PMS	-	Pregnant mare serum
TLC	-	Thin-layer chromatography
Tris	-	2-Amino-2-hydroxy methylpropane-1,3-diol

TRIVIAL NAMES OF STEROIDS USED IN THE TEXT

Androstanediol: 5α-androstane-3α,17β-diol

Androstanedione: 5α-androstane-3,17-dione

Androstanolone: 17β-hydroxy-5α-androstan-3-one

Androstenediol: androst-5-ene-3β,17β-diol

Androstenedione: androst-4-ene-3,17-dione

Androsterone: 3α-hydroxy-5α-androstan-17-one

Cholesterol: cholest-5-ene-3β-ol

Cyanoketone: 2α-cyano-4,4,17α-trimethyl-17β-hydroxy-androst-5-ene-3-one

Dehydroepiandrosterone: 3β-hydroxy androst-5-en-17-one

Deoxycorticosterone: 21-hydroxypregn-4-ene-3,20-dione

Dihydrotestosterone: 17β-hydroxy-5α-androstan-3-one

Epiandrosterone: 3β-hydroxy-5α-androstan-17-one

Epitestosterone: 17α-hydroxyandrost-4-ene-3-one

(20S)-20-Hydroxycholesterol: cholest-5-ene-3β,20α-diol

(22R),22-Hydroxycholesterol: cholest-5-ene-3β,22(R)-diol

(20R,22R)-20,22-Dihydroxycholesterol: cholest-5-ene-3β, 20α,22(R)triol

17α-Hydroxypregnenolone: 3β,17α-dihydroxypregn-5-ene-20-one

11β-Hydroxyprogesterone: 11β-hydroxypregn-4-ene-3,20-dione

17α-Hydroxyprogesterone: 17α-hydroxypregn-4-ene-3,20-dione

Oestradiol-17β: oestra-1,3,5 (10)-triene-3,17β-diol

Oestrone: 3-hydroxyoestra-1,3,5 (10)-triene-17-one

Pregnenolone: 3β-hydroxypregn-5-en-20-one

Progesterone: pregn-4-ene-3,20-dione

Testosterone: 17β-hydroxyandrost-4-en-3-one

CHAPTER 1

GENERAL INTRODUCTION

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GENERAL INTRODUCTION

The testis is an extremely complex organ which is comprised of many different types of cells, each having its own metabolic and physiological function. This has been one of the major obstacles in the study of the testis.

Although in some respects knowledge of the endocrinology of the testis has advanced more rapidly than that of other endocrine organs, the information on the biochemistry of steroidogenesis is comparatively sketchy. Many of the concepts concerning the regulation of steroid biosynthesis in the testis are assumed to be similar to that of the other steroidogenic organs viz adrenal and ovary, on which extensive research has been done.

The testis serves two distinct but related functions in the reproductive life of the adult male, namely the germinal function of providing sex cells and the endocrine function of secreting hormones, principally androgens - namely testosterone, androstenedione and dehydroepiandrosterone. These hormones are necessary for the maintenance of spermatogenesis and fertility (Ludwig, 1950; Clermont & Morgentaler, 1955; Davis et al., 1965; Tuleisnik et al., 1966; and Ellis & Berliner, 1969) and development of male accessory reproductive organs and the male secondary sexual characteristics (Walsh et al., 1934; Wood & Simpson, 1961; Clermont & Harvey, 1967). Early in foetal life, the secretions of the testis play an important role in the development of the male genital tract (Goldman et al., 1966a,b; Goldman, 1970). The testis however cannot achieve or maintain normal function without the support

of the pituitary gland (Greep et al., 1936; Greep & Fevold, 1937), the principal mediators being follicle-stimulating hormone (FSH) and interstitial cell stimulating hormone (ICSH) (Clermont & Harvey, 1967).

1.1 NATURE AND NUMBER OF TESTICULAR ANDROGENS

The testis secretes three main C_{19} steroids testosterone, androstenedione and dehydroepiandrosterone (DHA). The evidence that testosterone and androstenedione are true testicular hormones is unquestionable but evidence that DHA is secreted by the testis remains indirect (Eik-Nes & Hall, 1965a). In man it appears that testosterone is secreted by the testis alone but that the adrenal and testes secrete both DHA and androstenedione (Gurpide et al., 1965). In addition, a small amount of DHA is converted to testosterone and androstenedione in peripheral tissues (Horton & Tait, 1967).

Although cyclic activity has long been recognized as characteristic of the female reproductive organ, comparable cycles are less evident in the male. However, seasonal changes in the function of the testis are well known (Hall & Eik-Nes, 1962; Ewing et al., 1965; Niemi & Kormano, 1965). In man the secretion of androgens show diurnal changes (Resko & Eik-Nes, 1966).

Of the androgens, testosterone and androstenedione are considerably more potent than DHA (Dorfman & Shipley, 1956). However, certain qualitative differences have been observed, e.g. DHA has a relatively greater effect upon ventral prostate

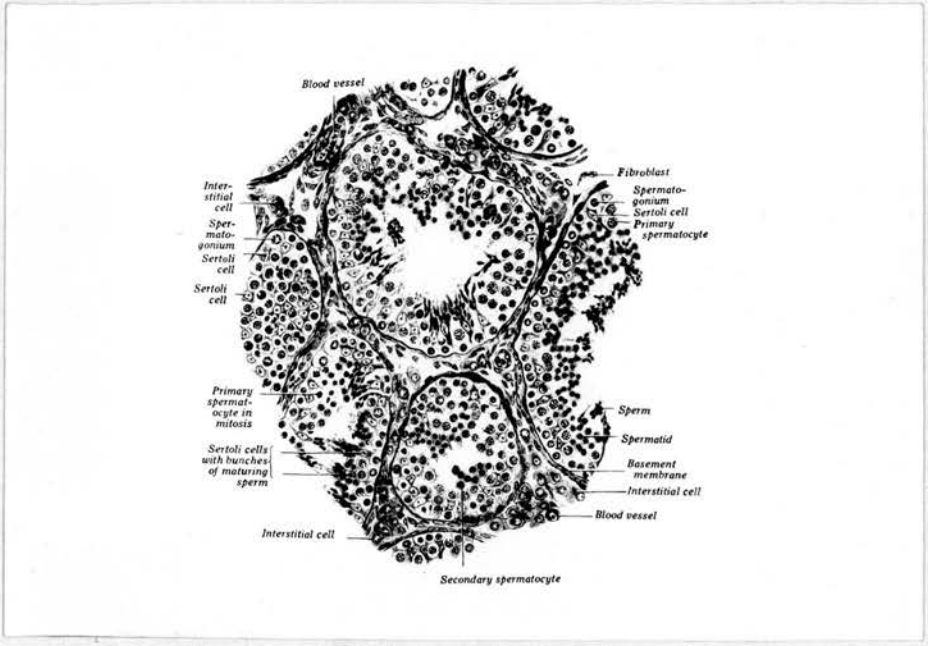


FIG. 1.1 DIAGRAMMATIC SECTION THROUGH THE TESTIS

than on the seminal vesicles when compared with testosterone. On the other hand testosterone and androstenedione are approximately equally potent as inhibitors of the adenohypophysis in rat, while DHA is approximately half as potent (Dorfman & Shipley, 1956). Androstenedione, although a potent anabolic agent, appears to be a weak androgen compared to testosterone, particularly in ruminants (Mann et al., 1960) where much of it is metabolized by red blood cells to the inactive epitestosterone (Lindner, 1961a).

Recently Baulieu (1970) suggested that testosterone per se is not the active compound. In the target cells testosterone was transformed into derivatives androstanolone and androstanediol, the active hormones. The effects of those metabolites on the secondary sex organs were different but the summation of their action reflected that of testosterone.

1.2 HISTOLOGY OF THE TESTIS IN RELATION TO STEROIDOGENESIS

The mature testis, which is enclosed within a sac called the tunica albuginea, is basically made up of two distinct functional parts (Fig. 1.1 shows a diagrammatic section through the testis):

(1) The seminiferous tubules which consist of
   (a) basement membrane formed by a single layer of epithelial cells.
   (b) Several layers of germinal cells at various stages of development. The development and maturation of these cells proceed from the periphery of the tubule (the basement membrane)

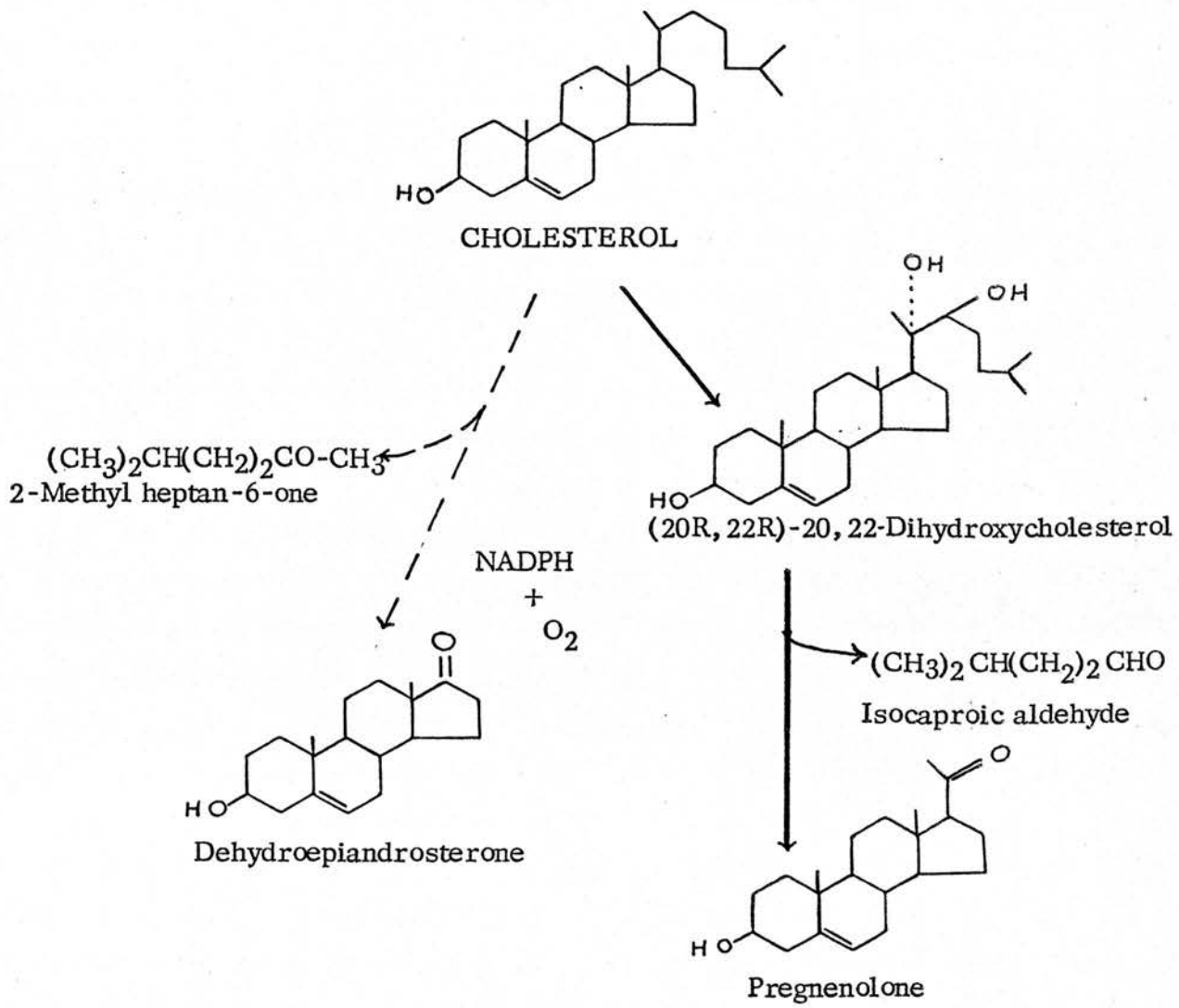


FIG. 1.2

PATHWAYS OF STEROID BIOSYNTHESIS FROM CHOLESTEROL

⟶ MAJOR PATHWAY

⇢ PROPOSED ALTERNATIVE PATHWAY

towards the lumen, where the formed spermatozoa are passed along into the excretory ducts.

(c) Sertoli cells - these are tall columnar, pillar-shaped nongerminal supporting cells which are evenly distributed in the seminiferous epithelium and around which the spermatocytes and spermatids are arranged. In man there are two types of Sertoli cells (Johnsen, 1969).

(2) The interstitial tissue which occupies the inter tubular spaces contain clusters of large polyhedral cells - the "Leydig cells" (or interstitial cells), around blood vessels. Interspersed among these clusters are macrophages and stem cells. Leydig cells differ from species to species not only in number and size but also in certain ultrastructural features (Fawcett et al., 1969; Christensen & Gillim, 1969).

It is now fairly well established that the Leydig cells of the testis are the principal source of testicular steroids. Evidence for this was obtained from a number of different direct and indirect observations such as (a) histochemical localization of the 3β- and 17β-hydroxy steroid dehydrogenases in these cells (Wattenberg, 1958; Baillie et al., 1966); (b) x-irradiation of the testis - this treatment completely damaged the spermatogenic germ cells. The remaining histologically intact Sertoli cells and Leydig cells were apparently capable of maintaining the weight of accessory sex organs such as seminal vesicles and prostate - the indices of the in vivo

produced and secreted androgens (Schoen, 1964; Inano & Tamaoki, 1968a; Abbott, 1959); (c) ability of interstitial cell tumors to produce androgens from several precursors *in vitro* (Dominguez *et al.*, 1958; Gual *et al.*, 1962; Inano *et al.*, 1968); (d) by a fluorescent antibody technique using antibodies against steroid-bovine serum albumin conjugates (Woods & Domm, 1966); (e) by immunological inactivation of endogenous LH (Wakabayashi & Tamaoki, 1966) and (f) by direct biochemical measurement of the *in vitro* ability of manually separated interstitial tissue and seminiferous tubules to metabolize progesterone (Christensen & Mason, 1965), cholesterol (Hall *et al.*, 1969) and endogenous precursors (Van der Vusse *et al.*, 1973) into androgens.

In addition to the interstitial cells the sertoli cells have also been reported to be involved in steroid production (Christensen & Mason, 1965; Lacy, 1967; Ellis, 1969; Lacy & Pettit, 1970; Bell *et al.*, 1968, 1971). However, the contribution was considerably smaller than that of the interstitial cells (Christensen & Mason, 1965). The functional significance of these findings remains uncertain.

Another relevant histological feature of the testis which is of physiological significance is the presence of well developed lymphatics between the seminiferous tubules and the Leydig cells (Fawcett *et al.*, 1970). The seminiferous tubules are not penetrated by blood vessels but are nourished by lymph formed in the peritubular spaces or exuded by capillaries applied to their basement membrane. It appears that part of the androgens secreted by the Leydig cells are

released into the testicular lymph and this probably provides a mechanism by which these hormonal secretions exert a local influence on target cells within the seminiferous tubules (i.e. in the maintenance of spermatogenesis) and on adjacent structures such as the epididymus (Lindner, 1969; Mason & Shaver, 1952).

It has been estimated that in ram, the concentration of testosterone in the lymph was about two thirds of the level in spermatic venous blood and five to ten times higher than that in peripheral venous or arterial blood (Lindner, 1966).

1.3 STEROID BIOSYNTHETIC PATHWAYS FROM CHOLESTEROL

The role of cholesterol as the physiological obligatory precursor of all steroid hormones seems now well established (Krum et al., 1964; Menon et al., 1965a). One exception to this generalization, however, has been reported: triparanol, i.e. 1-[p (β-diethylaminoethoxy) phenyl]-1-(p-tolyl)-2-(p-chlorophenyl)-ethanol, which by inhibiting the reduction of the double bond in the side chain of desmosterol (Avigan et al., 1960) prevents the conversion of acetate to cholesterol, did not block the biosynthesis of testosterone from acetate in slices of rabbit testis in vitro (Hall, 1964). Therefore, it was suggested that desmosterol could act as a precursor of steroid hormones in the testis.

1.3.1 Cleavage of cholesterol side-chain

In common with other steroidogenic organs like the ovary and adrenals, in testes the biosynthesis of steroids from cholesterol was shown (see Fig. 1.2) to involve the splitting

of the side chain of cholesterol resulting in the formation of the C_{21} steroid, pregnenolone and the 6 carbon side-chain fragment, isocaproic aldehyde isolated as the acid (Toren et al., 1964). This scission was postulated to involve hydroxylated cholesterol intermediates (Menon et al., 1965b; Hall & Young, 1968; Kobayashi & Ichii, 1967). The enzyme system (the cholesterol side-chain cleavage enzyme) catalysing this conversion of cholesterol to pregnenolone was localized in the mitochondrial fraction of the testis and found to have an absolute requirement for NADPH and molecular oxygen (Toren et al., 1964; Drosdowsky et al., 1965). This reaction has been suggested to be the rate limiting step in steroid biosynthesis and the site most likely to be affected by trophic hormones - LH and HCG (Menon et al., 1965a; Hall & Young, 1968; Hall, 1966; Hall & Eik-Nes, 1962; Eik-Nes & Hall, 1965a).

In addition to the above pathway, the existence of an alternative pathway involving the direct conversion of cholesterol to DHA without the intermediary formation of pregnenolone has been reported (Jungmann, 1968a,b). This by-pass step (see Fig. 1.2) was reported to involve the cleavage of a C_8 fragment of the side chain of cholesterol (2-methylheptan-6-one). However, the reaction could not be confirmed (Hochberg et al., 1971; Burstein et al., 1971).

1.3.2 Intermediate compounds in the conversion of cholesterol to pregnenolone

Information on the mechanism of transformation of cholesterol to pregnenolone has been based mainly on studies on the adrenal cortex. The exact nature of the intermediates and their sequence of formation remains unclear.

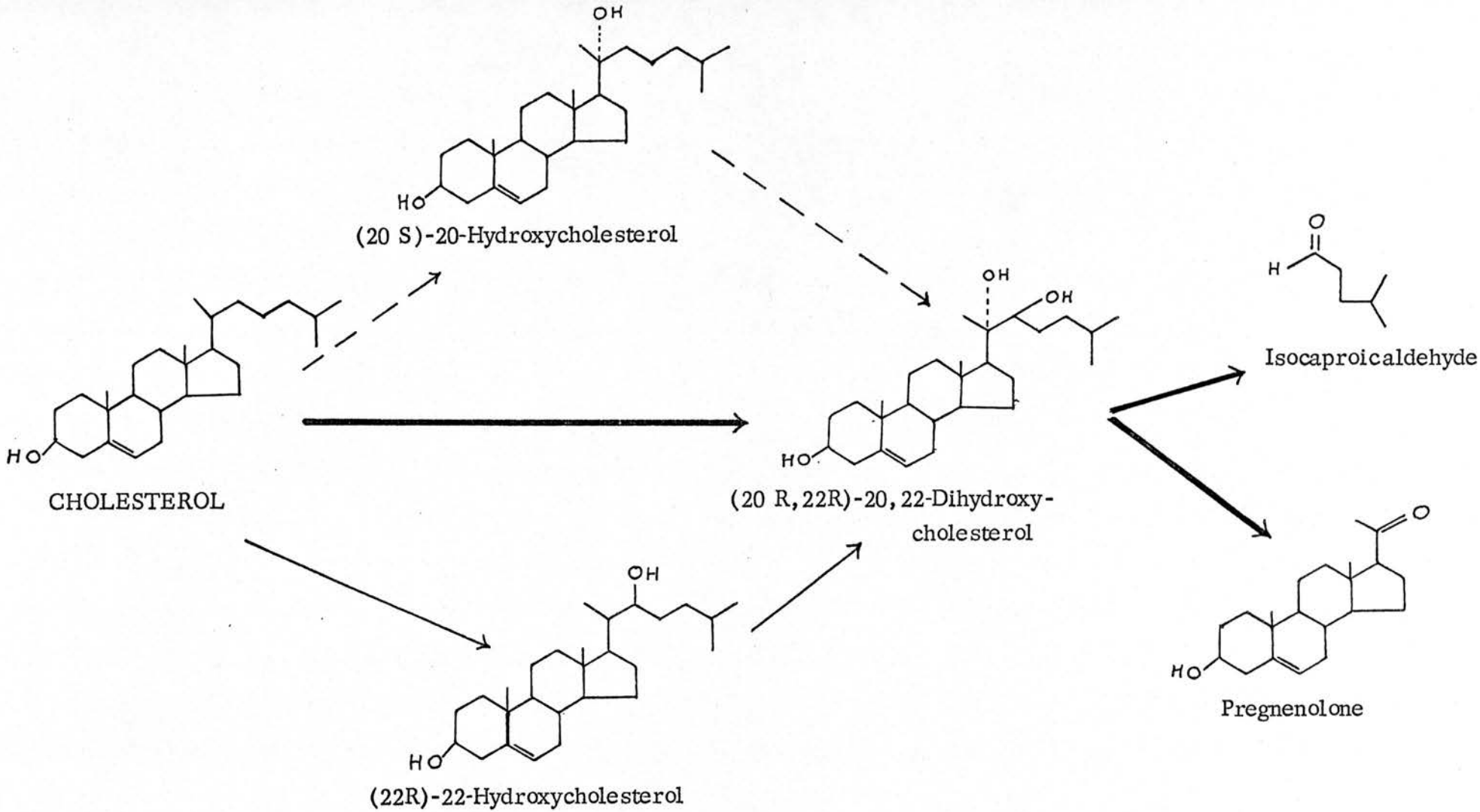


FIG. 1.3 POSTULATED PATHWAYS FOR CHOLESTEROL CONVERSION TO PREGNENOLONE IN ENDOCRINE ORGANS INVOLVING HYDROXYLATED CHOLESTEROL INTERMEDIATE(S) (after Burstein & Gut, 1969)

The pathway most commonly postulated for the conversion of cholesterol to pregnenolone in steroid-producing organs has been reported to involve two hydroxylated derivatives of cholesterol, (20S)-20-hydroxycholesterol and (20R,22R)-20, 22-dihydroxycholesterol (Shimizu et al., 1961, 1962; Constantopoulos et al., 1962; Constantopoulos & Tchen, 1961; Staple et al., 1956). Evidence for this hypothesis has generally been indirect. The studies of Koritz and Hall (1964), Hall and Koritz (1964a) and Simpson and Boyd (1967a) cast some doubt on whether the hypothesis was a valid mechanism for the conversion of cholesterol to pregnenolone. These workers were unable to demonstrate that (20S)-20-hydroxycholesterol was formed from cholesterol in vitro. Hall and Koritz (1964b) proposed that multienzyme complexes may exist, in which there occurs transfer of a product from one enzyme to the next without release of the intermediate into the medium. Recently, Burstein et al. (1970a,b) & Burstein & Gut (1969, 1971) have provided kinetic evidence that (22R)-22-hydroxycholesterol and not the 20S isomer is the more usual first intermediate in the process of the side-chain oxidation of cholesterol. They also suggested that formation of (22R)-22-hydroxycholesterol may apparently be by-passed in a concerted one-step production from cholesterol of the (20R,22R)-20,22-dihydroxycholesterol which then yields pregnenolone.

Recently the involvement of 20α-hydroperoxide of cholesterol in the cholesterol side-chain cleavage process has been strongly implicated by Van Lier and associates (Van Lier & Smith, 1970a,b; Van Lier & Kan, 1971): they

presented evidence for the incorporation of ^{3}H from cholesterol-1,2-^{3}H into 20α-hydroperoxy-cholesterol and demonstrated that this compound was readily converted to (20R,22R)-20,22-dihydroxycholesterol in an anaerobic enzymic reaction by adrenal mitochondria. The triol formed was then converted to pregnenolone by a much slower reaction which required oxygen and NADPH. Kraaipoel et al. (1974) proposed that a 20-22 cyclic peroxide of cholesterol may be an intermediate in cholesterol side-chain cleavage. However, this compound has not been isolated.

Lieberman et al. (1969), Luttrel et al. (1972) and Hochberg et al. (1974), suggested the existence of free radical or ionic intermediates in the transformation of cholesterol to pregnenolone.

$$R_3CH \longrightarrow R_3C. \longrightarrow R_3C\text{-}O\text{-}O. \longrightarrow R_3C\text{-}O. \xrightarrow{\text{Fragmentation}} R_2C{=}O + R.$$

1.3.3 Components of the cholesterol side-chain cleavage multi-enzyme

In testis comparatively little is known about the components of the cholesterol side-chain cleavage enzyme system. Since this testicular hydroxylase system is a mixed function oxidase, (i.e. involves the insertion of one atom of oxygen into the substrate with the other oxygen atom being reduced to water in the presence of an electron donor-NADPH), it has been assumed to be similar to those of other steroid-forming organs.

Most mammalian steroid mixed oxidases are associated with membranes within the cell, being located usually in the mitochondrial or microsomal subcellular compartments. The

cholesterol side-chain cleavage enzyme activity of all steroidogenic organs has been localized in the mitochondrial fraction (Sulimovici & Boyd, 1969). The particulate nature of these enzymes has made their solubilization and purification difficult to achieve. Omura et al., (1966) reported the fractionation of the adrenal mitochondrial steroid 11β-hydroxylase into three components - flavoprotein (diaphorase), iron sulphur protein (ISP) and cytochrome P450. Boyd and co-workers and other investigators accomplished a partial purification of the cholesterol side-chain cleavage system from mitochondria of adrenal cortex (Simpson & Boyd, 1967b; Bryson & Sweat, 1968), rat ovary (Sulimovici & Boyd, 1968a) and human placenta (Mason & Boyd, 1971). The enzyme appeared similar in all three tissues in that it could be resolved into an NADPH diaphorase, the iron sulphur protein and cytochrome P450. Furthermore, the adrenal mitochondrial cytochrome P450 associated with cholesterol side-chain cleavage was separated from the cytochrome P450 associated with 11β-hydroxylase activity (Jefcoate et al., 1970).

In testis, however, several attempts to solubilize the cholesterol side-chain cleavage enzymes have been unsuccessful (Green, 1963; Drosdowsky et al., 1965). Machino et al. (1969) failed to demonstrate the presence of cytochrome P450 in the testicular mitochondrial fraction from rat.

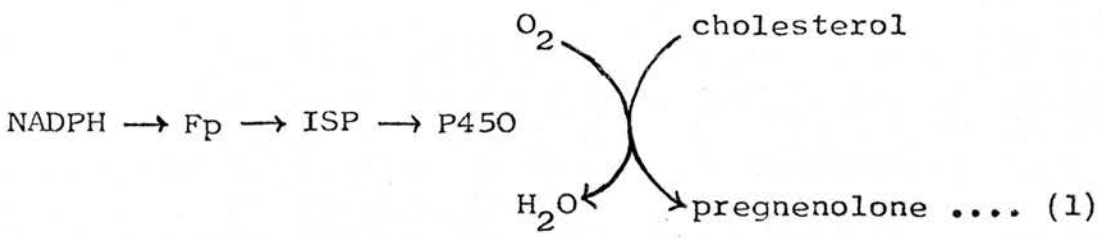
1.3.3.1 Role of components

The role of the flavoprotein in the electron transport chain appeared to be to catalyze the reduction of the non-heme iron protein by NADPH (Omura et al., 1966). The flavoprotein

associated with 11β-hydroxylase system was found to have a molecular weight of about 60,000 and to contain FAD as its prosthetic group.

Adrenodoxin, the iron sulphur protein (ISP) isolated from adrenals (Suzuki & Kimura, 1965; Kimura & Suzuki, 1965; Omura et al., 1965) and having a molecular weight of about 22,000 was shown to contain two atoms of iron and two moles of labile sulfur per mole of protein. The iron atoms were reduced in the presence of NADPH thus suggesting that they play an important role in the catalytic function of this protein (Omura et al., 1965; Watari & Kimura, 1966). Ohno et al. (1967) demonstrated the existence of a similar non-heme iron protein (testodoxin) in pig testis. Testodoxin, when substituted for adrenodoxin, also served as an oxidation-reduction component in the electron transfer system of adrenal hydroxylase.

Cytochrome P450, the hemoprotein possessing protoheme as a prosthetic group (Omura & Sato, 1964a,b) was demonstrated to be the terminal oxidase of the NADPH - requiring hydroxylase systems. This hemoprotein acted as the oxygen-activating enzyme as well as the substrate binding site (Estabrook et al., 1963; Omura et al., 1966). Reducing equivalents originating from pyridine nucleotides served as the primary source of electrons for the reduction of cytochrome P450 prior to the activation of oxygen for substrate hydroxylation. The inter-relationship of components involved in cholesterol hydroxylation in the mitochondria can be schematically depicted as follows:-



1.3.3.2 Cytochrome P450

The hemoprotein pigment cytochrome P450 was so called because of the intense absorption band at 450 nm produced by the carbon monoxide derivative of the reduced hemoprotein (Omura & Sato, 1964b). Cytochrome P450 has been detected in a large number of tissues of mammals and other animals such as fish, birds, amphibians (Ichikawa & Yamano, 1967) and house flies (Ray, 1967). Bacteria (Conrad et al., 1965; Cardini & Jurtshuk, 1970), yeast (Linenmayer & Smith, 1964) and pea seedling buds (Russell, 1971) have also been shown to contain cytochrome P450. In mammals, cytochrome P450 occurs in the lungs, liver, intestinal mucosa, kidney, pituitary and steroidogenic organs. These hemoproteins catalyse the oxidative metabolism of a broad spectrum of organic compounds (Estabrook et al., 1972a,b). Cytochrome P450 from different sources showed substrate specificity depending on the needs of the cell and source. The basis for the substrate specificity is not known. Certain differences in chemistry and function of cytochrome P450 could be observed by changes in the location of the spectral absorption maximum of the carbon monoxide derivative of reduced cytochrome P450 - which varied from 446 nm to 453 nm as well as the characteristics of the low spin form of the ferric hemoprotein as determined by EPR spectrometry.

TABLE 1.1

PROPOSED CLASSES OF CYTOCHROME P450

1. ADRENAL MITOCHONDRIA
   - 1. membrane bound P450
   - 1 soluble iron-sulfur protein
   - ? soluble flavoprotein

2. LIVER MICROSOMES
   - 12. membrane bound P450
   - 3 membrane bound b_5
   - 1 membrane bound flavoprotein

3. BACTERIAL SYSTEM
   - 1 soluble P450
   - 1 soluble iron sulfur protein
   - ? soluble flavoprotein

(after Estabrook et al., 1972b)

1.3.3.3 Multiplicity of cytochrome P450

The existence of different forms and types of cytochrome P450 has been demonstrated.

Depending upon the presence of various compounds capable of functioning as substrates or inhibitors of hydroxylation reactions ferric cytochrome P450 has been shown to exist in a variety of forms - reversible transitions between low and high spin states (Tsai et al., 1970; Peterson, 1971; Cammer et al., 1966). Optical and electron paramagnetic resonance spectral studies demonstrated the presence of at least three forms of the ferric state. These are a) the high spin form of the ferric hemoprotein observed in the presence of one class of substrate (Type I); b) the low spin form observed in the absence of substrate and c) a low spin form obtained when compounds such as aniline, pyridine or metyrapone interacted with the hemoprotein (Type II) (Remmer et al., 1966).

Like hemoglobin or myoglobin, ferrous cytochrome P450 could reversibly form distinguishable complexes with oxygen and carbon monoxide (Ishimura et al., 1971; Gunsalus et al., 1971; Estabrook et al., 1971a).

In addition the cytochrome P450's have been observed to differ depending on their localization within the cell, the nature of the primary electron donor, and the structural organization requisite for their function. Three general classes of cytochrome P450 have been distinguished as shown in Table 1.1.

(1) Adrenal mitochondrial type: This type of cytochrome P450 required the presence of an iron-sulfur protein as a mediator of electron transport between the flavoprotein

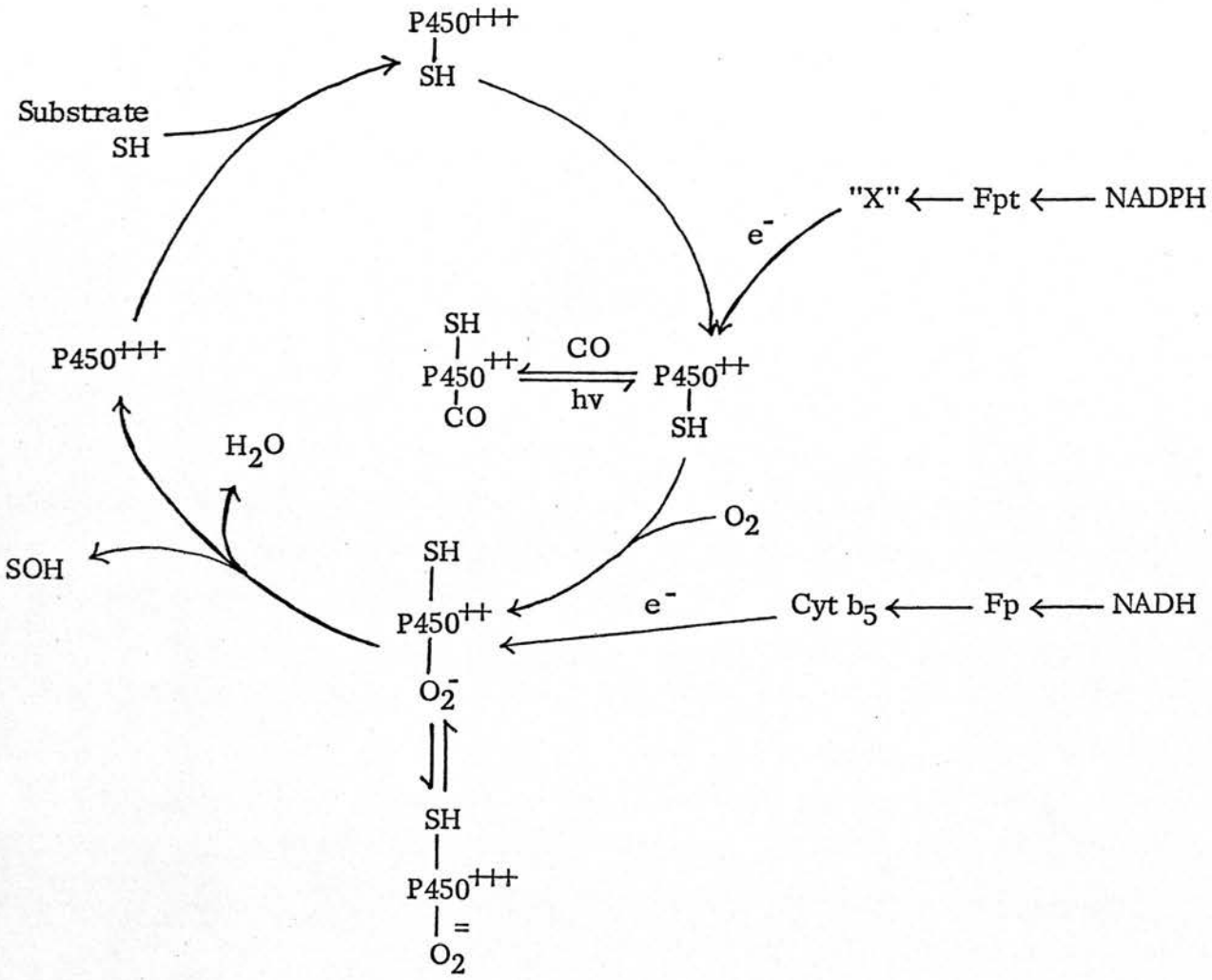


FIG. 1.4 POSTULATED PATHWAYS OF ELECTRON TRANSFER OCCURRING ON MICROSOMES DURING THE REDUCTION AND OXIDATION OF CYTOCHROME P450

Valence of heme iron of cytochrome P450 is indicated by the charge associated with Fe.

Fp = different flavoproteins

S = organic substrate to be hydroxylated

(after Hildebrandt & Estabrook, 1971)

dehydrogenase and cytochrome P450 (Harding et al., 1966; Omura et al., 1966; Cammer & Estabrook, 1967a). Here the membrane-bound cytochrome P450 exists in a 1:1 stoichiometry with the soluble iron-sulfur protein which in turn reacts with the readily soluble flavoprotein, NADPH-adrenodoxin reductase (see Fig. 1.5).

(2) Liver microsomal type - typifying the cytochrome P450 associated with the endoplasmic reticulum. Associated with this type of hemoprotein no iron-sulfur protein has been found to exist (Baron et al., 1972). Hildebrandt and Estabrook (1971) suggested that cytochrome b_5, as well as the flavoprotein, NADPH-cytochrome C reductase, may function in the capacity of electron donor. In this case, cytochrome P450 was reported to exist in a non-equivalent stoichiometric relationship with a second hemoprotein cytochrome b_5 and at least two flavoprotein dehydrogenases, NADPH-cytochrome C reductase and NADH-cytochrome b_5 reductase (Estabrook et al., 1971b).

It has been postulated as shown in Figure 1.4 that flavoprotein reductase or an unknown intermediate "X" participates by donating the first electron required for the reduction of cytochrome P450 resulting in the formation of reduced cytochrome P450 (Masters et al., 1971). Whereas cytochrome b_5, reduced by either the NADH or NADPH flavoprotein dehydrogenase serves as the donor of the second electron required for the reduction of the oxy-ferrous substrate complex of cytochrome P450 (Estabrook et al., 1972b).

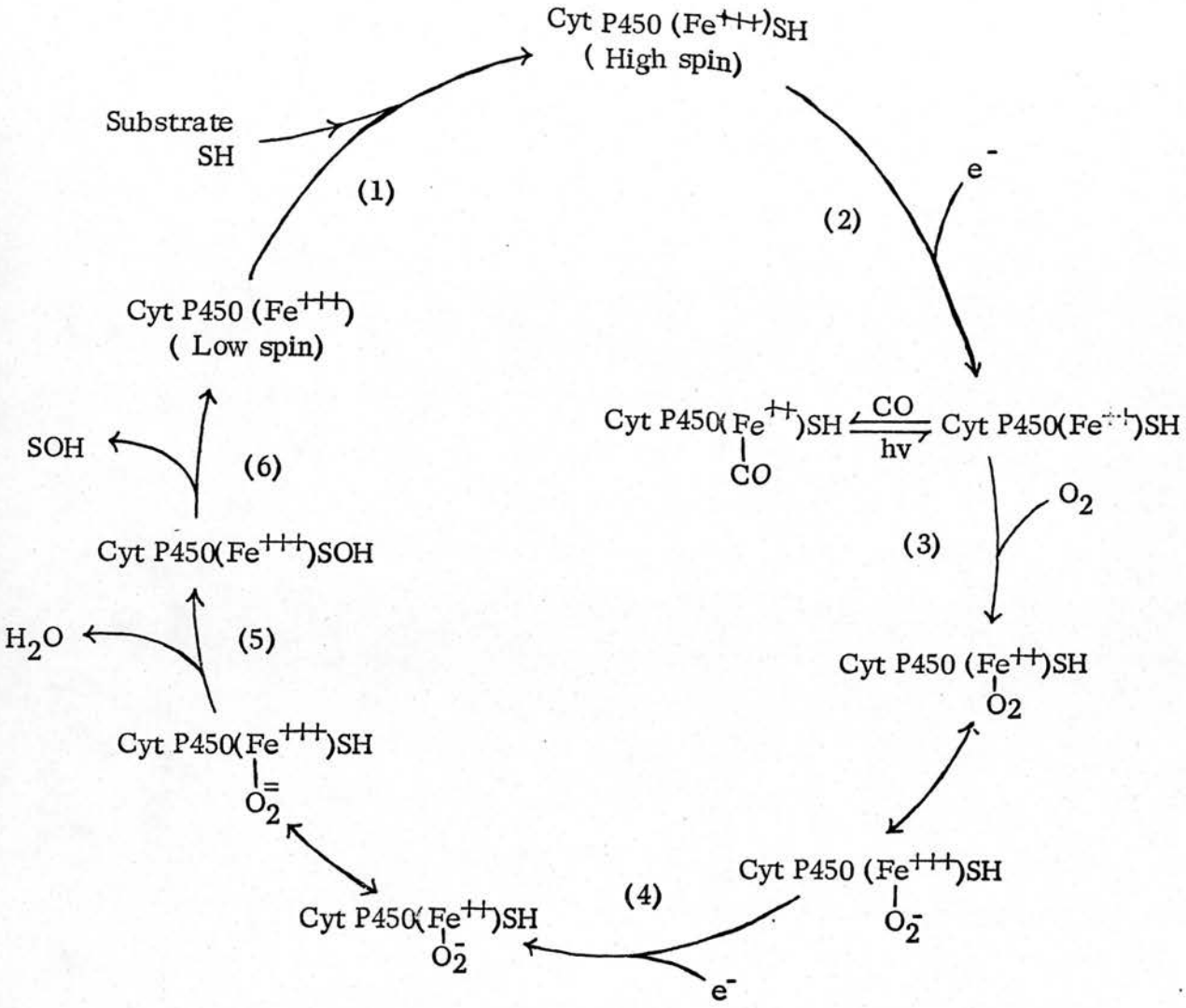


FIG. 1.5 A PROPOSED SCHEME FOR THE CYCLIC REDUCTION AND OXIDATION OF CYTOCHROME P450 DURING INTERACTION WITH A SUBSTRATE AND OXYGEN

(after Estabrook et al., 1973)

(3) Bacterial system - typified by the cytochrome P450 observed in the bacterium Pseudomonas putida. The bacterial system was composed of soluble proteins, readily isolated and purified. The bacterial cytochrome P450 was similar to the cytochrome P450 containing electron transport system present in mitochondria (Peterson, 1970). In this case the flavoprotein dehydrogenase was able to function with either NADPH or NADH (Cushman et al., 1967).

1.3.3.4 The mechanism of function of cytochrome P450

During the last few years a generalized scheme was proposed (Estabrook et al., 1971c, 1972a,b, 1973) describing the interaction of cytochrome P450 with its substrates oxygen and an organic substance (drug, steroid etc.) as well as with electron transport carriers necessary for its reduction. Direct evidence for the reactions proposed and some of the intermediates postulated were provided from studies with purified bacterial cytochrome P450 (Gunsalus et al., 1973; Ishimura et al., 1971; Griffin & Peterson, 1972). Experiments with the adrenal and liver cytochrome P450's are consistent with the proposed scheme. The cyclic reaction of cytochrome P450 involve at least six steps as shown in Figure 1.5.

Step 1. An organic substrate (S) reacts with the low spin form of the ferric (Fe^{3+}) cytochrome P450 to form a high spin ferric substrate complex (Fe^{3+})SH. This interaction is reversible.

Step 2. The ferric-substrate complex of cytochrome P450 undergoes a one electron reduction to form the ferrous-substrate complex of the pigment (Fe^{2+})SH.

Step 3. The ferrous substrate complex interacts reversibly with molecular oxygen to form an oxy-ferrous-substrate complex (Fe^{2+}-O_2.S), called oxycytochrome P450, the nature of which remains undefined.

In the presence of carbon monoxide, a competition for oxygen binding occurs resulting in the formation of the CO adduct of reduced cytochrome P450, characterized by an absorbance band in the blue portion of the spectrum with a maximum at about 450 nm.

Step 4. The oxycytochrome P450 presumably then interacts with an electron transfer component undergoing a second one-electron reduction, forming an unidentified intermediate. The intermediate could be a superoxide anion ferrous substrate complex or a hydroperoxide derivative of ferric-substrate complex of cytochrome P450.

Step 5. Presumably through an internal rearrangement, one atom of the bound molecular oxygen is introduced as a hydroxyl group into the substrate molecule with the concomitant formation of water.

Step 6. The hydroxylated product dissociates from the ferric cytochrome P450 regenerating the uncomplexed low spin form of the ferric hemoprotein.

1.4 CONVERSION OF PREGNENOLONE TO TESTOSTERONE

1.4.1 Pathways

Over the past ten years a considerable amount of data has been published on the biosynthesis of androgens from pregnenolone in vertebrate gonads. The possible pathways of

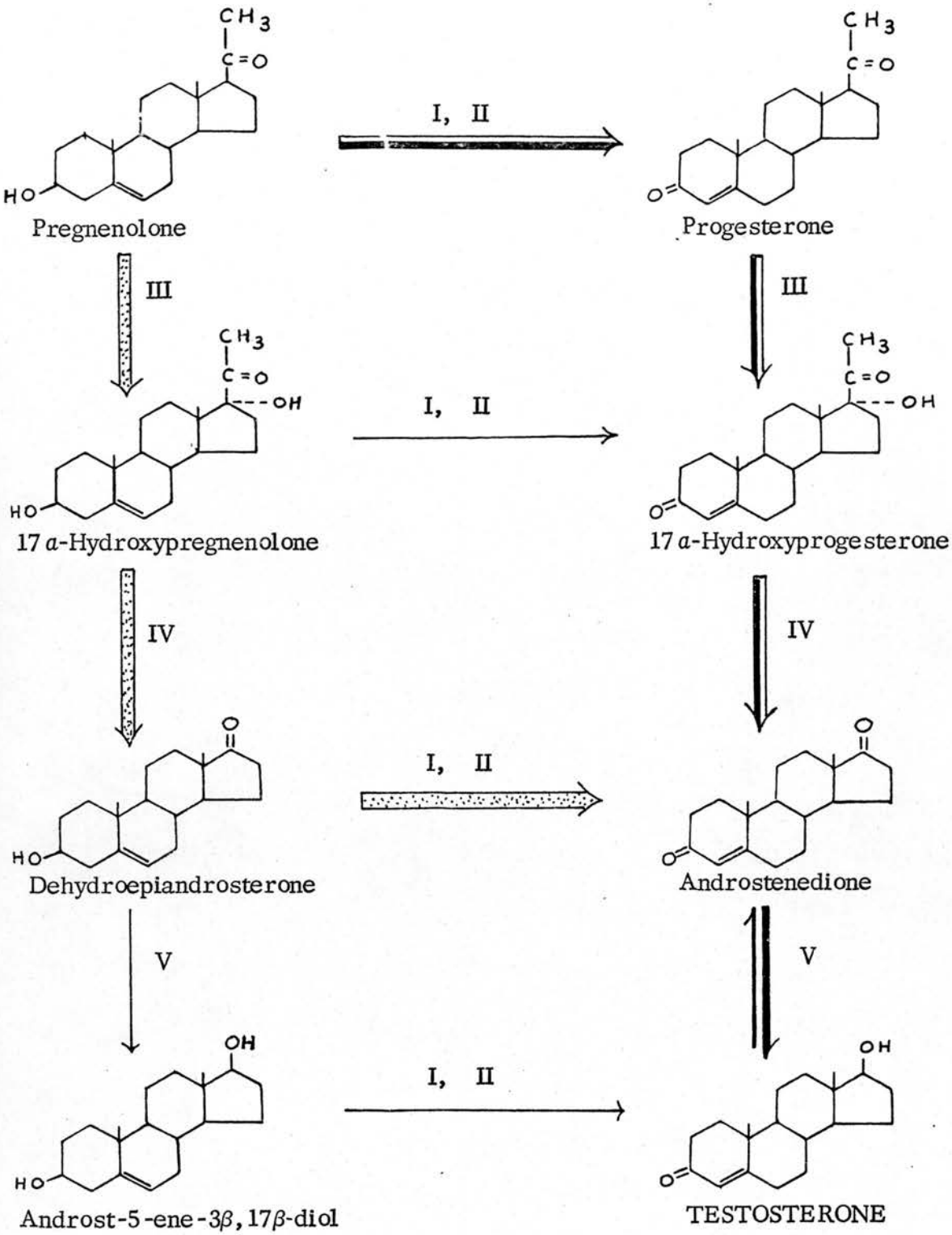


FIG. 1.6 SUGGESTED PATHWAYS OF TESTOSTERONE BIOSYNTHESIS FROM PREGNENOLONE IN TESTICULAR TISSUE IN VITRO AND IN VIVO

I. 3β-Hydroxysteroid dehydrogenase;
II. Δ^5-Δ^4-Hydroxysteroid isomerase;
III. 17α-Hydroxylase;
IV. 17α-Hydroxysteroid C-17,20 lyase
V. 17β-Hydroxysteroid dehydrogenase

in vitro pathway

in vivo pathway

testosterone production have been established mainly by results obtained by *in vitro* experiments and some confirmed by *in vivo* studies.

As shown in Figure 1.6 there are two major pathways to testosterone from pregnenolone. The dominant pathway (the 4-ene pathway), which utilizes 4-ene-3-oxo intermediates viz progesterone, 17α-hydroxyprogesterone and androstenedione was demonstrated clearly *in vitro* in the testes of rats (Shikita *et al.*, 1964; Shikita & Tamaoki, 1965a; Tamaoki & Shikita, 1966b; Bell & Vinson, 1968; Lynn & Brown, 1958; Slaunwhite & Samuels, 1956; Slaunwhite & Burgett, 1965), other rodents (Tamaoki & Shikita, 1966a; Murota *et al.*, 1965; Inano *et al.*, 1967a), rabbit (Tamaoki & Shikita, 1966), monkeys (Sharma *et al.*, 1967) and boars (Kahnt *et al.*, 1961); while the alternative pathway which involves the transformation of 5-ene-3β-hydroxysteroid intermediates such as 17α-hydroxy-pregnenolone and DHA to testosterone, has been demonstrated *in vivo* in dogs (Eik-Nes & Kekre, 1963; Ibayashi *et al.*, 1965).

Although the occurrence of this DHA pathway has been inferred for testes of rats (Ibayashi *et al.*, 1965; Bedrak *et al.*, 1973; Slaunwhite & Burgett, 1965), mouse (Ellis & Berliner, 1965; Grosso & Ungar, 1964), rabbit (Hall *et al.*, 1964), canine (Eik-Nes & Hall, 1965b), porcine, bovine (Kahnt *et al.*, 1961) and human foetus (Acevedo *et al.*, 1961; Ellis & Berliner, 1969), the evidence remains indirect (Eik-Nes & Hall, 1965b; Eik-Nes, 1971). Opinions differ concerning the

existence of the Δ^5 pathway and its significance in the biosynthesis of testosterone specially in rat testicular tissue (Shikita et al., 1964; Tamaoki & Shikita, 1966a). The inability to demonstrate the Δ^5 pathway in vitro has been attributed to the loss of enzymes during the in vitro studies (Ellis & Berliner, 1969).

A connection between the two pathways involving the conversion of 17α-hydroxypregnenolone to 17α-hydroxyprogesterone has been observed in vivo in canine testis (Hagen & Eik-Nes, 1964). The importance of this pathway is not known. However, it is of interest to observe that the formation of the progestational hormone - progesterone is by-passed. Progesterone has been reported to inhibit spermatogenesis (Erricsson & Dutt, 1965).

The conversion of DHA to testosterone can occur via two routes involving as an obligatory intermediate (a) androstenedione (Eik-Nes, 1969) or (b) androstenediol - another steroid with the 5-ene-3β-hydroxy configuration. The involvement of androstenediol has been observed in the in vitro conversion of DHA to testosterone by testes of certain species, e.g. rabbit (Rosner et al., 1964), monkey (Hoschoian & Brownie, 1967) and humans (Rosner & Macome, 1970). Androstenediol was observed in canine spermatic vein blood and its increase after LH administration reported (Yamaji et al., 1968). In rat, no evidence was obtained for androstenediol as an intermediate in testosterone biosynthesis (Tamaoki & Shikita, 1966a; Ellis & Berliner, 1969). However, in heat acclimatized rat testis, androstenediol was demonstrated as an obligatory intermediate

in the biotransformation of DHA to testosterone (Bedrak et al., 1973). In this respect the results of Dufau et al. (1971b) are of interest. They found that peritubular cell monolayers cultured from rat seminiferous tubules were able to convert DHA and androstenediol into androstenedione and testosterone.

1.4.2 Enzyme related to testosterone biosynthesis

The conversion of pregnenolone to testosterone requires five enzymic reactions 17α-hydroxylation, lyase (desmolase), 3β-hydroxysteroid dehydrogenation, 5-ene-3-oxo-steroid isomerization and 17α-hydroxysteroid dehydrogenation. It was demonstrated by cell fractionation studies that the enzymes catalyzing these reactions were concentrated mainly in the smooth surfaced microsomal fraction, i.e. smooth endoplasmic reticulum (Christensen & Gillim, 1969; Inano et al., 1970; 1967a,b; Shikita & Tamaoki, 1965; Shikita et al., 1964). The supernatant (105,000 x g) although essentially devoid of these five enzymic activities was capable of stimulating the conversion of pregnenolone to testosterone. This stimulation was not related to the generation of reduced pyridine nucleotides.

5-ENE-3β-HYDROXYSTEROID DEHYDROGENASE AND ISOMERASE

In co-operation with isomerase, 5-ene-3β-hydroxysteroid converts pregnenolone to progesterone, androstenediol to testosterone and also DHA to androstenedione in the presence of NADP or NAD (Goldberg et al., 1964; Slaunwhite & Burgett, 1965; Tamaoki & Shikita, 1966b; Wattenberg, 1958). The isomerase which transforms 5-ene-steroid to 4-ene-steroid was stimulated by NAD and NADH (Oleinick & Koritz, 1966). Indirect

evidence based on HCG stimulation suggest that two different 3β-hydroxysteroid dehydrogenases exist. The enzyme catalyzing the conversion of pregnenolone to progesterone was reported to be stimulated by HCG in immature animals (Inano & Tamaoki, 1966) whereas the dehydrogenase which transformed DHA to androstenedione was not affected (Frowein, 1973).

Although these above transformations were generally regarded as irreversible, it has proved possible under certain conditions to reverse the reaction pregnenolone —> progesterone (Rosner et al., 1965; Ward & Engel, 1966). The physiological significance of this reversal remains unknown.

17α-HYDROXYLASE. This enzyme catalyzes an irreversible reaction requiring NADPH as cofactor and molecular oxygen (Lynn & Brown, 1958; Inano et al., 1967b). One atom of oxygen is inserted as the 17α-hydroxy group of the steroid molecule (Nakano et al., 1968). Like other mixed function oxidases, this hydroxylation involves cytochrome P450 (Inano et al., 1970; Betz & Michels, 1973a,b).

C-17,20 LYASE which converts 17α-hydroxyprogesterone to androstenedione by the cleavage of the side-chain, plays a very important role in the production of C_{19} steroids (androgens) from C_{21} steroids (pregnene steroids). Like hydroxylases the lyase also requires molecular oxygen and NADPH (Nakano et al., 1966, 1967). This enzyme is not inhibited by cyanide or catalase (Lynn & Brown, 1958).

17β-HYDROXYSTEROID DEHYDROGENASE is responsible for the last step in the course of testosterone production, i.e. the reduction of the 17-ketone of androstenedione to the 17β-hydroxy group. The enzyme catalyzes a reversible reaction

which favours the reduced steroid-testosterone (Samuels, 1952). The preferred cofactor is NADP. The equilibrium of the 17β-hydrogenation is greatly influenced by the ratio of NADPH:NADP.

1.5 INVOLVEMENT OF STEROID SULFATES IN TESTOSTERONE BIOSYNTHESIS

Steroid sulfates have generally been reported as excretion by-products of steroid metabolism but there are an increasing number of reports suggesting that steroid sulfates may play an important role in steroid production in testis.

Dehydroepiandrosterone sulfate occurs in human peripheral blood as the predominant C_{19} steroid (Baulieu, 1960) and it was suggested that circulating DHA sulfate may be used by the testis in the synthesis of testosterone (Aakvaag et al., 1964; Dorfman & Ungar, 1965). Dixon et al. (1965) reported that DHA and testosterone were converted to their respective sulfates after incubation with normal human testes and the DHA sulfate (3β-yl sulfate) was converted to testosterone sulfate (17β-yl sulfate). After incubation with human feminizing Leydig cell tumor, the production of 3β-yl sulfates of pregnenolone, 17α-hydroxypregnenolone and DHA were observed (Pierrepoint et al., 1966). Recently, Raheja and Lucis (1970) reported that human testis failed to convert DHA-sulfate to free testosterone or free DHA in vivo. But in vitro, DHA sulfate was transformed to free DHA, testosterone and androstenedione. Aakvaag et al. (1964), after perfusing canine testes with labelled DHA sulfate, detected labelled testosterone in the spermatic venous effluent and perfused tissue. The discrepancies in these observations have been attributed to species differences.

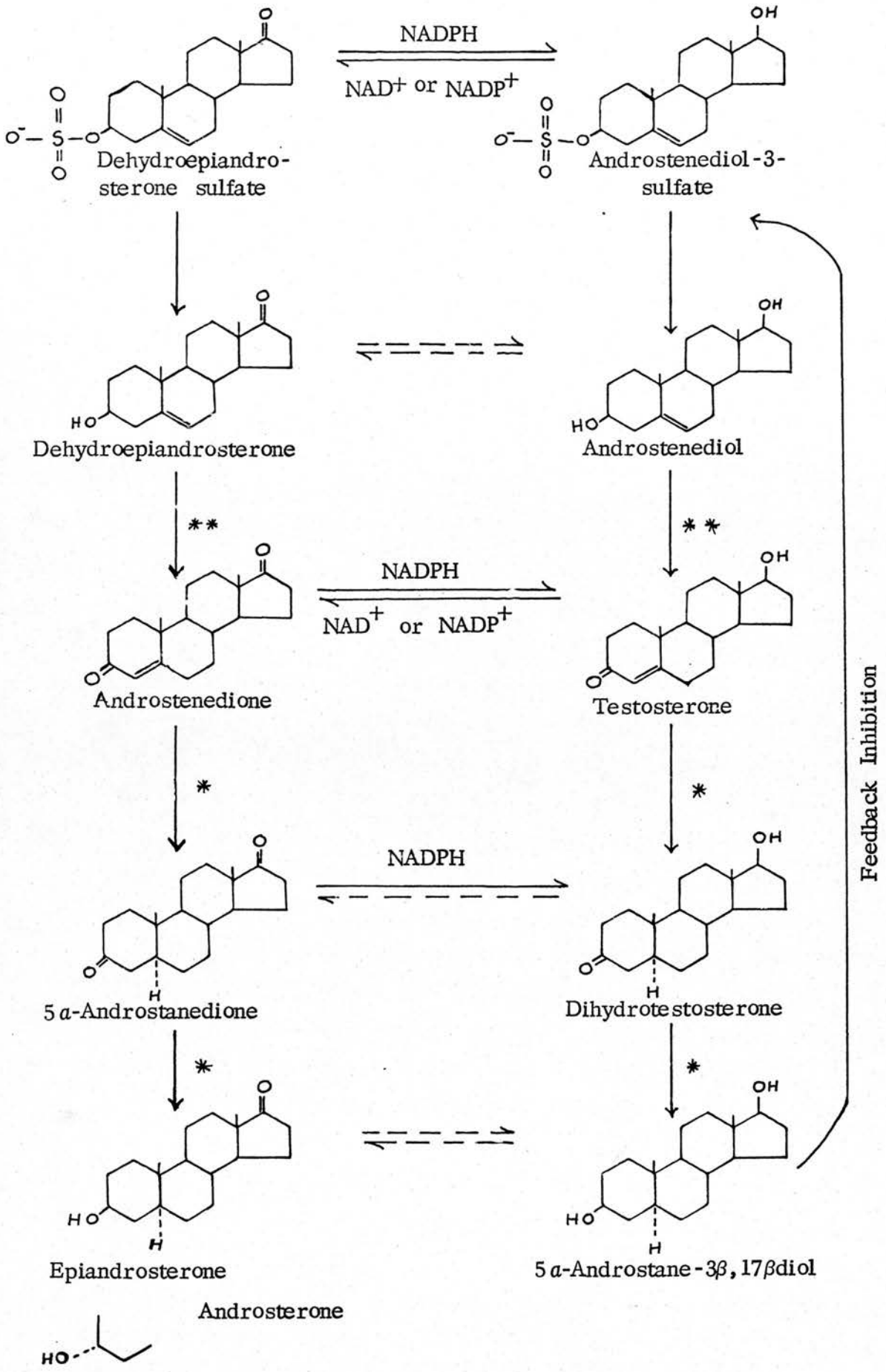


FIG. 1.7 PROPOSED PATHWAYS OF ANDROGEN SYNTHESIS AND METABOLISM IN RAT TESTICULAR MICROSOMES INVOLVING STEROID SULFATES

Cofactor involved * NADPH
** NAD^+

In rat testes, it was demonstrated that free steroids such as androstenedione and testosterone were produced from sulfated precursors — 17α-hydroxypregnenolone sulfate (Neher & Kahnt, 1965) and DHA sulfate (Burstein & Dorfman, 1963). A soluble extract of rat testes was able to transform DHA and estradiol-17β to their respective 3-yl-sulfates (Payne & Mason, 1965a,b). The testicular steroid 3β-sulfatase activity was found to be associated with the microsomal fraction (Payne & Jaffe, 1970; Burstein & Dorfman, 1963). Payne and Mason (1965a) observed that in rat the in vitro conversion of DHA sulfate to androstenediol-3-sulfate occurred four times more rapidly than that of DHA to androstenediol. The relative conversion by the microsomal preparations of DHA sulfate and androstenediol sulfate to testosterone and androstenedione in the presence of NAD, NADP and NADPH suggested that androstenediol-3-sulfate was the preferred precursor (Payne & Jaffe, 1970). On the basis of the 5α-reduced steroids isolated as additional products of both sulfated substrates and the kinetic studies (Payne et al., 1969; Notation & Ungar, 1969a), a general scheme for pathways of androgen synthesis involving sulfated steroids was proposed (Payne & Jaffe, 1970) as shown in Figure 1.7. It was suggested that the steroid sulfatases may act as control enzymes providing a regulatory mechanism for release of free steroids which could then be further metabolized to active hormone (Payne et al., 1969; Payne & Kelch, 1973). 5α-Androstane-3β,17β-diol the major distant metabolite of androstenediol-3-sulfate and a potent inhibitor of the testicular steroid sulfatase (Payne et al., 1969) may act by feedback inhibition to control the amount of

testosterone synthesized. Based on their response to gonadotrophins, the steroid sulfatase activity in seminiferous tubules was shown to be different from those of the interstitial tissue. The enzymic activity in the latter tissue was suggested to be under the control of both LH and FSH (Payne & Kelch, 1973), confirming the initial observations of Notation and Ungar (1968), that HCG administration *in vivo* to rats increased the *in vitro* steroid sulfatase activity. Although the contribution from steroid sulfates were small and highly variable, it was suggested to be an auxiliary biosynthetic route (Notation & Ungar, 1969b). Whether cholesterol sulfate can act as a precursor in this pathway remains to be demonstrated.

1.6 ESTROGEN BIOSYNTHESIS BY TESTIS

It has been known for some time that adult men excrete steroids with a phenolic A-ring in the urine. Until recently only indirect and conflicting evidence existed for the production and secretion of estrogens by mammalian testis (Leach *et al.*, 1956; Fishman *et al.*, 1967). Dynamic studies suggested that more than half the production rate of estradiol in blood was derived by peripheral aromatization of secreted testosterone (Longcope *et al.*, 1969; Baird *et al.*, 1969a). It was also claimed by other investigators that all the circulating estrogen in men could be accounted for by extra-glandular production (Lipsett, 1970; MacDonald, 1967, 1971). More recently estradiol was measured in higher concentrations in spermatic than in peripheral venous blood of men confirming that the testis secreted estrogens (Kelch *et al.*, 1972; Baird *et al.*, 1973).

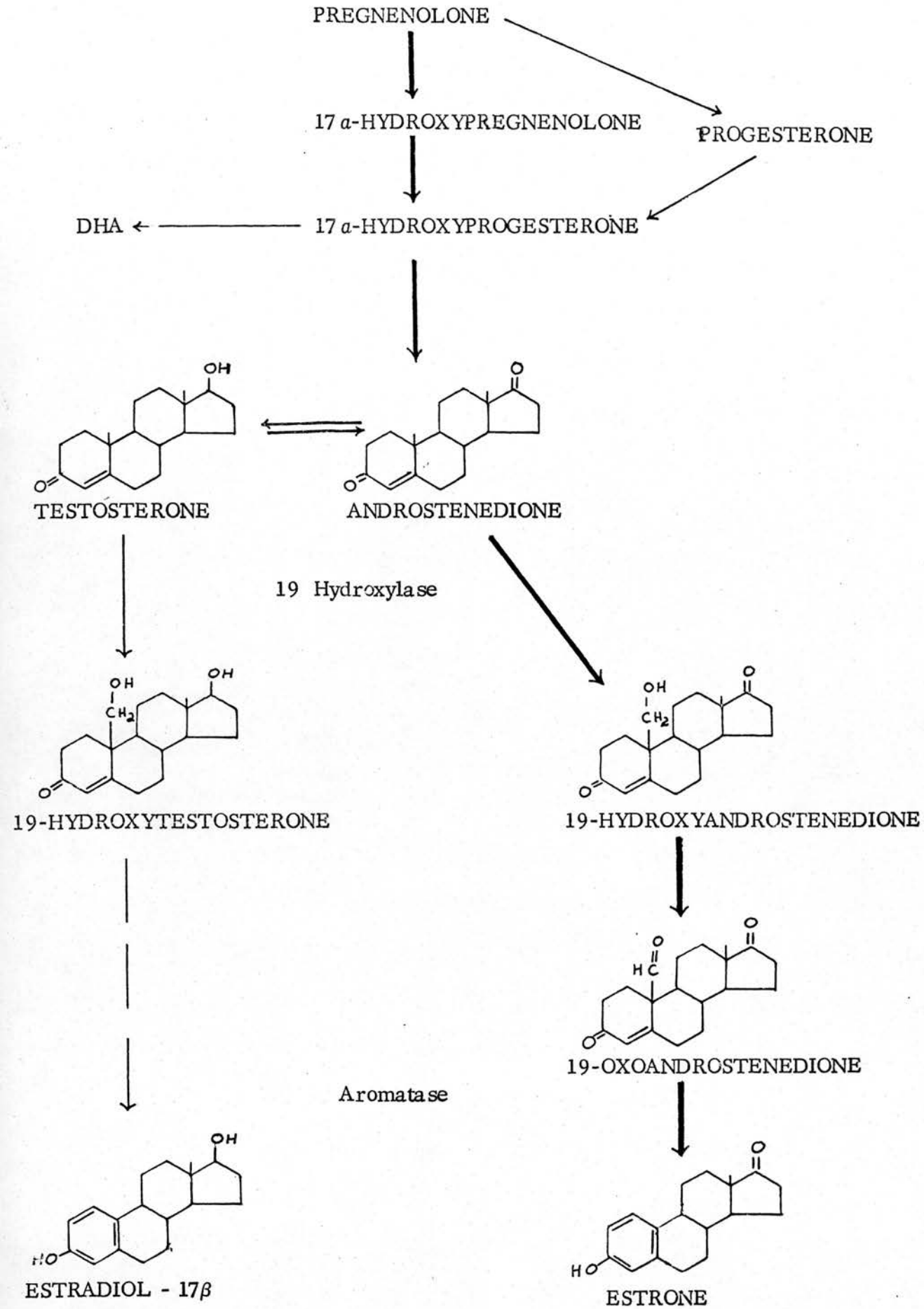


FIG. 1.8 PREDOMINANT PATHWAYS OF ESTROGEN BIOSYNTHESIS IN EQUINE TESTES
The width of arrows is proportional to activity.
----→ = more than one step may be involved. All the participating enzyme systems were localized in the microsomal fraction.
(After Bedrak & Samuels, 1969; Oh & Tamaoki, 1971)

Estrogens have been detected and their concentration measured in testicular tissue of humans (Goldzieher & Roberts, 1952; Anliker et al., 1957), horse (Beall, 1940; Raeside, 1969), and foetal sheep (Attal, 1969). The conversion of radioactive precursors to estrogens in vitro have been reported in testes of man (Axelrod, 1965; Sharma & Gabrilove, 1971), rabbit (Rabinowitz, 1956, 1958) and rat (Inaba et al., 1967; Ficher & Steinberger, 1971). However, Pierrepoint et al. (1967) could not show a significant conversion of pregnenolone or DHA and De Jong et al. (1973) of testosterone, androstenedione or DHA to estrogens in testes of normal dog and rat respectively. Eik-Nes (1967) demonstrated the in vivo conversion of radioactive androstenedione to estrone and estradiol during perfusion of dog testis.

The histochemical demonstration of estrone in Leydig cells (Ashbel et al., 1951) and the studies in which HCG was used to stimulate testis, strongly suggest that testicular estrogens were secreted by Leydig cells (Maddock & Nelson, 1952; De Jong et al., 1973). On the other hand, the feminizing influence of Sertoli cell tumors (Witschi & Mengert, 1942; Huggins & Moulder, 1945; Berthrong et al., 1949; Teilum, 1949; Lewis & Stockard, 1950) together with other indirect evidence has led to the view that estrogens arise from Sertoli cells (Lacy et al., 1968; Lacy & Pettitt, 1970; Bass et al., 1973).

The biosynthesis of estrogens in equine testes has been established in vitro (Bedrak & Samuels, 1969; Oh & Tamaoki, 1970). Figure 1.8 shows that estrone is produced from

androstenedione through 19-hydroxyandrostenedione and then 19-oxo-androstenedione. The aromatizing enzymes (19-hydroxylase and aromatase) were found to be concentrated in the microsomal fraction of the equine testis (Oh & Tamaoki, 1971, 1973).

1.7 REGULATION OF ANDROGEN BIOSYNTHESIS

1.7.1 Hormones

It has generally been observed that of the anterior pituitary hormones, only luteinizing hormone (LH) regulates the Leydig cells and the production of androgens in the testis (see Eik-Nes, 1964a). Recently, evidence based on the binding of radioiodinated LH (De Kretser *et al.*, 1971) and radioimmunoassay (Moudgal *et al.*, 1971) conclusively demonstrated that LH specifically binds to Leydig cells. Follicle stimulating hormone (FSH), which mainly affects spermatogenic activity (Dorrington *et al.*, 1972), was observed to increase testosterone synthesis *in vitro* (Hall & Eik-Nes, 1962) but this effect was suggested to be due to contamination by traces of LH (Mason & Savard, 1964; Moyle *et al.*, 1971). Currently, there is accumulating evidence that prolactin - a predominantly female hormone may also influence androgen biosynthesis in the testis (Bartke, 1971; Hafiez, 1971; Musto & Hafiez, 1972). The two placental gonadotrophins, human chorionic gonadotrophin (HCG; Brady, 1951; Hall & Eik-Nes, 1962) and pregnant mare serum (PMS; Eik-Nes & Hall, 1965b) stimulate the secretion of androgens by the testis, probably in a manner similar to

LH; but unlike LH, HCG has been reported to also increase the activities of microsomal 17α-hydroxysteroid,C-17,20 lyase (Schoen & Samuels, 1965) and 3β-hydroxysteroid dehydrogenase (Shikita & Hall, 1967). It has been demonstrated that LH does not increase the conversion of pregnenolone to testosterone but primarily enhances the oxidation of cholesterol to pregnenolone in the mitochondria (Hall, 1966).

The mechanism of LH action in the testis is not clear. Based on ovarian and adrenal studies, there are several ways in which the trophic hormone could effect the metabolism of cholesterol by the mitochondria. The rate of pregnenolone production could be limited by controlling the availability of the various substrates for cholesterol side-chain cleavage namely:-

(a) NADPH - by affecting the cytoplasmic glucose 6-phosphate dehydrogenase activity (Koritz & Peron 1958; Savard & Casey, 1964; Haynes, 1958; McKerns, 1969), mitochondrial nucleotide trans-hydrogenase or malic enzyme (Simpson et al., 1972).

(b) Cholesterol - by affecting synthesis or mobilization of cholesterol from cell stores (Behrman & Armstrong 1969; Behrman & Greep, 1972; Trzeciak & Boyd, 1973) or the synthesis of a "labile protein factor" (Garren et al., 1965; Simpson et al., 1972).

(c) Oxygen - by altering the blood flow to the organ (Ellis, 1961, Wurtman, 1964).

(d) The accumulation of pregnenolone could result in product inhibition of cholesterol metabolism (Koritz & Hall, 1964a,b; Koritz & Kumar, 1970).

Current concepts suggest that in part LH stimulates the accumulation of cyclic AMP which in turn promotes steroidogenesis via intermediary factor(s) (Robison et al., 1971; Moyle et al., 1971).

1.7.2 Nucleotides

Until recently the evidence for cyclic-AMP being the mediator in the action of trophic hormones on steroidogenesis, in testis, were generally indirect. In as much as cyclic-AMP and its dibutyryl derivative have been shown to stimulate steroidogenesis when added to rat testicular slices (Sandler & Hall, 1966); whole testis (Connell & Eik-Nes, 1968; Dufau et al., 1971b, 1972; Rommerts et al., 1972), or to mouse Leydig cell tumors (Moyle et al., 1971) or administered via the spermatic artery of anaesthetized dogs (Eik-Nes, 1971, 1967); it has been widely accepted that the LH-induced testosterone biosynthesis is mediated by cyclic-AMP (Robison et al., 1971). Eik-Nes (1969) demonstrated by infusion experiments that the effect of cyclic-AMP on the rates of secretion of testosterone was very specific since administration of 5-AMP, 2',3'-AMP or 3'-AMP had no effect on testosterone secretion. It was observed that the trophic stimulation of cyclic-AMP accumulation in testis (Murad et al., 1969; Kuehl et al., 1970; Eik-Nes, 1971; Dorrington et al., 1972) was not due to inactivation of cyclic-AMP phosphodiesterase but an augmented synthesis of cyclic AMP from ATP by adenylcyclase localized in the nuclear membrane of the testicular tissue (Murad et al., 1969; Pulsinelli & Eik-Nes, 1970; Eik-Nes, 1971). More recently, direct experimental evidence to support

the view for cyclic AMP being the second messenger (Robison et al., 1971) in the trophic stimulation of steroidogenesis in testis, was provided. Van der Molen and co-workers (Rommerts et al., 1972; Cooke et al., 1972a) demonstrated that in HCG stimulated rat testes and isolated interstitial tissue, the increase in testosterone production was preceded by increase in cyclic-AMP levels. LH, HCG but not FSH increased the cyclic AMP content in rat Leydig cell preparations (Cooke et al., 1972a,b; Moyle & Ramachandran, 1973) and mouse tumor Leydig cells (Moyle & Ramachandran, 1973). LH failed to stimulate cyclic AMP synthesis in the seminiferous tubules.

The mechanism for the role of cyclic AMP in steroidogenesis is obscure and some of the evidence conflicting. It has been reported that LH acts at the site involved in the hydroxylation of cholesterol (Menon et al., 1965a). However, Pulsinelli & Eik-Nes (197o) reported that testicular mitochondria will not enhance the conversion of endogenous or exogenous cholesterol to pregnenolone in the presence of cyclic-AMP. Furthermore, it has been observed that higher concentrations of trophic hormone were required for maximal stimulation of cyclic-AMP accumulation and the amount of cyclic AMP synthesized was far in excess of that needed for maximal stimulation of steroidogenesis. The stimulation of cyclic-AMP synthesis was readily terminated by dilution of the LH whereas the steroidogenic response to LH was not affected (Moyle & Ramachandran, 1973).

1.7.3 Prostaglandins

Prostaglandins occur in and are synthesized in the testis (Carpenter & Wisman, 1970; Michael, 1973). The significance of these findings are not clear and evidence of the action of prostaglandin in testes are conflicting. In other steroidogenic organs, prostaglandin E_2 for example stimulated steroidogenesis (Speroff & Ramwell, 1970) probably via cyclic-AMP (Marsh, 1970). In minced testes from normal rats the addition of prostaglandin E_1 was observed to increase the formation of cyclic-AMP. In contrast, Kuehl et al. (1970) reported that prostaglandin E_1 and $E_2\alpha$ will not promote the accumulation of cyclic-AMP from ATP in homogenates of rat testes. Eik-Nes (1969) observed that the infusion of large amounts of prostaglandin E_2 via the spermatic artery of dogs increased the secretion of testosterone. This finding was attributed to a possible increased blood flow in the testis (Bergstrom, 1967; Eik-Nes, 1964b).

1.7.4 Serotonin and melatonin

Ellis et al. (1972) suggested that serotonin and melatonin may be involved in the normal regulation of testicular function in rats. Serotonin and melatonin were both found to non-competitively inhibit the 17α-hydroylase and 17α-hydroxy-pregnene-C-17,20 lyase activities. In addition it was observed that serotonin increased and melatonin reduced the 17β-hydroxysteroid dehydrogenase activity.

1.8 INFLUENCE OF AGE ON TESTICULAR STEROIDOGENESIS

A considerable body of evidence has accumulated showing that the testes of foetal and immature rats produce androgenic substances and that this capacity varies with the growth and differentiation of the Leydig cells both pre- and post-natally. In rats the Leydig cells rapidly multiply and differentiate from day 15 of foetal life (when the cells are clearly distinguished) until day 19 when the cells reach the climax of their quantitative development (Roosen-Runge & Anderson, 1959; Niemi & Ikonen, 1963). This phase is then followed by a drastic regression or de-differentiation of the Leydig cells until day 4 after birth, when the cells are virtually absent. From about the fourth post-natal week, concomitant with the appearance of the first spermatids, a second generation of Leydig cells differentiate and develop to the adult level by the third month (Roosen-Runge & Anderson 1959; Niemi & Ikonen, 1963, 1966; Clegg, 1966; Hitzeman, 1962; Niemi & Kormano, 1964). The appearance of this second generation of Leydig cells coincides with the secretion of gonadotrophins shortly before puberty (Niemi & Ikonen, 1961, 1962). In mouse only a single generation of Leydig cells has been reported to occur (Baillie, 1961, 1964).

1.8.1 Foetal testes

The influence of steroid androgens on mammalian sexual differentiation has been well documented by biological experiments (Jost, 1965; Price & Oritz, 1965; Goldman, 1970) and the presence of testosterone and other neutral steroids in foetal testis demonstrated (Attal, 1969; Huhtaniemi et al.

1970). Although there are some conflicting reports, in general, at about the time Leydig cells first appear, the foetal testes acquire certain steroidogenic enzymes and can convert pregnenolone to androgens - the predominant androgen being testosterone. During this period differentiation of the genital ducts occur. The presence of the enzymes required for the conversion of pregnenolone via progesterone to androstenedione and testosterone have been established by several investigators (Acevedo et al., 1963; Bloch, 1964, 1967; Lipsett & Tullner, 1965; Noumura et al., 1966; Serra et al., 1970). It has also recently been reported that rat foetal testes possess the enzymes necessary for de novo synthesis of testosterone from acetate (Warren et al., 1972, 1973; Serra et al., 1970).

In rat, the ability of foetal testes to metabolize radioactive acetate and radioactive pregnenolone to labelled testosterone could not be detected before 15.5 days of intrauterine existence (Warren et al., 1972, 1973; Bloch et al., 1971). However, testosterone formation from progesterone was demonstrated as early as 13.5 days of gestation (Noumura et al., 1966) and a small amount of pregnenolone conversion to progesterone at 14.5 days (Bloch et al., 1971). The observation that testes at 14.5 days of foetal age formed no radioactive testosterone when incubated in vitro with radioactive acetate or pregnenolone supported the lack of enzymes needed for testosterone biosynthesis from these precursors before 15.5 days of foetal life (Warren

et al., 1973) and suggested that the main precursors utilized in the production of androgens by 13.5-14.5 day foetal testes may not be acetate or pregnenolone but progesterone derived from maternal blood (Grota & Eik-Nes, 1967; Warren et al., 1973). From day 15 after gestation, the steroid biosynthetic activity as well as testicular testosterone levels in the foetal rat was observed to increase sharply and to reach a maximum on day 18. This activity was maintained at the same high level up to a few hours after birth, when a precipitous drop was recorded (Noumura et al., 1966; Warren et al., 1973). The factors responsible for regulating testosterone synthesis in the foetal testes are currently unknown but maternal or placental gonadotrophins are assumed to be involved.

1.8.2 Post-natal testes

Information in literature indicates that post-natal testes at different stages of development produce different steroids and show a characteristic change in the pattern of the enzymes. These changes appear to be a reflection of the post-natal regression and prepubertal redifferentiation of the Leydig cells.

In a systematic study using rat testes at different stages of development, Steinberger and Ficher (1968, 1969) demonstrated a biphasic character of the capacity of differentiating testes to convert progesterone to testosterone. A high rate of conversion, equal to and possibly higher than that obtained with testicular tissue from an adult animal,

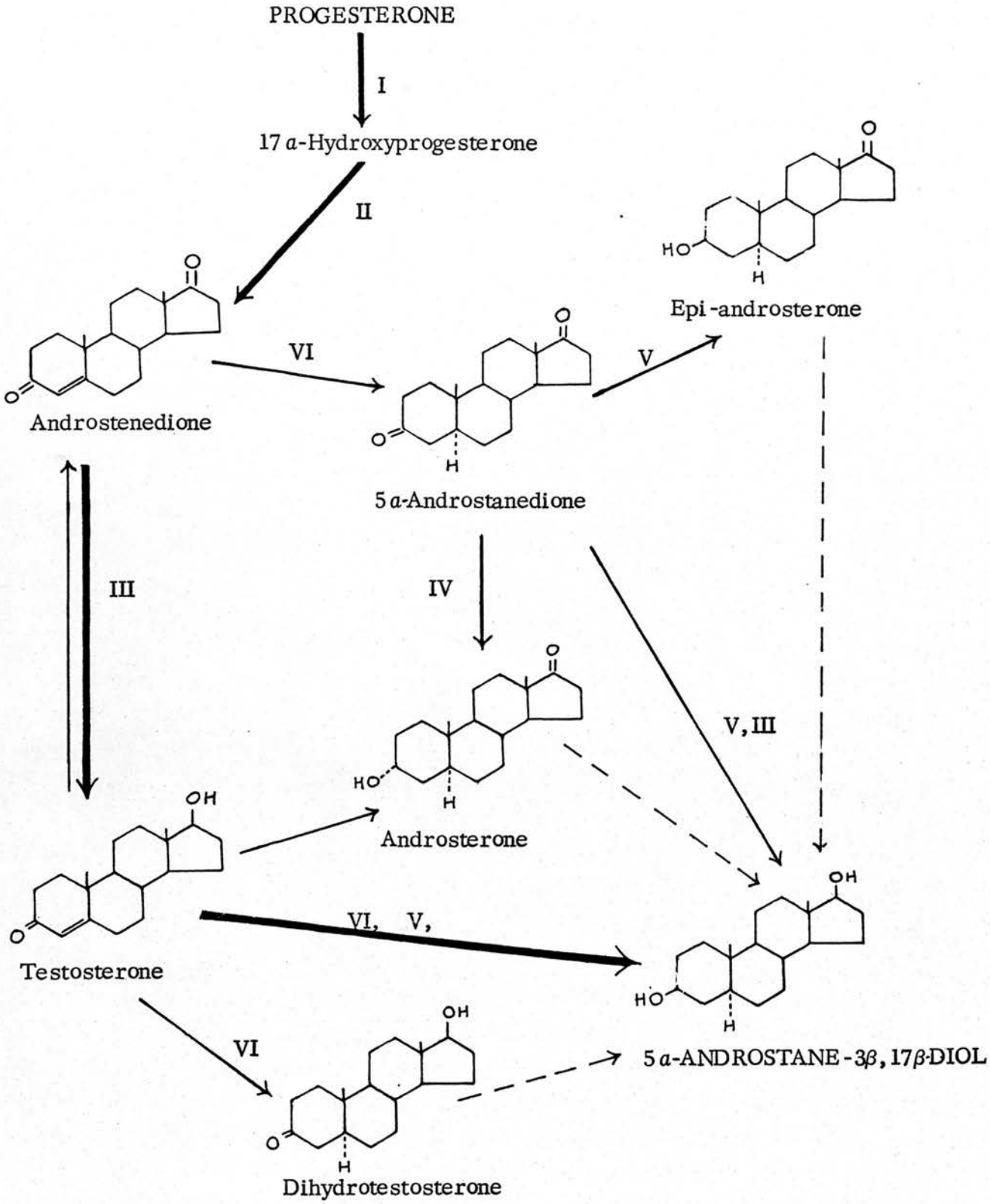


FIG. 1.9 POSTULATED PATHWAYS OF PROGESTERONE METABOLISM IN TESTICULAR TISSUE CHARACTERISTIC OF IMMATURE RATS

Thick arrows indicate the relatively major pathway of steroid conversion. All the enzymes involved were found to reside in the microsomal fraction of testes

I	17α-Hydroxylase
II	C-17,20 LYASE
III	17β-Hydroxysteroid dehydrogenase
IV	3α-Hydroxysteroid dehydrogenase
V	3β-Hydroxysteroid dehydrogenase
VI	5α-Reductase

was observed in testes from newborn rats (Steinberger & Ficher, 1968; Noumura et al., 1966). The rate diminished progressively as the testes developed reaching essentially zero at the age of 20 days. With further development, the capacity for testosterone synthesis gradually increased, reaching high levels in 90 days (Steinberger & Ficher, 1968; Inano et al., 1967c). Resko et al. (1968) and Knorr et al. (1970) found that testosterone levels in plasma and testicular tissue of rats followed a similar biphasic pattern.

The pattern of formation of androstenedione was similar to that of testosterone. However, androstenedione appeared earlier than testosterone in the course of sexual maturation and resulted in an androstenedione/testosterone ratio of greater than 1. As the testis developed, the conversion rate to testosterone increased, while androstenedione formation did not change significantly, causing a reversal in the ratio (Inano et al., 1967c; Hashimoto & Suzuki, 1968). Such a transition from androstenedione to testosterone dominance has also been observed in guinea pig (Snipes et al., 1965; Becker & Snipes, 1968) and bull (Lindner, 1961b; Lindner & Mann, 1960). The enzyme responsible for converting androstenedione to testosterone thus appears to arise late in the course of the differentiation of the Leydig cells in post-natal life.

The testes of 20-day-old rats, while not capable of accumulating testosterone, metabolized progesterone very efficiently to two major 5α-reduced C_{19} steroids, androsterone and androstanediol (Steinberger & Ficher, 1969; Coffey et al., 1971) (see Fig. 1.9). Androstanediol was reported to appear

earlier (at day 13) than androsterone (at day 17) (Ficher & Steinberger, 1971). A number of other investigators (Sylianou et al., 1961; Nayfeh et al., 1966; Ficher & Steinberger, 1968, 1971; Inano et al., 1967c; Yamada & Matsumoto, 1974) also demonstrated the conversion of suitable precursors to 5α-reduced androgens by testicular tissue from immature rats. As the capacity for converting progesterone to testosterone diminished with progressive maturation of the testes, the formation of androsterone gradually increased and reached a peak at the time when testosterone was detected in only trace amounts. As maturation of the testes progressed further and progesterone conversion to testosterone increased, the formation of the 5α-reduced C_{19} steroids diminished.

Hall et al. (1969) reported that in vitro conversion of cholesterol to androgens by isolated Leydig cells from testes of rats aged 20 days was less than with cells from mature animals.

Inano et al. (1967c) investigated the enzyme pattern of immature rat testes and observed that the concentration of 3β-hydroxysteroid dehydrogenase, 17α-hydroxylase, C-17,20-lyase and the 17β-hydroxysteroid dehydrogenase gradually increased between the ages of 20 and 60 days post-natal and levelled off at the time approaching maturity. In contrast, the 4-ene-5α-reductases (pregn-4-ene and androst-4-ene-5α-reductases sharply increased between days 20 and 30 and then dropped rapidly between days 40 and 60. This disappearance was observed to coincide with a sharp increase in the growth

rate of the seminal vesicles and prostate (Lindner, 1968). The predominance of the catabolic pathway (Δ^4-5-reductases) appears to be characteristic for certain stages in the development and maturation of the testis. Thus testicular tissue of post-natal rats of all ages contain the enzymes involved in the biosynthetic pathways from progesterone to androgens - a functional capacity acquired during foetal life and retained in post-natal life. The process of differentiation therefore appears to involve mainly induction or suppression of the ring A reductase system which regulates the accumulation and catabolism of testosterone, rather than with enzymes dealing with the synthesis of the androgen from progesterone.

The physiological significance of 5α-reductase activity during differentiation of the androgen biosynthetic pathways remains to be determined. The cell type containing the 5α-reductase system and the factors which regulate this enzymic activity are also not known. However, it has been suggested that the high serum FSH levels observed in the immature rats may play a role in the increased reductase activity (Coffey et al., 1971). Swerdloff et al. (1971) demonstrated that serum LH levels were only slightly lower and FSH levels two to three times higher in immature than in mature rats. FSH levels were maximal at 35 days of age and low at about 50 days of age corresponding with the decline in reductase activity. Recently, Rivarola and Podesta (1973) reported that androstanediol was the main in vitro metabolite of testosterone and was found in high concentrations only in the isolated seminiferous tubules of immature 20-26 day old rats but not in the mature ones.

1.9 AIMS OF PRESENT STUDY

Although the pathways related to the synthesis of androgens in testes have been demonstrated there is comparatively little information on the biochemistry of steroidogenesis. The first step in steroid biosynthesis, namely the hydroxylation of cholesterol, has been suggested to be the rate-limiting step and the site most likely to be affected by trophic hormones, hence special interest has been attached to this reaction. Many of the concepts are assumed to be similar to those of the adrenal gland or ovaries from which much of the thinking and speculation has evolved. The aim of the present study therefore was to investigate and characterise the properties of the cholesterol side-chain cleavage reaction in rat testes in the following way:

(1) To evolve a sensitive and quantitative method for the assay of the enzymatic activity.

(2) To re-investigate the subcellular localization, cofactor requirements and optimal conditions for cholesterol side-chain cleavage activity.

(3) To investigate whether cytochrome P450, the oxygen activating and substrate binding component of hydroxylation reactions is present in testes mitochondria and to establish whether this enzyme component participates in cholesterol side-chain cleavage activity.

(4) To attempt to establish whether the seminiferous tubules and Leydig cells are both capable of *de novo* steroid biosynthesis from cholesterol.

(5) To attempt to investigate the mode and site of action of gonadotrophin in the control of steroidogenesis.

CHAPTER 2

EXPERIMENTAL PROCEDURES

CONTENTS

EXPERIMENTAL PROCEDURES

CHAPTER 2

EXPERIMENTAL PROCEDURES

In this chapter the techniques which form the basis for much of the work presented in subsequent sections will be described.

2.1 TISSUE SOURCE

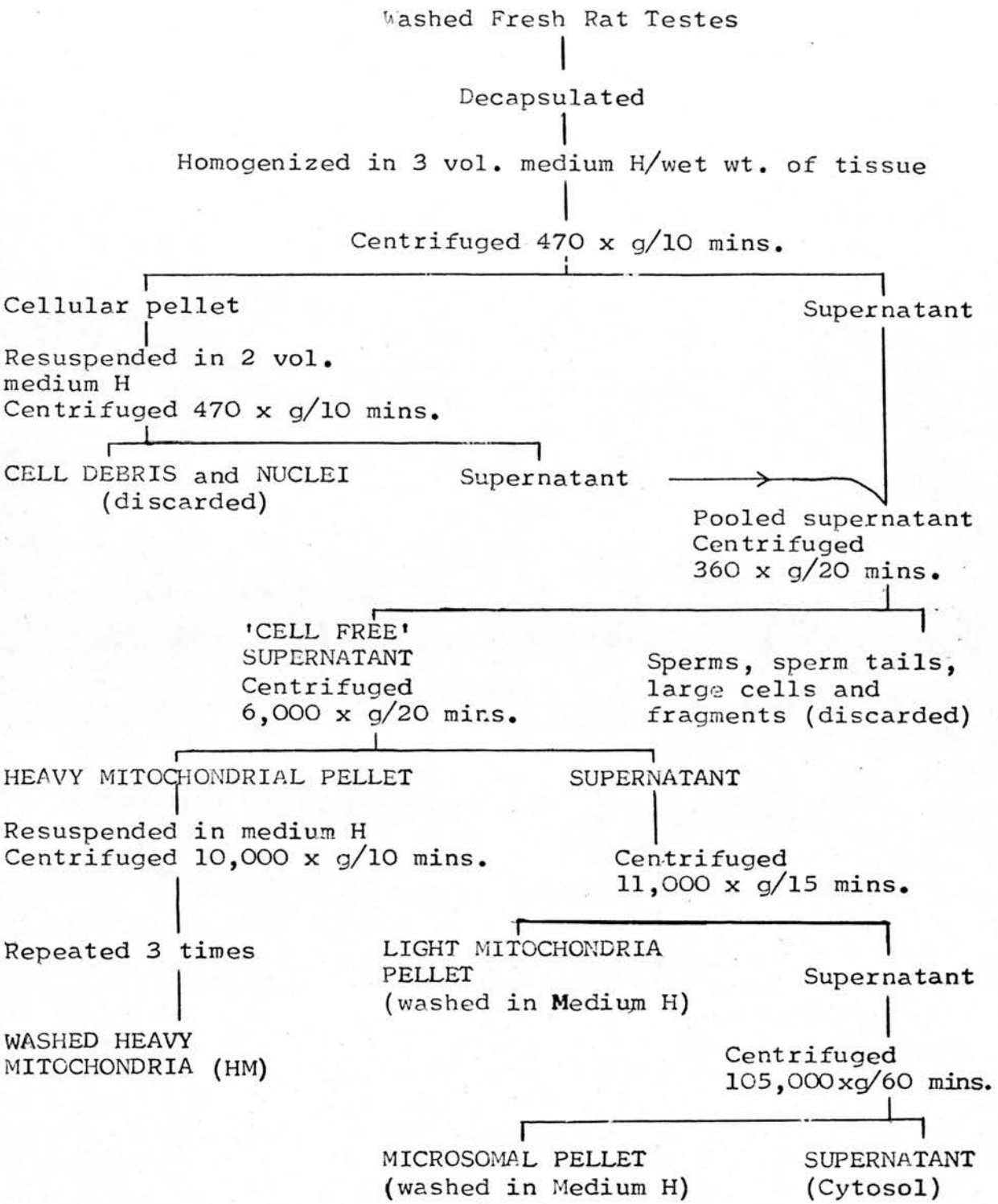
Testes from normal rats of the Wistar strain were used. The rats were bred in the Clinical Endocrinology Unit, Medical Research Council, Edinburgh. Unless otherwise stated, each experiment consisted of a group of 8 to 12 animals - all of the same age. The rats were killed by a blow on the head followed by cervical dislocation. The testes were then quickly removed via a ventral approach, dissected away from the adhering epididymus and superficial fat, and placed in ice cold isotonic KCl. Subsequent fractionation procedures were carried out at $4^{o}C$.

2.2 SUBCELLULAR FRACTIONATION

Gonads thus obtained within 20 minutes of the death of the animals were washed several times with isotonic KCl, (until free of adhering blood) and finally rinsed in homogenizing medium. The testes were weighed and the tunica albuginea (sac) and testicular artery carefully removed. The remaining tissue was then homogenized in three volumes (vol/gm wet weight) of medium consisting (unless stated otherwise) of 250 mM sucrose-0.2 mM EDTA and 10 mM Tris (final pH 7.4) (Medium H). The homogenization was carried out using a loose-fitting Potter-Elvehjem type teflon pestle - glass homogenizer. The teflon pestle was motor driven and the glass homogenizer kept on ice to avoid

TABLE 2.1

CELL FRACTIONATION PROCEDURE



(All fractionation procedures were carried out at 4°C)

heating. No more than 8 passes of the pestle were made. This crude homogenate was then subjected to differential centrifugation as outlined in Table 2.1. The pink homogenate was centrifuged at 470 x g av. for 10 minutes in an M.S.E. High Speed 18 centrifuge to sediment the nuclei, seminiferous tubular fragments, unbroken cells, cell debris and sperms. The pale supernatant was carefully decanted and retained while the pellet was re-suspended in 2 volumes of medium H and centrifuged again at 470 x g for 10 minutes. The resulting pellet was discarded, and the supernatant was combined with that of the first centrifugation and recentrifuged at 360 x g for 20 minutes. This low speed centrifugation was necessary to remove most of the remaining spermatids, sperm tails and heads and other cellular fragments. This 'cell and fragment free' supernatant was carefully removed with a Pasteur pipette and then centrifuged at 6,000 x g for 20 minutes to sediment the 'heavy mitochondria' (HM). The thin white film at the top of the tube was first aspirated off and the inner walls wiped. The supernatant fluid was decanted and retained, while the packed light brown heavy mitochondrial pellet was washed three times by re-suspending in medium H (with gentle strokes by hand in a loose-fitting Teflon-glass homogenizer) and then resedimented at 10,000 x g for 10 minutes. This washed heavy mitochondria was suspended in Medium H giving a protein concentration of between 8 to 10 mg/ml. The light mitochondria (probably consisting of mitochondria, broken mitochondria and heavy microsomes) was separated from the 6,000 x g supernatant by centrifuging at 11,000 x g for 15 minutes. The resulting

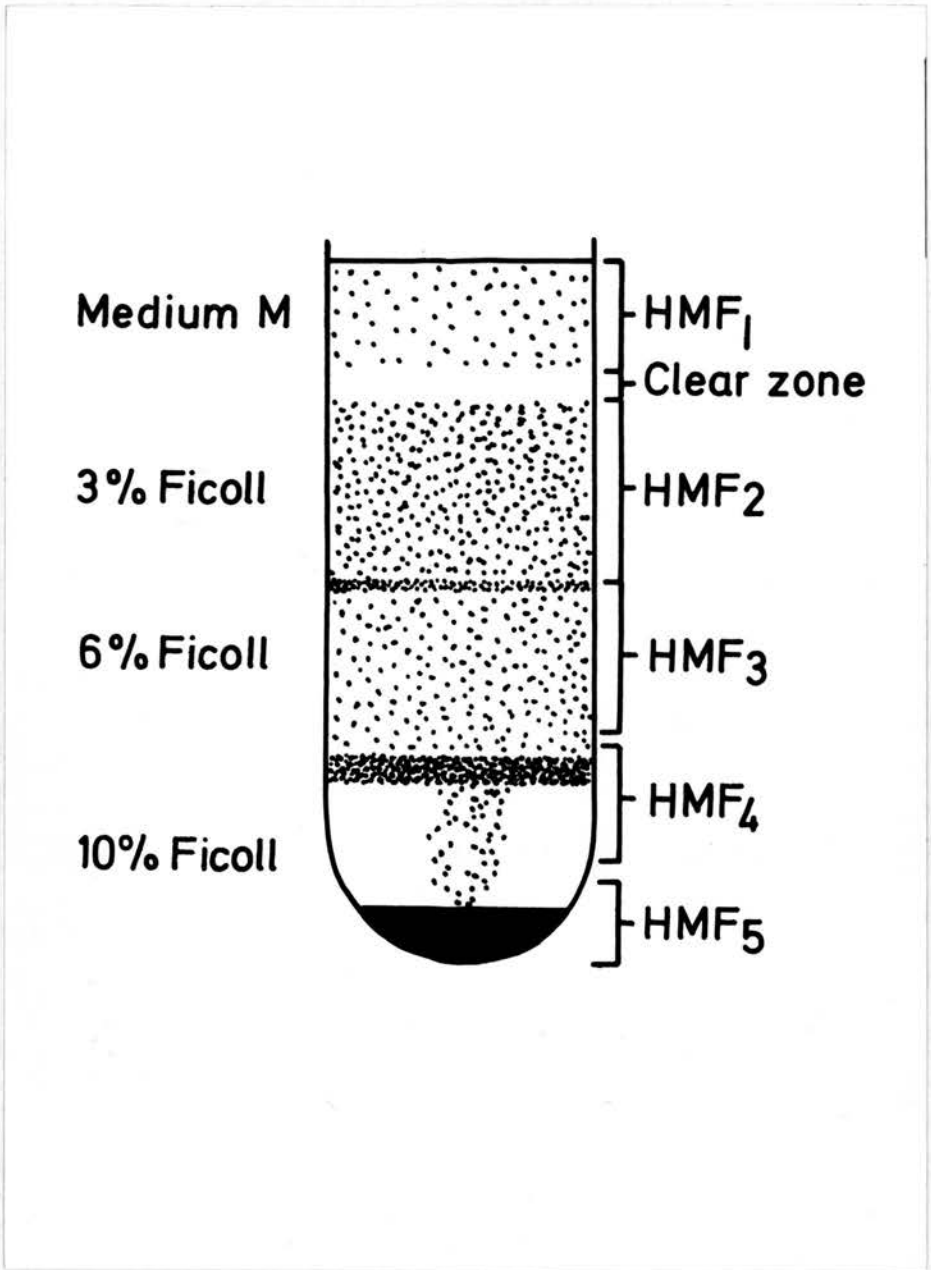


FIGURE 2.1 Diagrammatic representation of the fractionation achieved after centrifugation of crude heavy mitochondria on a discontinuous 3-10% ficoll density gradient.

supernatant was removed and further centrifuged at 105,000 x g in a Beckman Ultracentrifuge for 1 hour, yielding a white microsomal pellet and pale pink supernatant (cytosol). Both the light mitochondrial and microsomal fractions were washed once and suspended in medium H. This subcellular fractionation procedure was carried out between 0 to 5°C.

2.3 FURTHER FRACTIONATION OF HEAVY MITOCHONDRIA

In an attempt to obtain a clean homogeneous mitochondrial preparation rich in cholesterol side-chain cleavage activity, the crude heavy mitochondrial fraction obtained by differential centrifugation (section 2.2), was subjected to density gradient centrifugation on ficoll. The following operations were carried out at 4°C.

Using a 60 ml centrifuge tube, 8 to 10 ml of the heavy mitochondrial fraction suspended in medium H, was carefully layered over a discontinuous ficoll density gradient consisting of 14 ml each of 10% (bottom), 6% (middle) and 3% ficoll (top): the ficoll was dissolved in medium H in order to maintain isotonicity. After centrifugation at 10,000 x g for 1 hour in a Beckman swing-out rotor (Model S.W. 25.2) five clearly discernible particulate fractions were obtained. These heavy mitochondrial sub-fractions (HMF) were designated, as indicated in Figure 2.1 (in order, from the top) as:-

HMF 1 - a white light turbid layer corresponding to medium H and separated by a thin clear layer from

HMF 2 - a broad white dense turbid layer corresponding to 3% ficoll.

HMF 3 - a broad yellowish brown layer corresponding to 6% ficoll.

HMF 4 - a floating brownish narrow band at the interface between the 6% and the almost clear 10% ficoll layers, and

HMF 5 - the dark brown hard packed pellet at the bottom of the tube.

These fractions were carefully removed separately with the aid of a Pasteur pipette and retained. All the fractions were diluted with, or suspended in medium H and then centrifuged at 12,000 x g in an M.S.E. High Speed 18 centrifuge for 15 minutes. This process was repeated once more to wash off the ficoll. The final pellets of the different fractions were again suspended in medium H.

It was observed (see Chapter 3 section 3) that most of the cholesterol side-chain cleavage activity occurred in fractions 4 and 5. However, only fraction 5 was collected routinely and used for the studies on cholesterol side-chain cleavage and related aspects of testes metabolism.

Ficoll - a nonionic synthetic high polymer of sucrose and epichlorohydrin (Mol. wt. 400,000 ± 100,000) was chosen for the fractionation of heavy mitochondria because it provides high density solutions of low viscosity and osmotic pressure.

2.4 DETERMINATION OF CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY

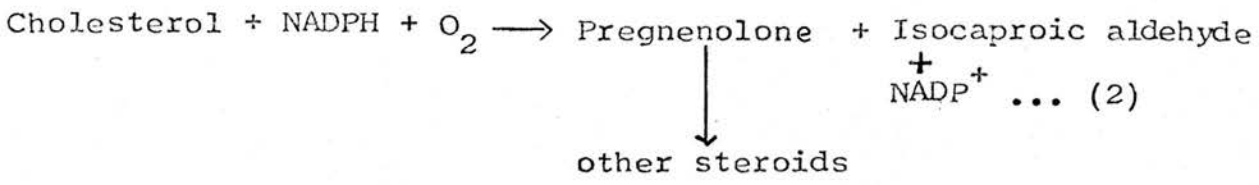
THE BASIS OF THE ASSAY

The reaction being studied can be written simply as follows:-

TABLE 2.2

COMPOSITION OF ROUTINELY USED INCUBATION MEDIA

	Final Molarity (mM)
Medium A	
Tris buffer pH 7.5	60
Magnesium chloride	10
Medium B	
Sucrose	100
Tris buffer pH 7.5	50
Potassium chloride	20
Potassium phosphate buffer, pH 7.5	10
EDTA (pH 7.0)	0.3
Bovine serum albumin (fatty acid free)	0.15% (wt/vol)
Electron Source	
1. DL Isocitrate	5
2. Extra-Mitochondrial NADPH Generating System	
$NADP^+$	5
Glucose-6-phosphate	40
Glucose-6-phosphate dehydrogenase	0.2 I.U.



This reaction can be followed by measuring the conversion of cholesterol either to the steroid products or to isocaproaldehyde. The enzymic assay used throughout this study was based on the conversion of added [4-^{14}C] cholesterol to [4-^{14}C] steroid products, the electrons being supplied either by isocitrate or extramitochondrial NADPH generating system (see Table 2.2). The activity was therefore expressed as the percentage conversion of [4-^{14}C] cholesterol to the labelled steroid products.

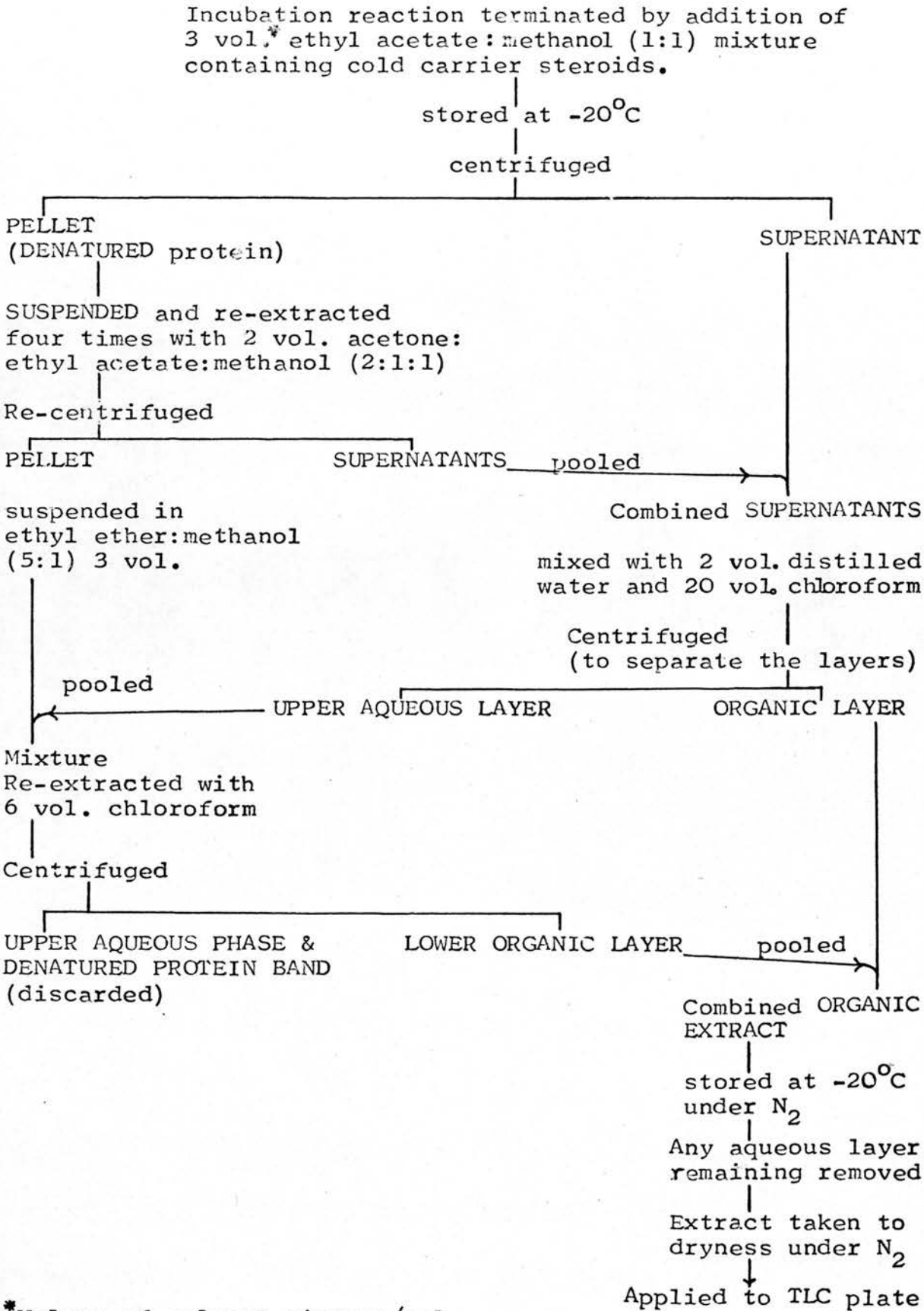
2.4.1 Incubation procedure

Immediately before use aliquots of [4-^{14}C] cholesterol (58 μCi/mg) were subjected to thin-layer chromatography on silica gel G using di-isopropyl ether:petroleum ether (boiling point 60-80°C):acetic acid (75:35:2 by vol.) as solvent system to remove any auto-oxidation products. The radio-active area corresponding to the authentic reference cholesterol spot was eluted from the plate with redistilled acetone.

Heavy mitochondria (0.5 mg protein) were incubated in a final volume of 200 μl (in 10 x 75 mm test tubes) in either medium A or B (Table 2.2) together with either DL isocitrate 5 mM or an extra-mitochondrial NADPH generating system ($NADP^+$ 5 mM, glucose-6-phosphate 40 mM and glucose-6-phosphate dehydrogenase 0.2 I.U.) as electron source. The reaction was initiated by the addition of 200,000 cpm of [4-^{14}C] cholesterol

TABLE 2.3

EXTRACTION PROCEDURE



*Volume of solvent mixture/vol. of incubation.

(approx. 2 µg) in 2 µl acetone. Incubations were carried out 37°C in an atmosphere of air using a Dubnoff incubator with constant shaking. The period of incubation varied as indicated in the text but was usually 1.5 or 2 hours. The enzymic reaction was terminated by the addition of 600 µl of ethyl-acetate: methanol (1:1) containing 3 µg each of the following carrier standard steroids and sterols:- pregnenolone, dehydro-epiandrosterone (DHA), androstenediol, progesterone, 17α-hydroxy-pregnenolone, androstenedione, testosterone, 17α-hydroxy-progesterone, cholesterol, 20α-hydroxycholesterol and cholesterol oleate (representing cholesteryl esters). Tubes were sealed and kept at -20°C until extraction. Control samples were complete reaction mixtures either stopped at zero time or incubated with a boiled enzyme fraction (i.e. enzymic preparation denatured by placing the tubes in a water bath for 2 minutes).

Unless stated otherwise, all incubations were routinely carried out in duplicate.

2.4.2 Extraction procedure

The extraction procedure is outlined in Table 2.3. The denatured protein precipitate was sedimented using a bench centrifuge. The clear supernatant was removed with a fine tipped Pasteur pipette and reserved in a glass stoppered conical centrifuge tube. The pellet was broken up and extracted 4 times with 400 µl of hot (approx. 50°C) solvent mixture consisting of acetone: ethyl acetate: methanol (2:1:1). The supernatants were combined, 400 µl distilled water added mixed and then extracted with 4 ml chloroform. After a thorough mixing, the layers were separated by centrifugation. The upper aqueous

and any remaining precipitate at the interface was carefully removed and transferred to the original incubation tube which contained the final pellet now suspended in 600 µl ethyl ether: methanol (5:1), and mixed. This mixture was re-extracted (twice) with 1.5 ml of chloroform and centrifuged. The lower organic layer was removed with a Pasteur pipette and pooled with the main organic fraction in the conical centrifuge tube and stored under nitrogen at -20°C. The remaining upper aqueous layer and denatured protein band were discarded. Just before thin layer chromatography, any remaining aqueous layer was removed with a fine tipped Pasteur pipette and the organic extract taken to dryness under a stream of nitrogen at 45°C.

This entire extraction procedure was carried out in two tubes - the original incubation tube and the extraction conical glass centrifuge tube. No significant amount of material was lost during the procedure. Recoveries could be monitored at each stage and any radioactivity remaining re-extracted if necessary. Under these conditions recoveries were complete (see Table 2.6).

2.4.3 Thin layer chromatography of organic extract

To separate the steroids, thin layer chromatography was performed at room temperature (21-23°C). The inner walls of the TLC chamber were lined with filter paper to increase saturation for a more even migration of steroids and solvent across the plates. Thin layer glass plates (5 x 20 cm or 10 x 20 cm) precoated with silica gel F_{254}, layer thickness 0.25 mm were used. The extracts were dissolved twice successively in each of the following solvents: a) chloroform

b) chloroform : methanol (2:1) c) acetone : ethyl acetate : methanol (2:1:1) and d) ethyl ether : chloroform : methanol (2:2:1), and spotted on the TLC plate 1.5 cm from the bottom and near the left hand edge (i.e. plates were divided into two vertical lanes). Non-radioactive steroid standards were applied at the other edge (right hand) of the same plate. The chromatoplates were developed in di-isopropyl ether : petroleum spirit (b.pt. 60-80°C) : acetic acid (80:35:2 v/v), three or four times (as indicated) in the same direction with air drying for one minute between each run.

After chromatography, the 3-oxo-4-ene steroids were located under 254 nm U.V. light and marked. Then the side with the radioactivity was masked off while the opposite edge of the plate bearing the reference steroids was visualized by spraying with a freshly prepared mixture of sulfuric acid : methanol (2:1 v/v). The heat generated by this solution was sufficient to ensure colour development without warming at 110°C. All radioactive peaks were detected by scanning the plates under a Panax windowless, gas flow, Geiger-Muller type radiochromatogram scanner (Panax Equipment Ltd., System E.0111/P7900A). A carrier gas mixture of 98% argon and 2% propane was employed.

It was initially observed that lipid like material in testicular extracts from large incubations of 1.0 ml or more, interfered with the spotting and mobility of steroids on the precoated chromatoplates. Several preliminary attempts to remove the interfering material by treating the extracts with 1% Na_2CO_3, petroleum ether (40-60°C) and 80% ethanol, ethanolic

TABLE 2.4

EFFECT OF SILICA GEL F254 ON RADIOACTIVE COUNTING EFFICIENCY

	EXPERIMENT		
Exptl. Set	A No Silica gel	B Silica-gel $6cm^2$	C Spotted plate scraped
1	5,311 ± 50	5,232 ± 145	5,296 ± 81
2	11,798 ± 102	11,785 ± 99	11,675 ± 130
3	49,331 ± 110	49,403 ± 189	49,469 ± 95
4	105,096 ± 111	104,920 ± 160	104,898 ± 405
5	150,728 ± 257	150,802 ± 210	150,698 ± 380

Five sets of estimations were performed with different amounts of [4-^{14}C] cholesterol as indicated. Each set containing the same quantity of radioactivity throughout the different experiments.

In experiment A [4-^{14}C] cholesterol was added directly into scintillation vials and counted

B Silica gel equivalent to $6cm^2$ from the TLC plate was added to vials containing [4-^{14}C] cholesterol and counted.

C [4-^{14}C] cholesterol was spotted on TLC plates and silica gel then scraped into vials and counted by liquid scintillation spectrometry.

TLC plates were obtained from E. Merck, Darmstadt, Germany, precoated with silica-gel F254 (layer thickness C.25 mm).

The mean values represent the averages of six samples per set ± standard deviation of this mean.

$MgCl_2$ or column chromatography were not satisfactory. These procedures all resulted in loss of radioactive compounds (20-30%). This problem was overcome by the use of the micro-incubation system of 200 µl final volume described above. No further preliminary purification of the extracts was necessary.

2.4.4 Measurement of radioactivity

TLC plates were lightly sprayed with water (this allows the gel to be scraped off as a flake rather than powder) and the radioactive zones corresponding to the radioactive peaks and standard steroids were scraped directly into vials, dried in a warm oven and then suspended in 10 ml of scintillation fluid consisting of 4 gm 2,5-diphenyloxazole (PPO) and 30 mg 1,4-bis-(5-phenyloxazoly-2-)-benzene dissolved in 1 litre of dry toluene containing 2% redistilled methanol. The radio-activity of the samples were assayed in a Packard Tricarb liquid scintillation spectrometer, model 314-Ex. Samples were counted until the standard deviation of the counts obtained was below 1% of the mean. Under the conditions employed, the counting efficiency for the ^{14}C isotope was 82%.

It was ascertained that up to 6 cm^2 of silica gel could be added to the scintillation solution without impairing the counting efficiency as shown in Table 2.4. These results confirm previous observations of Mitton (1967) with silica gel H and Robinson (1971) with silica gel G. In routine assays the area of silica gel scraped into each vial did not exceed 3 cm^2.

TABLE 2.5

RECOVERY OF RADIOACTIVITY THROUGHOUT THE EXPERIMENTAL PROCEDURE

Steroids	A Radioactivity Added		B Radioactivity Recovered from TLC plates	
	c p m mean ± SD	% of total cpm added	c p m mean ± SD	% of total cpm recovered
[4-^{14}C] Cholesterol	181,578 ± 154	92.71	179,006 ± 351	92.70
[4-^{14}C] Progesterone	4,916 ± 80	2.51	4,847 ± 101	2.51
[4-^{14}C] Pregnenolone	2,605 ± 20	1.33	2,588 ± 45	1.34
[4-^{14}C] DHA	1,606 ± 25	0.82	1,560 ± 93	0.81
[4-^{14}C] Testosterone	2,997 ± 42	1.53	2,935 ± 28	1.52
[4-^{14}C] Cholesterol oleate	2,154 ± 15	1.10	2,163 ± 30	1.12
TOTAL	195,856 ± 326	100.00	193,103 ± 446	100.00

% of total cpm are based on the mean cpm

cpm are mean ± SD of six determinations.

2.4.5 Recovery of [4-^{14}C] products throughout the experimental procedure

This was ascertained by adding the [4-^{14}C] steroids and sterols listed in Table 2.5A, to a series of six incubation tubes containing medium B and heavy mitochondria. Further metabolism of these added compounds was prevented by the immediate addition of ethyl acetate:methanol (1:1) and the 'incubates' then subjected to the same procedure previously described for extraction, TLC, scanning and counting.

At different stages of the extraction procedure (Table 2.6) samples were taken and their radioactivity estimated by liquid scintillation counting. Aqueous layers were taken to dryness and the protein pellets digested with 500 µl soluene (Packard Instruments Co.) before the addition of scintillator fluid. The results in Table 2.6 show that recovery after extraction was greater than 95%. Table 2.5B shows that the ratio of labelled compounds estimated after TLC and scintillation counting were similar to that added initially. This indicates that when losses did occur they would not affect the calculations of the cholesterol side-chain cleavage activity when this was expressed as the percentage of [4-^{14}C] cholesterol converted to [4-^{14}C] steroid products. Most of the loss which could occur could be due to the material being left in the centrifuge tube when the plates were spotted.

2.5 PROCEDURES USED IN THE IDENTIFICATION OF STEROID PRODUCTS

2.5.1 Chemical conversion

A. OXIDATION: To the dried steroid extract was added 0.5 ml of a 5% solution (w/v) of chromic acid in glacial acetic acid.

T A B L E 2 . 6

RECOVERY OF RADIOACTIVITY THROUGHOUT THE EXPERIMENTAL PROCEDURE

RADIOACTIVITY RECOVERED	c p m mean ± SD	% of Total cpm added
1. During Extraction Procedure		
In extracted pellet	4,504 ± 500	2.3
In organic layer	190,758 ± 300	97.4
In upper aqueous layer	509 ± 100	0.3
In combined pellet and aqueous layer after re-extraction	606 ± 90	0.3
In final organic extract	195,055 ± 450	99.6
2. After spotting, TLC	193,104 ± 108	98.6

cpm are mean and SD of six determinations

% of total cpm are based on the mean cpm

After 4 hours at room temperature 2 ml of water was added and the steroid extracted twice with 4 ml chloroform:ether (1:1). The washed extracts were pooled, concentrated and spotted on TLC plates.

B. REDUCTION: The steroid samples dissolved in 500 µl methanol was incubated with 5 mg sodium borohydride for 1 hour. 1.0 ml water was then added followed by a few drops of hydrochloric acid to decompose the unreacted borohydride. The steroids were extracted with chloroform and the extracts washed twice with water before being submitted to TLC.

C. ACETYLATION: 200 µl of a mixture of pyridine-acetic anhydride (1:1 v/v) was added to the sample of the dry steroids. After an overnight incubation at room temperature under N_2, the reaction was terminated by the addition of 200 µl methanol. This mixture was evaporated to dryness under a stream of nitrogen. The steroids were then extracted with chloroform: methanol (3:1) and submitted to TLC.

2.5.2 Enzymatic conversion

Steroids were incubated with a steroid-3β-ol dehydrogenase preparation from Pseudomonas testosteroni and an excess of NAD^+ for 30 minutes at 37°C. The incubation system consisted of 200 µl Tris buffer pH 7.5 (50 mM), 50 µl of steroid 3β-ol dehydrogenase and 10 µl NAD^+ (2 mM). Steroids were added in acetone (3 µl). Reaction was terminated by the addition of methanol:acetone (1:2) and the steroids extracted as previously described (section 2.4.2).

Steroid 3β-ol dehydrogenase was prepared by homogenizing 50 mg of dried cell preparation of Pseudomonas testosteroni

(Type I) in 5 ml of 20 mM Tris buffer, pH 7.5. After 1 hour at 5°C, the suspension was centrifuged at 20,000 x g for 10 minutes and the supernatant used without further purification (Marcus & Talalay, 1956).

To check for recoveries in all the above studies, controls were run using authentic [4-^{14}C] steroids diluted with cold steroid standards treated identically as the extracted radioactive compound under investigation.

2.6 ASSAY FOR 3β-HYDROXYSTEROID DEHYDROGENASE ACTIVITY

The assay procedure was essentially the same as that described for cholesterol side-chain cleavage except that [4-^{14}C] pregnenolone (specific activity 55.7 mc/mM) was used instead of [4-^{14}C] cholesterol as substrate. The TLC plates were usually developed four times. Activity is expressed as the percentage conversion of [4-^{14}C] pregnenolone to radioactive steroid products which are progesterone, 17α-hydroxyprogesterone, androstenedione, and testosterone.

Before use [4-^{14}C] pregnenolone was purified by TLC in solvent systems VII and VI (see Table 3.4, page 69). The radioactive area corresponding to the authentic reference spot was eluted from the silica-gel with redistilled acetone.

2.7 ASSAY FOR CHOLESTEROL ESTERASE (HYDROLYTIC) ACTIVITY

When this activity was determined in heavy mitochondria the assay conditions were exactly the same as for cholesterol side-chain cleavage, except that [4-^{14}C] cholesterol oleate

(specific radioactivity 46 μCi/mg) was used instead of [4-^{14}C] cholesterol. TLC plates were developed twice. [4-^{14}C]Cholesterol oleate was purified before use by TLC in a manner similar to that described for [4-^{14}C] cholesterol (see section 2.4.1).

2.8 DETERMINATION OF ENDOGENOUS FREE AND ESTERIFIED CHOLESTEROL

2.8.1 Extraction and separation of cholesterol and cholesterol esters.

Testicular fractions equivalent to 4 mg protein were extracted as described for steroids (section 2.4.1). [4-^{14}C] Cholesterol (5,000 cpm approx. 0.01 μg) and [4-^{14}C] cholesterol oleate (5,000 cpm approx. 0.01 μg) were added as internal standards (for recovery corrections and as markers) just before extraction. For TLC separation, plates were prepared with silica gel G without binder (a slurry of silica gel G in distilled water was spread on glass plates and then activated at about 110°C for 1 hour). The plates were developed twice in the di-isopropyl ether solvent system (see section 2.4.2).

The zones containing the radioactive peaks corresponding to cholesterol (near the middle of the plate) and cholesterol oleate (solvent front) were scraped from the glass plate. The silica gel powder thus obtained was packed into small columns over glass wool and the sterols eluted successively with 15 ml each of mixtures of chloroform:ethyl acetate:methanol (3:1:1) and ethyl ether:methanol (3:1). The eluted samples were taken to dryness under nitrogen.

TABLE 2.7

GLC SEPARATION OF STEROIDS ON 3% QFI

COMPOUNDS	Retention (minutes)
7α-Hydroxycholesterol-3,7-diacetate	7
Pregnenolone acetate	17
Pregnenolone	11
DHA	9
Cholesterol	15
3β,5α-Cholestanol	16

The conditions for GLC are described in the text

3β,5α-cholestanol: 5α-cholestan-3β-ol

2.8.2 Hydrolysis of cholesterol esters

Hydrolysis of cholesterol esters was achieved by the procedure described below. To the samples of cholesterol ester dissolved in 1 ml acetone: methanol (1:1), 50 µl saturated KOH solution in 50% methanol was added and the tube incubated at 37°C for 2 hours. The solution was then neutralised with HCl and extracted with 4 ml chloroform, twice. The chloroform extracts were washed three times with 1 ml water and then evaporated to dryness under nitrogen. This hydrolyzed material was purified by TLC and eluted from absorbant as described above. Under these conditions 90-95% of cholesterol esters were hydrolyzed.

Both samples of cholesterol (i.e. free cholesterol and that obtained after hydrolysis of ester) were dissolved in redistilled acetone. Small aliquots were taken for radioactive measurements by liquid scintillation spectrometry to check for recoveries during the above extraction procedures. (Final recoveries of 93-96% were obtained.) To the remainder of the sample, dehydro-epiandrosterone was added as an internal standard to correct for losses during gas-liquid chromatography. Suitable aliquots of each sample were injected into the gas-liquid chromatography column, described below.

2.8.3 Gas liquid chromatography

Gas liquid chromatography was carried out on a Pye 104 gas chromatograph equipped with a hydrogen flame ionization detector. A glass column silanized with dichloro-dimethyl silane, 1.75 metres long and 4 mm i.d. containing 3% QF1 as

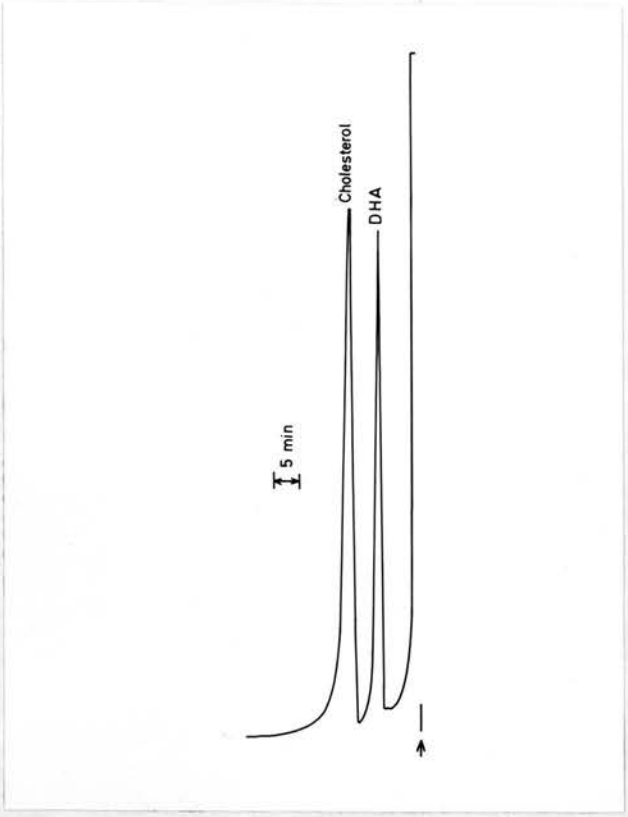


FIGURE 2.2 A typical gas liquid chromatogram tracing showing the separation of cholesterol and dehydroepiandrosterone. Column: 1.75 metres x 4 mm I.D. glass packed with 3% QF1 on 100-200 mesh Gas Chrom Q. Column temperature: 240°C. N_2 flow rate 30 ml/min at 30 lb/sq.in. A standard mixture containing cholesterol (1.6 µg) and DHA (0.8 µg) in 4 µl of acetone was injected onto the column. (Actual weight ratio 2:1.) Arrow indicates the point of injection of the sample mixture. The observed peak height ratio of cholesterol/DHA = 1.06. Detector: flame ionization at 2 x 10^{-10} AFS.

stationary phase with 100-200 mesh Gas Chrom Q (Applied Sciences Lab. Inc., Pennsylvania, U.S.A.) as column support was employed. The column oven temperature was kept at 240°C, and the injection port temperature 300°C. High purity nitrogen was used as carrier gas at a flow rate of 30 ml/min. Gas chromatographic retention times were measured from the initial excursion of the recorder pen upon injection of the sample to the maximum pen response of the eluted sample component. The retention times of dehydro-epiandrosterone was 9 minutes and of cholesterol 15 minutes (Table 2.7). A typical gas chromatographic tracing is shown in Figure 2.2.

Solutions made up of known ratios of cholesterol and DHA were subjected to gas liquid chromatography and the ratios of the observed peak heights calculated. Figure 2.3 shows a typical calibration curve produced from known ratios by weight of cholesterol and DHA. To measure the amount of cholesterol in an unknown sample, known amounts of DHA were added to the sample and the actual amount of cholesterol present determined from the peak height ratios obtained. 2 µg cholesterol and 1.3 µg DHA produced a full scale deflection on the gas chromatographic trace.

2.9 PROTEIN ESTIMATION

The amount of protein in the subcellular fractions were routinely determined by the method of Lowry et al. (1951) using a solution of bovine plasma albumin (fraction V) as standard.

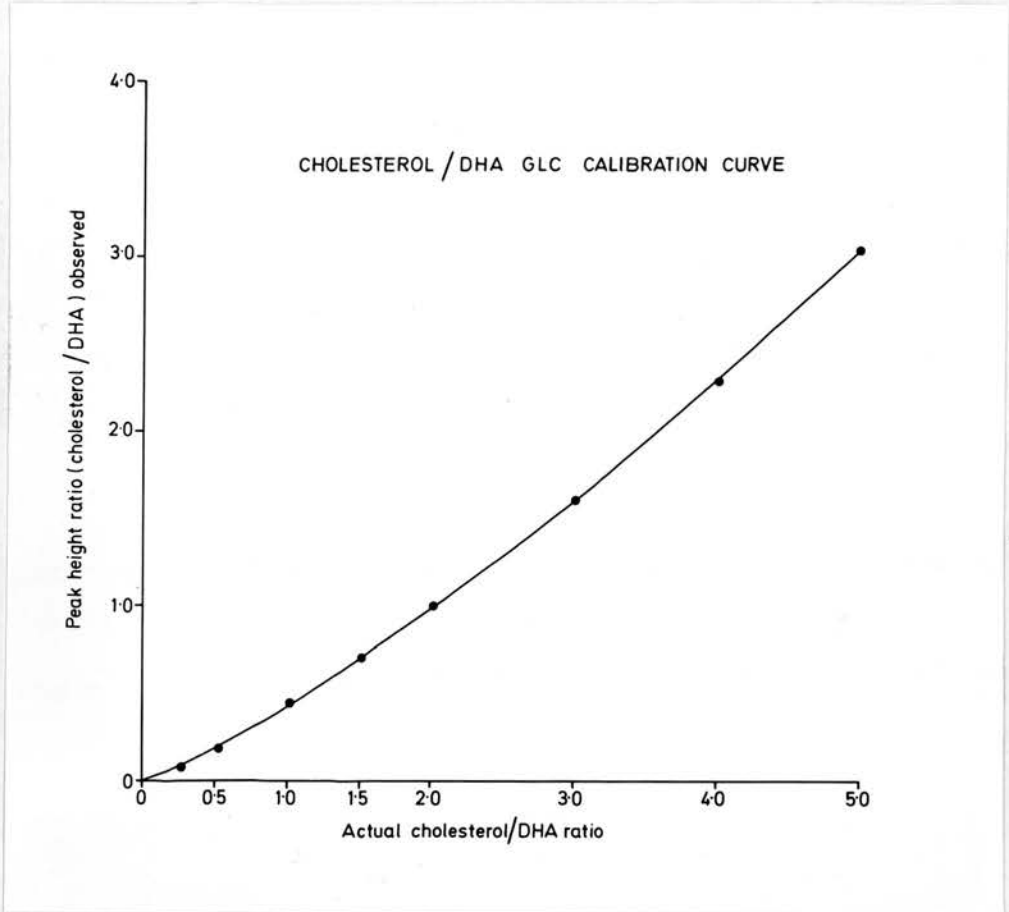


FIGURE 2.3 A typical gas liquid chromatographic calibration curve constructed for assay of cholesterol on 3% QF1. The actual weight ratio of cholesterol/ DHA (the internal standard) in the solution is plotted against the ratio of the observed heights of the peaks of the steroids produced on GLC.

2.10 DETERMINATION OF RESPIRATORY CONTROL AND ADP:O RATIOS

Oxygen uptake was determined essentially as described by Estabrook (1967). The apparatus consisted of a Clark membrane-covered oxygen electrode inserted into a closed lucite chamber designed to exclude air. The polarographic measurements were performed at 30°C in a magnetically stirred solution which consisted of 100 mM sucrose, 20 mM KCl and 25 mM Tris buffer pH 7.5 (Basic Incubation Medium). In addition to this basic incubation medium variable amounts of potassium phosphate, ethylene glycol-bis-(β-aminoethyl ether)-N_2N^1 tetra acetic acid (EGTA), ethylene diamine tetra acetic acid (EDTA), $MgCl_2$ and bovine serum albumin (free of fatty acid) were added as indicated in the text or legends to tables and figures. Mitochondrial protein concentration was 1-2 mg. The final volume of the medium was 3.0 ml. The substrate, unless otherwise stated was 5 mM succinate. The concentration of ADP employed was either 0.083 mM or 0.166 mM. These additions were made in 5-10 µl aliquots. The respiratory control ratios (RCR) were calculated as described by Chance and Hess (1959) and ADP/O ratios determined as defined by Chance and Williams (1955b).

Each data point shown in figures and tables represents the mean of at least two determinations. Each experiment was repeated twice with different groups of rats all of the same age.

The oxygen content of the complete incubation medium H was calibrated by using sub-mitochondrial particles supplemented with limiting amounts of NADH as recommended by Estabrook (1967) and was found to be 243 µM O_2 at 30°C.

The oxygen consumed by the electrode itself over an average experimental period of 15-20 minutes was less than 1% of the total oxygen present in the 3.0 ml reaction medium. Back diffusion of oxygen from the atmosphere into the solution was negligible and the amount of oxygen introduced in the 5-10 µl additions of substrate or ADP was small enough to be ignored.

SUMMARY

1. A method for the isolation of rat testicular mitochondria which is essentially free of sperms and sperm fragments is described.

2. A procedure for the fractionation and purification of the heavy mitochondria on a discontinuous ficoll density gradient was developed.

3. A micro-procedure for the assay of the following enzymatic activities are presented:-

(a) Cholesterol side-chain cleavage activity based on the conversion of [4-^{14}C] cholesterol to [4-^{14}C] steroid products.

(b) Steroid 3β-hydroxysteroid dehydrogenase activity based on the conversion of [4-^{14}C] pregnenolone to labelled steroids.

(c) Cholesterol esterase activity based on the enzymatic hydrolysis of [4-^{14}C] cholesterol oleate to [4-^{14}C] cholesterol.

4. The procedures for incubation of the enzyme source with the radioactive substrate; the quantitative extraction of the resulting labelled products and their subsequent separation by TLC on a single plate are described.

5. Endogenous free and esterified cholesterol were determined by GLC using a column containing QF1.

6. The use of the Clark electrode to measure respiratory control and ADP:O ratios is also described.

CHAPTER 3

PRELIMINARY STUDIES ON THE CHOLESTEROL SIDE-CHAIN CLEAVAGE ENZYME SYSTEM

CONTENTS

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In this section the subcellular distribution of the cholesterol side-chain cleavage enzyme complex and the nature and characteristics of the organelles involved will be presented. In addition the development of the systems used for the separation of the steroids formed from cholesterol during *in vitro* incubations will be discussed.

3.1 SUBCELLULAR DISTRIBUTION OF THE ENZYME ACTIVITY

The subcellular distribution of cholesterol side-chain cleavage enzyme was determined by subjecting freshly homogenized testes to differential centrifugation as described in Chapter 2 (Section 2.2; Table 2.1). Four subcellular fractions were obtained viz:- (a) a particulate fraction sedimenting between 470-6,000 x g/20 minutes (washed three times) termed "Heavy mitochondrial" fraction. (b) The material sedimenting between 6,000-11,000 x g/15 minutes (twice washed) termed "Light mitochondrial" fraction. (c) "Microsomal" fraction - endoplasmic reticulum sedimenting between 11,000-105,000 x g/hour (washed once) and (d) Supernatant (cytosol) - the fluid remaining after centrifugation at 105,000 x g/hour.

Aliquots of these fractions equivalent to 0.4-0.6 mg of protein were separately incubated with [4-^{14}C] cholesterol at 37°C for 2 hours in medium B (see Table 2.2) in the

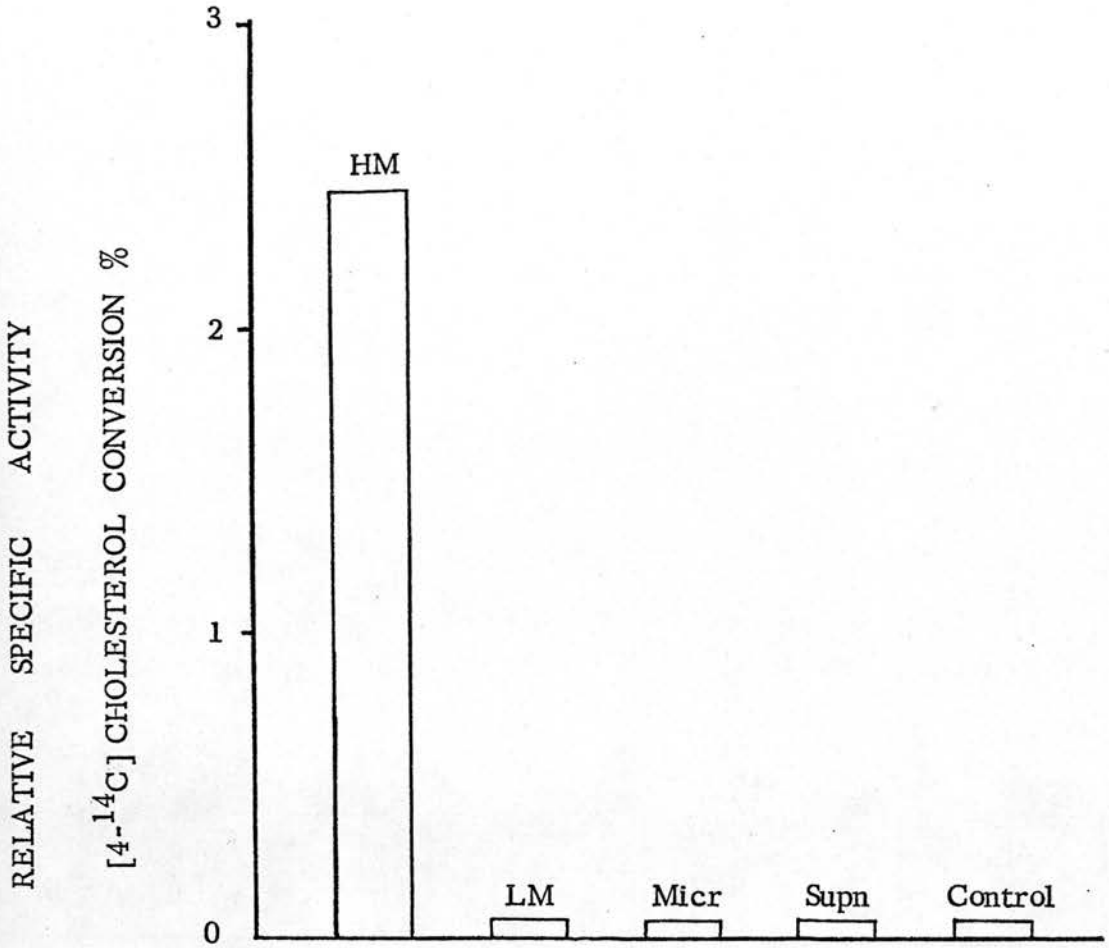


FIG. 3.1 TYPICAL SUBCELLULAR DISTRIBUTION OF CHOLESTEROL SIDE-CHAIN CLEAVAGE ENZYME SYSTEM AFTER DIFFERENTIAL CENTRIFUGATION

HM = Heavy mitochondrial fraction (470-6,000 x g/20 min)

LM = Light mitochondrial fraction (6,000-11,000 x g/15 min)

Micr = Microsomal fraction (11,000-105,000 x g/60 min)

Supn = Supernatant fraction remaining after 105,000 x g/60 min)

Control = Boiled heavy mitochondria.

Aliquots of the above fractions (0.5 mg/tube) were incubated with [4-^{14}C] cholesterol at 37°C for 2 hours in medium B in the presence of extramitochondrial NADPH-generating system. Similar results were obtained with isocitrate as the electron source.

presence of either DL isocitrate or extramitochondrial NADPH generating system as the electron source. The cholesterol side-chain cleavage activity was then assayed as previously described (see section 2.4.1-2.4.4). The results in Figure 3.1 show that all the cholesterol side-chain cleavage enzyme activity is localized exclusively in the heavy mitochondrial fraction of rat testes. The light mitochondrial fraction and the cytosol fraction were found to be completely devoid of cholesterol side-chain cleavage activity. This observation is in accord with the general view that the cholesterol oxygenase enzyme resides in the mitochondria of all steroid producing tissues (see Sulimovici & Boyd, 1969).

3.2 INFLUENCE OF CHEMICAL AND PHYSICAL TREATMENTS OF THE TESTICULAR HEAVY MITOCHONDRIA UPON CHOLESTEROL SIDE-CHAIN ACTIVITY

The cholesterol side-chain cleavage activity in rat testes mitochondria was found to be relatively low - the percentage conversion to steroids being of the order of 2-6% of the added labelled cholesterol. In adrenal and ovaries very much higher conversions (20-50%) have been obtained. The low activity in the testes could be attributed to the presence of large amounts of endogenous substrates.

With a view to improving the cholesterol side-chain cleavage activity the rat testes heavy mitochondria were subjected to the different chemical and physical treatments described below.

Acetone powder: washed heavy mitochondria (suspended in 2 volumes distilled water) were added dropwise to 20 volumes of acetone (at -40°C) with continuous stirring. The precipitated material was filtered using a Buchner apparatus, washed with 3 volumes of acetone (at -20°C) followed by 4 volumes of ether (at -20°C). The material was finally dried in a vacuum desiccator. This testicular mitochondrial acetone powder was suspended in 0.25 M sucrose-10 mM Tris pH 7.5 (at concentration of 10 mg/ml) and centrifuged at 105,000 x g for 60 minutes. The resulting supernatant and pellet and a combined mixture of these two fractions were assayed for cholesterol side-chain activity using NADPH generating system (in medium A) as source of electrons. None of these fractions showed any significant enzyme activity. (<0.3%). The addition of BSA to t he suspending medium before acetone treatment, or to the dried acetone powder and/or to the assay medium, or extraction with 0.1 M KCl or 0.05 M phosphate buffer pH 7.0, did not result in the recovery of cholesterol side-chain cleavage activity.

Ultrasonication: the heavy mitochondrial fraction suspended in Tris buffer pH 7.4 was subjected to ultrasonication for five periods of 2 minutes duration with intervals for cooling, using an M.S.E. ultrasonic disintegrator. The suspension was cooled in ice throughout the sonication process. After centrifugation at 105,000 x g for 30 minutes, the supernatant, the pellet and the mixed fractions were found to be devoid of cholesterol side-chain cleavage activity. Exogenous NADPH generating system was used as the electron source. Addition of BSA before sonication had no effect.

Freezing and Thawing: heavy mitochondria suspended in Tris buffer pH 7.5 was frozen in a bath of methanol and solid carbon dioxide and then thawed in a water bath. This procedure was repeated 5-10 times. After this treatment no cleavage enzyme activity was observed in either the pellet or supernatant fluid obtained at 105,000 x g/30 minutes.

All these procedures proved unsuccessful. In each case cholesterol side-chain cleavage activity was <5% of that present in an equivalent amount of original 'native' heavy mitochondria.

These observations are similar to those of Drosdowsky et al. (1965). These authors were unable to solubilize this enzyme complex by using such methods as dialysis against n-butanol, and addition of egg or beef lecithin to acetone powder. The failure in solubilizing the cholesterol side-chain cleavage enzyme emphasizes the labile nature of this enzyme complex from testes in contrast to the stability of the corresponding enzyme complexes in bovine adrenal (Contantopaulos & Tchen, 1961; Simpson & Boyd, 1966), hog adrenal (Ichii et al., 1967), bovine corpus luteum (Ichii et al., 1963), human placenta (Morrison et al., 1965; Mason & Boyd, 1971) and immature rat ovarian tissue (Sulimovici & Boyd, 1968b).

3.3 DISTRIBUTION OF CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY AFTER FICOLL GRADIENT CENTRIFUGATION

Unlike the corpora lutea and adrenal cortex which are almost entirely composed of steroid forming cells only 3-5% of the cells of rat testis synthesize steroids (Christensen &

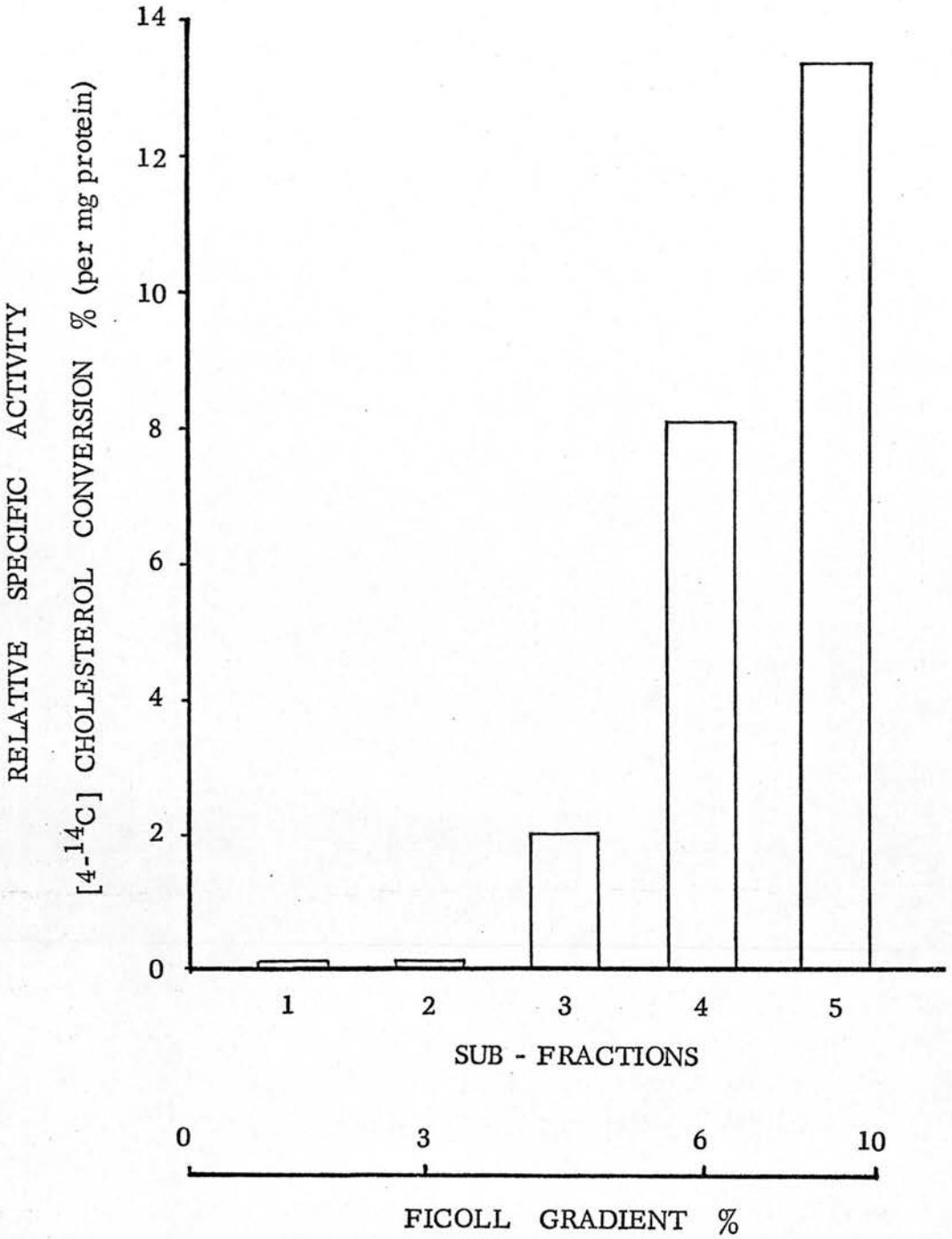


FIG. 3.2 TYPICAL SUBCELLULAR DISTRIBUTION OF CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY AFTER FICOLL GRADIENT FRACTIONATION OF TESTICULAR HEAVY MITOCHONDRIA

Heavy mitochondria obtained after differential centrifugation were further subfractionated by density gradient centrifugation on 0-10% ficoll as described in the text. Cholesterol side-chain cleavage activity in each fraction was assayed using medium A and an NADPH-generating system (see Chapter 2). Similar results were obtained with isocitrate as the electron donor.

Mason, 1965). By virtue of the heterogeneous cellular nature of the organ, the bulk of mitochondria derived from whole testes would not only be non-steroidogenic but also of different kinds and sizes. Differential centrifugation, as described above, achieved an initial separation of the mitochondria into a fraction rich in cholesterol side-chain cleavage activity (heavy mitochondria) and a fraction (light mitochondria) not containing the enzyme activity. A further fractionation of the heavy mitochondrial fraction was achieved by centrifuging the cholesterol side-chain cleavage enzyme rich fraction through a 3-6-10% discontinuous ficoll gradient as described in Chapter 2.3. The five subfractions thus obtained were assayed for cholesterol side-chain cleavage activity. A typical result presented in Figure 3.2 shows that most of the enzyme activity is located in the heavy sedimenting subfraction 5 (HMF5). Subfraction 4 and subfraction 3, both contained some cleavage activity but accounted for less than 15% of the total activity in HMF5 (see Table 3.1 and Figure 3.2). As the mitochondria from subfraction 3 and 4 had a lower "apparent" relative specific activity, only HMF5 was used as source of the cleavage enzyme. Subfractions 1 and 2 did not exhibit any cholesterol side-chain cleavage activity. Based on cholesterol side-chain cleavage activity and specific cytochrome P450 content (see Chapter 5) such a fractionation generally resulted in a 2-4-fold purification over the original 'native' heavy mitochondria.

TABLE 3.1

PROTEIN DISTRIBUTION PATTERN OF SUBCELLULAR FRACTIONS IN 3-10% FICOLL GRADIENT

FICOLL SUB-FRACTIONS	SUBCELLULAR FRACTIONS (mg protein/subfraction)		
	HEAVY MITOCHONDRIA	LIGHT MITOCHONDRIA	MICROSOMES
1	3 (1.2)	6 (14)	60 (94)
2	65 (27)	30 (68)	
3	48 (20)	5 (11)	4 (6)
4	16 (7)	<1 (2)	0
5	110 (45)	2 (5)	0

After ficoll gradient centrifugation each subfraction was carefully removed, diluted with medium H up to 50 ml and centrifuged at 15,000 x g for 20 minutes. The pellet was suspended in a small known volume of medium H and the protein content measured by the Lowry procedure. Figures in ( ) indicates the percentage of total protein applied on the gradient.

3.3.1 Behaviour of subcellular fractions on ficoll gradient

Table 3.1 shows a typical protein distribution pattern obtained when heavy mitochondria, light mitochondria and microsomal fraction of testes were each subjected to ficoll gradient. Most of the protein from the heavy mitochondrial fraction was recovered in the cholesterol side-chain cleavage rich subfraction 5. This fraction accounted for approximately 45% of the protein applied on the gradient. Most of the remaining protein was distributed between subfractions 2 (27%) and 3 (20%). Light mitochondria consisted mainly of a fraction corresponding to subfraction 2 of heavy mitochondria. Microsomes, on the other hand, occupied the region corresponding to sucrose and the 3% ficoll layers, (i.e. subfractions 1 and 2) as one broad, white diffuse layer, i.e. subfractions 1 and 2 were not distinguishable.

These results show that heavy mitochondria, although a distinct fraction, contains material with characteristics similar to light mitochondria and microsomes and these contaminants can be readily separated by the 3-10% ficoll gradient.

3.3.2 Further fractionation of heavy mitochondria subfraction 5

Since the enzymatically active HMF5 sedimented as a pellet, the suitability of this fraction for further subfractionation was examined. After centrifuging HMF5 at 10,000 g for 60 minutes through a second discontinuous gradient consisting of 6%, 10%, 15% and 20% ficoll, three fractions were obtained corresponding to 10%, 15% and 20% ficoll layers. Most of the protein was found in the 15% layer. Cholesterol side-chain

cleavage enzyme activity was present in all the three layers. The apparent specific activities of the three fractions were similar. These observations suggest that the mitochondria from rat testes capable of steroidogenesis are not uniform in size and density. Since this second gradient fractionation did not appear to be much of an improvement over the first 0-10% ficoll gradient fractionation, further fractionation studies were not pursued.

3.4 DETERMINATION OF RESPIRATORY CONTROL AND ADP:O RATIOS

It is known that in mammalian systems respiratory control and coupled oxidative phosphorylation cannot be demonstrated unless the mitochondria are intact.

It was initially observed that mitochondria incubated in a media consisting of sucrose KCl-triethanolamine-potassium phosphate and $MgCl_2$ (Simpson et al., 1972b) or 250 mM sucrose - 10 mM potassium phosphate, did not exhibit any respiratory control. Since the demonstration of respiratory control (expressed as a ratio of the maximum rate of oxygen uptake in the presence of ADP [state 3] to that obtained when ADP was absent [state 4] is usually regarded as the most sensitive indicator of mitochondrial integrity, the conditions and composition of the incubation medium required to induce coupling were investigated.

3.4.1 Composition of medium required to induce coupling

Washed heavy mitochondria were prepared as previously described from testes of 17 week old male rats and homogenized in 250 mM sucrose. These mitochondria were incubated in the basic incubation medium to which potassium phosphate (10 mM),

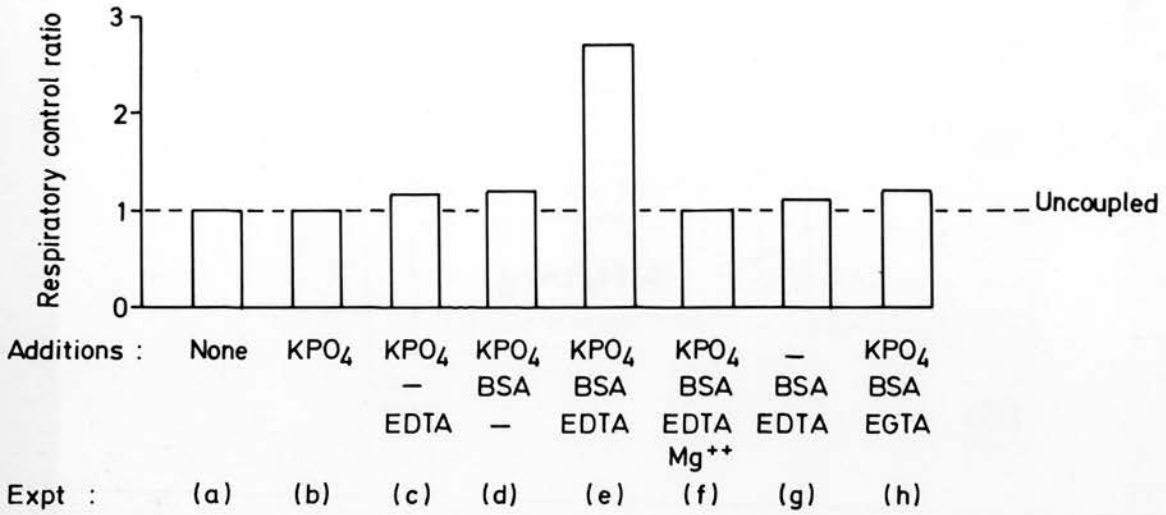


FIGURE 3.3 Composition of the incubation medium necessary to induce coupled mitochondria.

Testis were homogenized in 0.25 M sucrose and washed heavy mitochondria prepared. To the 'basic incubation medium' the following compounds were added as indicated:-

Potassium phosphate buffer pH 7.4 (10 mM), EDTA (1 mM), EGTA (1 mM), Mg^{++} (5 mM) and BSA (0.5% w/v). Respiratory control ratios were measured as described in the text using succinate as substrate.

EDTA (1 mM), EGTA (1 mM) or BSA (0.5%) were added either individually or in different combinations. The effect of these compounds on respiratory control are shown in Figure 3.3. It was observed that mitochondria were coupled only when all three factors, potassium phosphate, EDTA and BSA were present together - in an "all or none" type of effect. With other combinations no appreciable respiratory control was obtained. These specific requirements hint at a complex relationship between the factors involved.

The addition of 2-5 mM Mg^{2+} to the above coupled system completely depressed respiratory control (as shown in Figure 3.3, Experiment F) so that the ADP/O ratio was unmeasurable (i.e. completely uncoupled the mitochondria). This observation contrasts with those reported for pig corpora lutea (Robinson, 1971) and superovulated rat ovaries (Arthur & Boyd, personal communication) in which the presence of exogenous Mg^{2+} was required for coupling. A further addition of EDTA (up to 10 mM) to these Mg^{2+} uncoupled mitochondria, failed to relieve the inhibition. This effect might be explained as follows:- a potent Mg^{2+} dependent ATPase present in the heavy mitochondria was reconverting the ATP formed back to ADP which thereby continued to stimulate the oxygen uptake. The ATPase activity of these mitochondria was therefore measured by a modified Gomori (1942) procedure (Rebello et al., 1969). It was found that Mg^{2+} stimulated the ATPase activity two-fold. The mean rate of inorganic phosphate released from ATP was 3.5 µ moles/mg protein/hour. This was increased to 8.1 µ moles Pi released/mg

protein/hour by 5 mM Mg^{2+}. Similar effects of Mg^{2+} on oxidative phosphorylation were observed in rat adrenal mitochondria (Purvis et al., 1968; Sauer & Mulrow, 1969; Simpson et al., 1972). Sauer and Mulrow (1969) suggested that this Mg^{2+} stimulated ATPase may be extramitochondrial. However, when mitochondria purified on ficoll gradient (HMF5) was used instead of heavy mitochondria, a similar uncoupling effect of Mg^{2+}, to those described above was obtained. Therefore, if the ATPase activity represents contaminants (microsomal or lysosomal), then these contaminants may be closely associated with the mitochondria. Vanha-Perttula (1971) reported that rat testes mitochondria, compared to other testicular subcellular fractions, was rich in acid phosphatase activity. In addition, Pressman and Lardy (1956) observed that adrenal mitochondrial ATPase activity was stimulated by fatty acids and that this activation was a mitochondrial membrane effect. In view of the abundance of fatty acids in the testis (Carpenter, 1971; Davis et al., 1966) the depression of respiratory control may not be due solely to the stimulation of ATPase by Mg^{2+}.

3.4.2 Effect of varying concentrations of potassium phosphate, EDTA and BSA on respiratory control ratio

The optimal concentration of potassium phosphate, EDTA and BSA required for the display of maximal respiratory control by rat testis mitochondria were investigated.

The observations on respiratory control ratio of several preliminary experiments indicated that irrespective of the composition of the homogenizing medium (whether 250 mM sucrose,

FIGURE 3.4

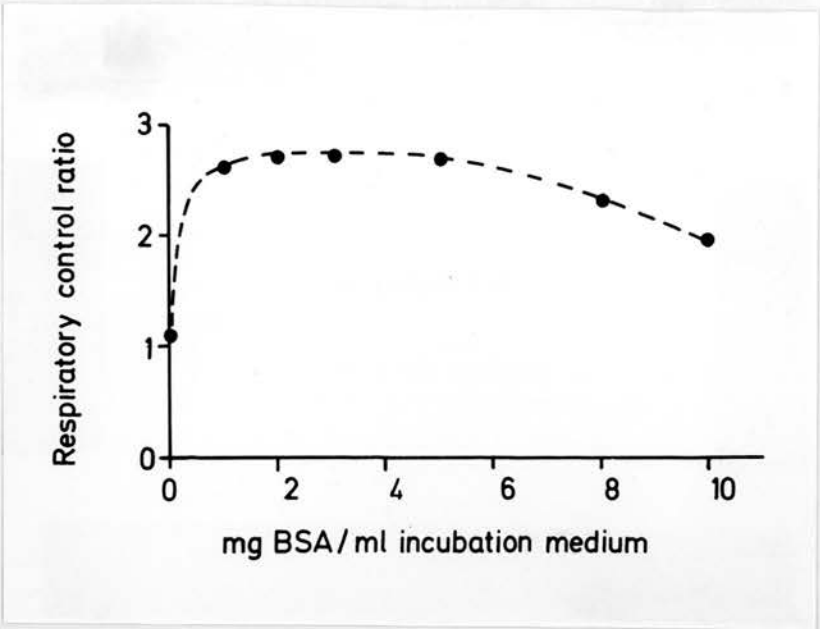
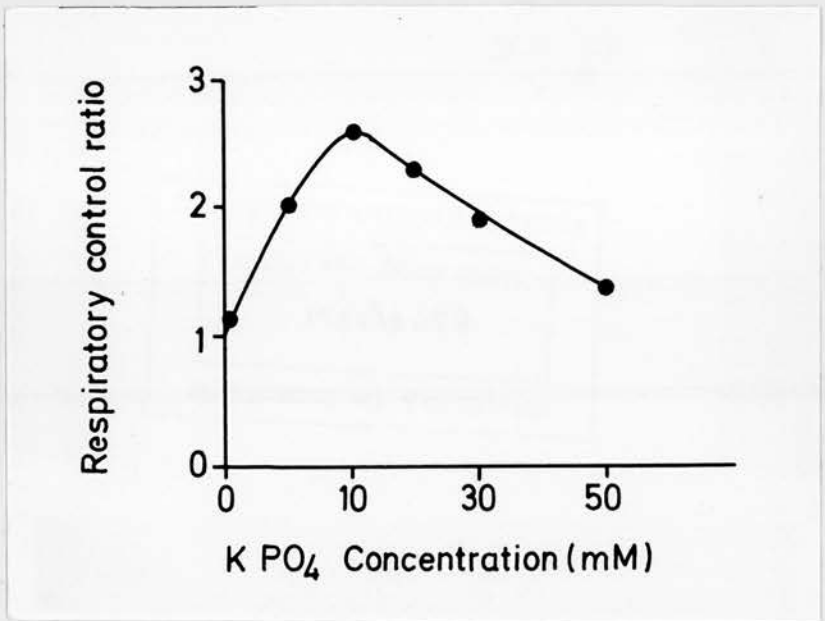


FIGURE 3.5



Effect of varying amounts of potassium phosphate and BSA on mitochondrial respiratory control ratios.

Testes were homogenized in 0.25 M sucrose. RCR were measured as described in the text after suspending the washed heavy mitochondria (2 mg protein) in 'basic incubation medium' containing EDTA (0.3 mM) and:-

FIG. 3.4 BSA (0.1% w/v) and varying concentration of potassium phosphate.

FIG. 3.5 Potassium phosphate (10 mM) and varying amounts of BSA (fatty acid free).

250 mM sucrose plus 10 mM Tris (pH 7.4), 250 mM sucrose plus 1 mM EGTA (pH 7.4), or 250 mM sucrose plus Tris 10 mM plus 0.3 mM EDTA (pH 7.4) the results obtained with either heavy mitochondria or ficoll fractionated mitochondria (HMF_5) were essentially similar provided potassium phosphate, EDTA and BSA were all present in the incubation medium. The results presented below were typical of those observed.

A. The effect of different concentrations of potassium phosphate on respiratory control is shown in Figure 3.4. The optimal concentration of potassium phosphate was found to be 10 mM. Increasing the concentration beyond this maximum led to a decrease in the respiratory control ratios. This effect resembles substrate-enzyme inhibition. The reason for this phenomenon is not clear. It may in part be related to energized phosphate binding (Packer & Utsumi 1969; Allmann et al. 1970a). It is well established that the respiration of tightly coupled mitochondria is strikingly dependent on the presence of inorganic phosphate and the phosphoryl acceptor system-ADP.

B. The effect of varying amounts of BSA on mitochondrial respiratory control ratios presented in Figure 3.5 shows that in the absence of BSA the mitochondria were uncoupled. Respiratory control was however induced by the presence of small amounts of BSA 0.05%. Maximal values were obtained with BSA concentrations of 0.1-0.5%. Higher amounts (1%) had an inhibitory effect. These observations suggest that in addition to the well established protection against fatty acid induced inhibition of mitochondria respiratory control (Allmann et al., 1970a; Bjorntorp et al., 1964) BSA may have direct

FIGURE 3.6

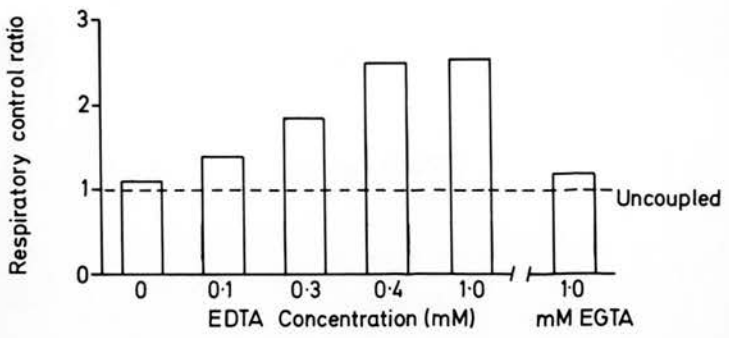


FIGURE 3.7

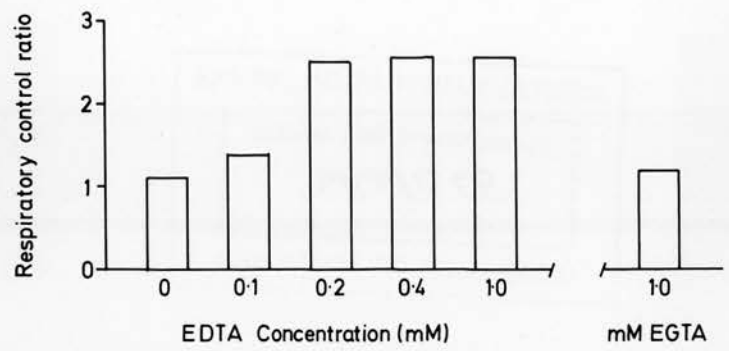


FIG. 3.6 Effect of different concentrations of EDTA and EGTA on mitochondrial respiratory control ratio. Testes were homogenized in 0.25 M sucrose. Washed heavy mitochondria (2 mg protein) were suspended in 'basic incubation medium' plus potassium phosphate buffer (10 mM), BSA (0.1%) and varying concentrations of EDTA or EGTA.

FIG. 3.7 Same as Fig. 3.6 except that in this experiment testes were homogenized in 0.25 M sucrose containing EGTA (1 mM).

mitochondrial structural effects (Helinski, 1960; Strehler, 1966). Harding et al. (1968) and Satre et al. (1969) showed that the cristae of adrenal mitochondria, isolated in a sucrose medium free of albumin, were in the orthodox (uncoupled) configuration.

C. The variation of RCR with EDTA concentrations is illustrated in Figure 3.6. The minimum amount of EDTA which produced maximal respiratory control, in the absence of an additional chelator in the homogenizing medium, was 0.4 mM. Above this minimum concentration (i.e. 0.4-1 mM) respiratory control appeared to remain constant. When mitochondria were isolated from testes homogenized in 250 mM sucrose-1 mM EGTA, a further addition of EDTA in the incubation medium was still essential. The results in Figure 3.7, while qualitatively similar in pattern to Figure 3.6, show a lowered minimum requirement for EDTA of 0.2 mM. EGTA (1 mM) could not be substituted for EDTA. EDTA protects the mitochondria against the uncoupling effects of free fatty acids and Mg^{++} stimulated ATPase.

The effect of the specific Ca^{++} chelating agent (EGTA) in the homogenizing medium, in lowering the effective minimum concentration of EDTA necessary to produce maximum respiratory control could be attributed to the removal of Ca^{++} ion. If initially no EGTA was present then the amount of EDTA in the incubation medium would be expected to be higher - to remove both Mg^{2+} and Ca^{2+} (Settlemire et al., 1968). Ca^{2+} ion which is known to cause mitochondrial swelling could depress

TABLE 3.2

EFFECT OF EGTA ON MITOCHONDRIAL STABILITY TO STORAGE AT 0-4°C

HOMOGENISING MEDIUM	RESPIRATORY CONTROL RATIO			
	HOURS			
	1	6	25	48
250 mM sucrose (5)	2.4 ± 0.2	2.3 ± 0.3	-	1.3 ± 0.2
250 mM sucrose + 1 mM EDTA (5)	2.5 ± 0.2	2.4 ± 0.2	1.4 ± 0.2	-
250 mM sucrose + 1 mM EGTA (5)	2.5 ± 0.3	2.5 ± 0.2	2.5 ± 0.3	2.4 ± 0.3

Reaction conditions were similar to those described for Figure 3.8, succinate was the substrate used. Results are expressed as mean ± SEM; number of determinations are in parenthesis.

respiratory control by inducing the mitochondria to the orthodox configuration through the generation of non-esterified fatty acids (Allmann et al., 1970a). Allmann et al. (1970a,b) observed the existence of a critical balance between Mg^{2+} and Ca^{2+}.

The respiratory control ratios of freshly prepared mitochondria were not significantly affected by the variations in the composition of the homogenizing medium. However, it was observed that the presence of EGTA in the homogenizing medium appeared to affect the stability of the mitochondria to storage at 0-4°C for several hours and its ability to show respiratory control. Data presented in Table 3.2 illustrates this point. Testes mitochondria isolated in a sucrose medium containing 1 mM EGTA were much more stable to storage (up to 48 hours at least) compared to those containing EDTA or no chelator. The reason for these results is not clear. It could in part be attributed to the loss of endogenous K^+. The removal of limited amounts of Mg^{2+} by EDTA increased the Na^+ uptake and K^+ loss. In contrast, EGTA, which has a high affinity for Ca^{2+}, does not remove Mg^{2+} and does not result in the loss of endogenous K^+ (Settlemire et al., 1968). On the basis of the above results the composition of medium B (Table 2.2) was arrived at. This medium was used in both the studies on ADP/O ratios and mitochondrial steroidogenesis.

3.4.3 Oxidative, phosphorylative and respiratory control capacities of rat testis heavy mitochondria

The polarographic determination of ADP:O and respiratory control ratios were carried out in 3.0 ml of medium B as described in Chapter 2, section 2.10.

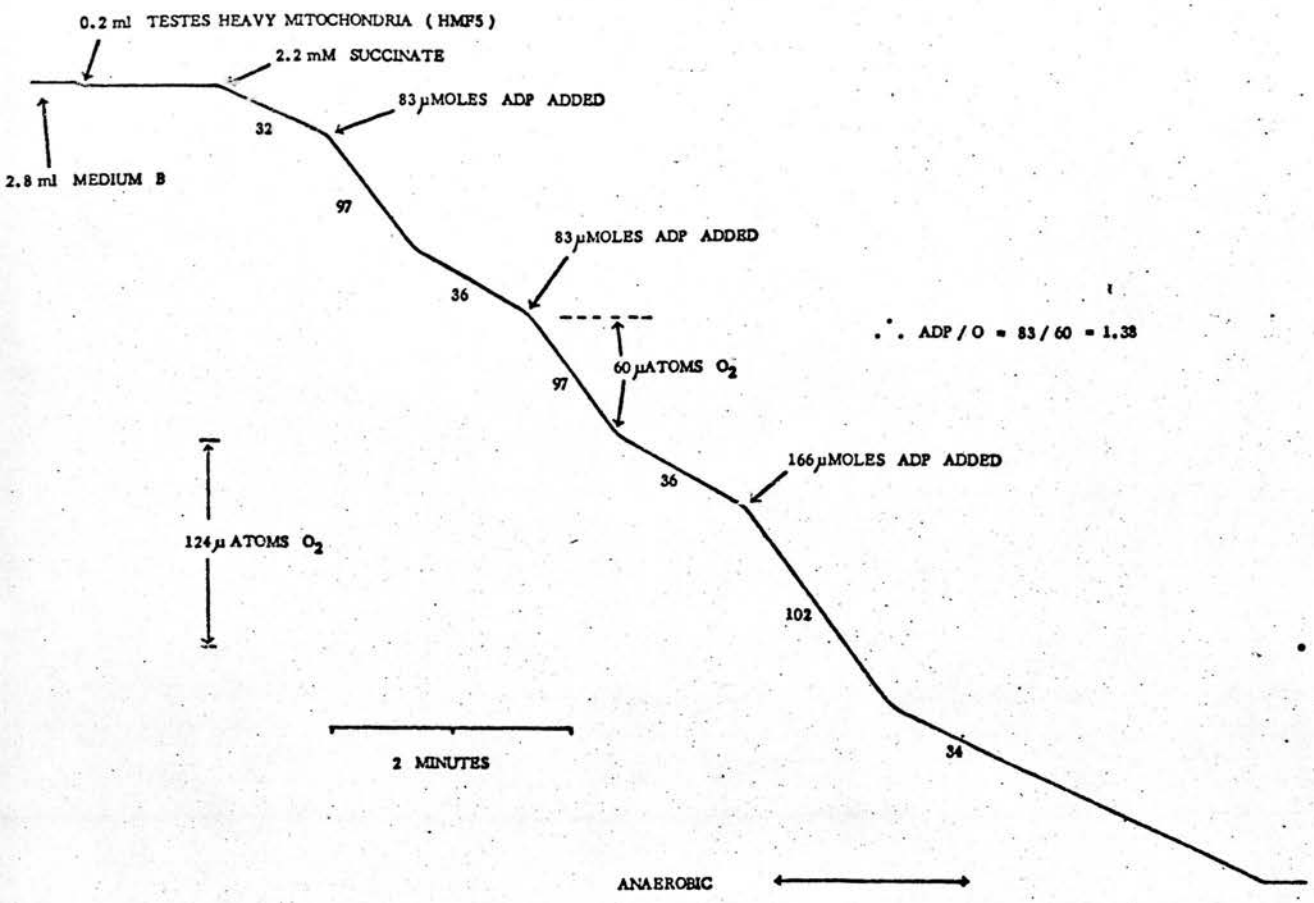


FIG. 3.8 ILLUSTRATION OF RESPIRATORY CONTROL IN TESTICULAR HEAVY MITOCHONDRIA

The numbers below the traces refer to the rate of oxygen utilization in micro atoms of oxygen per minute. Conditions used were similar to those described in Chapter 2.10

A typical polarographic trace is shown in Figure 3.8. After adding mitochondria the addition of succinate increased the rate of oxygen utilization from the low level, attributable to endogenous substrates to the ADP-deficient state (state 4). The subsequent addition of limiting amounts of ADP caused a three-fold increase in the rate of respiration (state 3). After conversion of the added ADP into ATP, as indicated by the cut-off in the polarographic trace, the respiratory rate returned to the low level indicative of the ADP deficient state (state 4). Respiratory rates could be stimulated to the same extent by further additions of ADP. These classical state 4 - state 3 transitions in response to ADP are characteristic of intact, well preserved mitochondria (Chance, 1956). The ADP:O ratios, i.e. the amount of ADP phosphorylated divided by the amount of oxygen used during this process, were calculated as shown in Figure 3.8.

The ADP:O and respiratory control ratios and the rates of oxygen utilization in the presence (state 3) or absence (state 4) of ADP observed in a typical heavy mitochondrial preparation isolated from rat testes are summarized in Table 3.3. Testes heavy mitochondria were able to oxidize various tricarboxylic acid cycle intermediates, with succinate being oxidized at the highest rate. Malate, which was less effective, was a much better substrate than isocitrate. The respiratory rates of the three Krebs cycle intermediates tested were dependent on the presence (state 3) or absence (state 4) of added ADP. By contrast NADPH or NADH were not oxidized to any appreciable extent by the mitochondria even in the

TABLE 3.3

OXIDATIVE, PHOSPHORYLATIVE AND RESPIRATORY CONTROL CAPACITIES OF RAT TESTES HEAVY MITOCHONDRIA

SUBSTRATE	OXYGEN UPTAKE n atoms/mg protein/min		ADP/O	RESPIRATORY CONTROL RATIO
	STATE 4 (-ADP)	STATE 3 (+ADP)		
SUCCINATE (2.2 mM) 1	46 ± 3 (8)	96 ± 5 (3)	1.4 ± 0.3 (8)	2.1 ± 0.4 (8)
2	36 ± 2 (8)	98 ± 4 (8)	1.4 ± 0.2 (8)	2.7 ± 0.3 (8)
L-MALATE (2.2 mM)	11 ± 2 (5)	31 ± 6 (5)	2.1 ± 0.3 (5)	2.8 ± 0.3 (8)
DL ISOCITRATE (2.2 mM)	9 ± 3 (3)	13 ± 3 (3)	3.5 ± 0.4 (3)	1.3 ± 0.2 (3)
NADH (33 μM)	< 1 No detectable oxygen uptake			
NADPH (35 μM)	< 1 No stimulation on addition of ADP			

Respiratory control ratios and ADP:O ratios were determined as described in Chapter 2. Reaction conditions were similar to those shown in Figure 3.8. In 1 washed heavy mitochondria was used, in all other experiments ficoll fractionated heavy mitochondria 5 (HMF_5) was used. Results are expressed as mean ± S.E.M.; number of determinations are in parenthesis.

presence of ADP. Had these mitochondria been permeable to exogenous nucleotides, the rate of oxygen uptake would have increased. These observations attest to the biochemical "intactness" of these mitochondria.

The respiratory and phosphorylative activities of the heavy mitochondria and ficoll purified mitochondrial fraction 5 were found to be similar.

With succinate as substrate the addition of Mg^{2+} completely uncoupled the mitochondria, as discussed earlier, by markedly stimulating state 4 oxygen uptake so that ADP:O ratios were unmeasurable.

The low ADP:O and respiratory control ratios observed in this study with testicular heavy mitochondria compared to reported values for liver does not appear to be unusual. Similar low ratios, especially with succinate as substrate, have been reported in other steroidogenic tissues: bovine adrenal cortex (Cammer & Estabrook, 1967), porcine corpus luteum (Robinson, 1972), rat adrenal (Purvis et al., 1968; Sauer & Mulrow, 1969). The observations in this study on testes mitochondria closely resemble those of rat adrenal (Sauer & Mulrow, 1969). In both cases the low ADP:O and respiratory control ratios were sensitive to Mg^{2+} ions; omission of Mg^{2+} from the incubation medium increased the values.

3.5 IDENTIFICATION OF THE PRODUCTS OF CHOLESTEROL OXIDATION

3.5.1 Thin layer chromatography

Cholesterol metabolism by mitochondria isolated from most steroidogenic tissues, including the corpus luteum

TABLE 3.4

SOLVENT SYSTEMS INVESTIGATED FOR THE SEPARATION OF INTERMEDIATES OF TESTOSTERONE SYNTHESIS

No. of System	SOLVENT	Ratio
I	= Chloroform :Ethanol	99:1
II	= Benzene :Ethanol	96:4
III	= Cyclohexane :Ethyl acetate	50:50
IV	= Chloroform :Acetone	90:10
V	= Benzene :Acetone	80:20
VI	= Toluene :Methanol	92:8
VII	= Di-isopropyl ether:Petroleum ether (60^{o}-80^{o}C): Glacial acetic acid	80:35:2

(Robinson & Stevenson, 1971), adrenal cortex (Simpson & Boyd, 1967a) and the placenta (Mason & Boyd, 1971), resulted in the formation of only two major products, pregnenolone and progesterone. However, "heavy mitochondria" prepared from the rat testes catalysed the formation of a number of steroid products from [4-^{14}C] cholesterol when an NADPH generating system was used as a source of reducing equivalents. The total yield of steroid products formed was low, usually not more than 5% of the added [4-^{14}C] cholesterol used. The necessity therefore, to reduce procedural losses of compounds during routine analysis prompted the development of a suitable technique for extraction of the steroids from testicular incubates (described in Chapter 2.4.2, Table 2.3). Since most of the chromatography systems described were suited to the separation of only a few steroids from the biological extracts, a reproducible system for separating the multiple steroid intermediates in testosterone biosynthesis from cholesterol on a single TLC chromatoplate was developed.

A number of different solvent systems (listed in Table 3.4) were investigated using authentic steroids and silica-gel GF254 precoated glass plates (0.25 mm layer thickness). The conditions for TLC were the same as described in Chapter 2, section 2.4.3. Because of the multiple developments the Rf values were not reproducible from run to run. However, provided the plates were each developed the same number of times the relative migration values (relative to progesterone, i.e. R progesterone) were very reproducible (± 0.5). Hence the results were expressed as

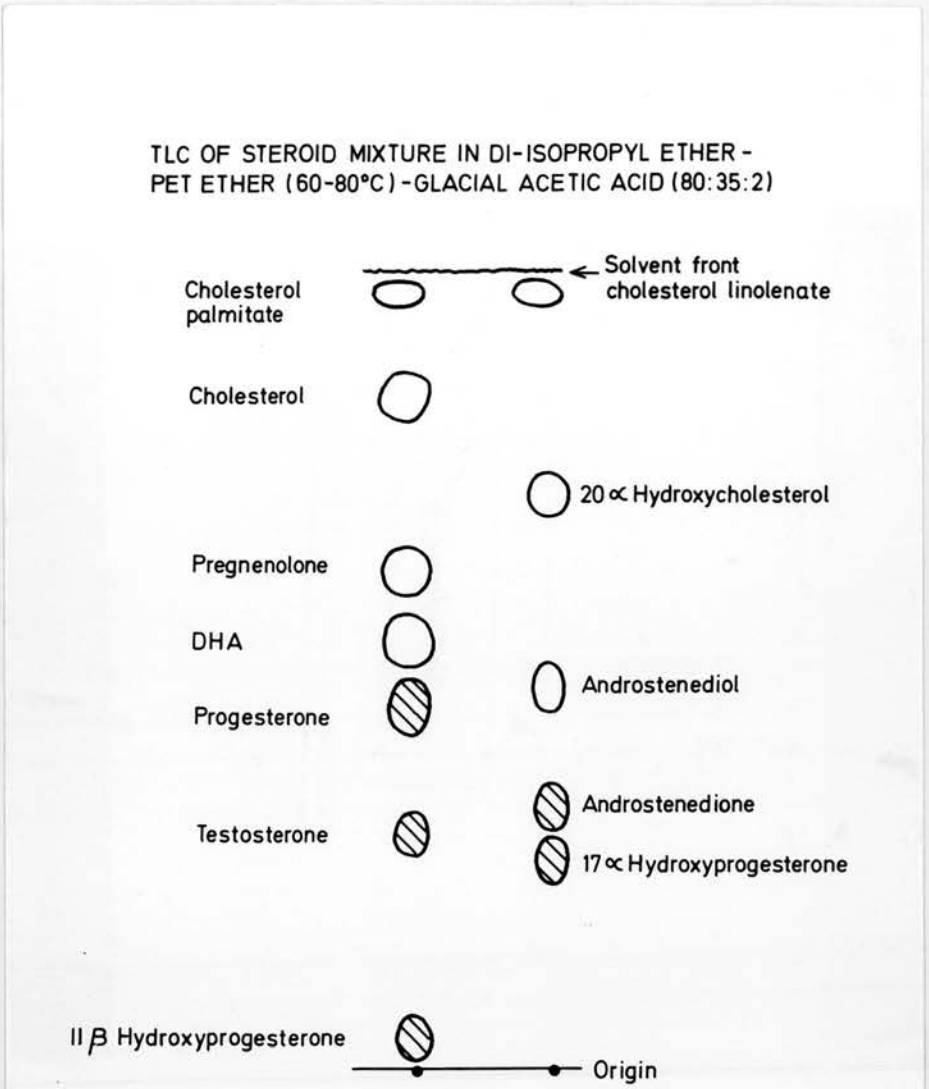


FIGURE 3.9 Trace of a typical TLC resolution of steroids in di-isopropyl ether:petroleum ether (BP 60-80°C): glacial acetic acid (80:35:2) developed four times. Silica gel GF 254 precoated plates (E. Merck) were used. Shaded spots are steroids visualized under u.v. Unshaded spots are steroids located after spraying the chromatoplate with sulfuric acid: methanol (2:1).

$$R\ \text{progesterone} = \frac{\text{Migration distance of spot}}{\text{Migration distance of progesterone}} \times 100$$

The resolution achieved with the different solvent systems are shown in Table 3.5.A. It can be seen that the best separation was obtained in solvent mixture VII di-isopropyl ether: petroleum ether (BP 60°-80°C): glacial acetic acid (80:35:2 by vol.). Two developments in this solvent system gave moderately good resolution of the steroid mixture, but separation was much improved by four consecutive developments (Table 3.5.B). Cholesterol esters, cholesterol, 20α-hydroxycholesterol, pregnenolone, dehydroepiandrosterone and progesterone were all very well separated from each other and from the 4-ene3-oxo steroid group, 17α-hydroxyprogesterone, testosterone and androstenedione (as depicted in Figure 3.9). These latter three steroids, which were poorly resolved when visualized with ultra violet light, could be delineated if ^{14}C-labelled steroids were run and their distribution examined by means of a radiochromatogram scanner. The separation of dehydroepiandrosterone as a discrete spot from pregnenolone and progesterone, with which it otherwise very closely overlapped, was of particular importance in view of the role of dehydroepiandrosterone as a possible intermediate in the alternative route in testosterone biosynthesis from cholesterol (Jungmann, 1968).

The complete separation of the multiple steroids by this system VII was slow since each run took 2 hours (i.e. a total development time of 8 hours). However, an equivalent separation of the intermediate steroids involved in testosterone formation from cholesterol, was not obtained with any of

TABLE 3.5

SEPARATION OF AUTHENTIC STEROIDS AND STEROLS

IN SOLVENT SYSTEMS DESCRIBED IN TABLE 2

STEROIDS	A SOLVENT MIXTURES R Progesterone x 100							B SOLVENT MIXTURE VII Apparent R_f x 100	
	I	II	III	IV	V	VI	VII	VII	VII
11β-hydroxyprogesterone	35	45	26	33	37	24	8	2	4
17α-hydroxyprogesterone	78	70	83	72	82	54	58	15	26
Testosterone	71	63	71	63	72	46	64	16	28
Androstenedione	96	94	87	95	93	91	70	18	32
Androstenediol	58	58	79	52	66	44	106	27	48
Progesterone	100	100	100	100	100	100	100	26	45
Dehydroepiandrosterone	75	74	90	76	83	62	118	31	54
Pregnenolone	79	79	96	78	86	68	137	36	62
20α-hydroxycholesterol	79	79	109	72	88	67	159	45	72
Cholesterol	85	89	116	80	96	89	186	56	84
Cholesterol esters	114	130	136	127	120	135	216	94	98
Number of runs	2	2	2	1	2	2	4	2	4

$$\text{R progesterone} = \frac{\text{migration distance of spot}}{\text{migration distance of progesterone}} \times 100$$

$$\text{Apparent } R_f = \frac{\text{migration distance of spot}}{\text{(top end of plate - line of origin)}} \times 100$$

Values presented for solvent mixtures I-VI are averages of at least 5 determinations and those of solvent VII, 12 determinations

the other solvent systems tried, or with those reported in literature (Stahl, 1965; Levin et al., 1969). Furthermore, the separation of the steroid mixture on one TLC plate during routine investigations greatly reduced the loss of accuracy and eliminated the use of tedious recovery techniques involving elution and rechromatography in different solvent systems, which would have been necessary if incomplete resolution was obtained.

Examples of the use of this method for the separation of steroids extracted from rat testicular heavy mitochondria after incubation with [4-^{14}C] cholesterol and different electron donors are depicted in Figure 3.10. It can be seen that when electrons for cholesterol side-chain cleavage were supplied by an NADPH generating system many products were formed. The radioactive peaks shown in the radiochromatogram (Trace 1) correspond with the authentic standards pregnenolone, progesterone, testosterone, 17α-hydroxyprogesterone, androstenedione and cholesterol esters, run on the same plate and visualized as described in Chapter 2. All these steroid products are known intermediates in testosterone biosynthesis. When isocitrate was the electron source pregnenolone and progesterone were the major products (Trace 2). Trace 3 shows that when $NADP^+$ was added together with isocitrate, the pregnenolone formed was converted to progesterone and another 4-ene-3-oxo-steroid (testosterone). Finally, in the presence of cyanoketone, an inhibitor of 3β-hydroxysteroid dehydrogenase, pregnenolone was the only significant labelled product of cholesterol metabolism (Trace 4). It is of interest to

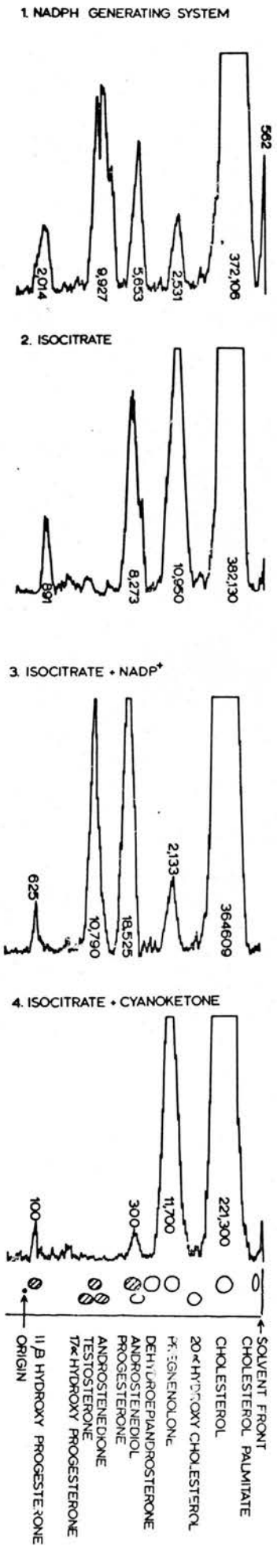


FIGURE 3.10

Typical traces of Thin layer radiochromatogram scans of extracts from incubated testicular "heavy mitochondria". Heavy mitochondria were incubated with [4-^{14}C]cholesterol in the presence of an electron source and other compounds (see below) and the steroids formed extracted and separated by TLC in solvent VII as described in text. The radioactive steroids formed from cholesterol were located by scanning the developed plates under a Panex windowless gas flow, Geiger-Muller type radiochromatogram scanner. A carrier gas mixture of 98% argon and 2% propane was employed. The radioactivity of each zone was subsequently determined by liquid scintillation spectrometry.

Trace 1 NADPH generating system
2 DL-isocitrate
3 DL-isocitrate plus $NADP^+$
4 DL isocitrate plus cyanoketone
5 standard authentic steroids and sterols.

note that in all the above examples there was no detectable radioactive peak corresponding to dehydroepiandrosterone.

The above observationsindicate that the rat testes heavy mitochondrial fraction possess in addition to the cholesterol desmolase, other enzymes involved in the further metabolism of pregnenolone and progesterone.

Solvent system VII described in this study was a modification of that previously described by Simpson and Boyd (1967) for the separation of postulated intermediates in cholesterol oxidation to pregnenolone in adrenal cortex and that used by Manners et al. (1969) for separating the products of fat digestion.

Although solvent systems I-VI were unsatisfactory for these testicular studies, they were adequate for the separation of a few steroids when present in the biological extracts. The separation of some of the steroids from mixtures achieved by these solvents are summarized in Table 3.6.

3.5.2 Derivative formation

A large number of identical incubations were carried out to provide sufficient material for analysis and identification of the products. After TLC in solvent VII, the radioactive areas on the chromatoplate corresponding to standard pregnenolone, progesterone, cholesterol oleate (individually) and testosterone, androstenedione and 17α-hydroxyprogesterone (as a group) were scraped off, packed into small columns and eluted as described in Chapter 2.8.1. The extracts were concentrated under nitrogen and aliquots then taken for analysis.

TABLE 3.6

STEROID MIXTURES RESOLVED

SOLVENTS SYSTEMS	STEROID MIXTURES
I	(a) Pregnenolone, progesterone and androstenediol (b) 17α(OH)-progesterone, androstenediol, testosterone, androstenedione or progesterone. (c) DHA, androstenediol and androstenedione
II	(a) Pregnenolone, progesterone and androstenediol or testosterone (b) Progesterone, 17α-hydroxyprogesterone, androstenediol and androstenedione (c) DHA, androstenedione and testosterone
III	(a) Progesterone, 17α-hydroxyprogesterone and testosterone (b) Androstenedione and testosterone
IV	(a) Progesterone, pregnenolone, androstenediol and testosterone (b) DHA, androstenediol, testosterone and androstenedione or progesterone (c) 17α-hydroxyprogesterone, androstenediol, progesterone and androstenedione
V	(a) Pregnenolone, progesterone and testosterone or androstenediol (b) Pregnenolone, androstenedione, testosterone or androstenediol (c) 17α-hydroxyprogesterone, androstenedione, testosterone and androstenediol (d) Progesterone, DHA and testosterone or androstenediol
VI	(a) Pregnenolone, androstenediol, 17α-hydroxy-progesterone, testosterone and progesterone or androstenedione (b) DHA, testosterone, androstenediol and androstenedione or progesterone (c) Testosterone, 17α-hydroxyprogesterone, 17α-hydroxypregnenolone and androstenedione

1. Pregnenolone and Progesterone

The identity of these steroids was confirmed by

(a) rechromatography in solvent systems I, II, IV, V and VI (see Table 3.5A). In all instances the radioactive material behaved as homogeneous fractions having identical chromatographic mobilities with those of the corresponding standards - pregnenolone and progesterone. 90-95% of the radioactivity applied on the thin layer chromatoplate was recovered in the peaks. There was no significant radioactivity in the area corresponding to androstenediol. Androstenediol separated from progesterone in these solvents.

(b) On oxidation with chromium trioxide (see Chapter 2.5.1A) pregnenolone was converted to an ultra violet light absorbing spot which behaved like progesterone in all TLC systems examined. Progesterone remained unchanged.

(c) On acetylation (see Chapter 2.5.1C) only pregnenolone acetate was formed.

In both the above cases 85-90% of the radioactivity was recovered in the converted compounds after TLC. Authentic steroid controls too gave similar results.

(d) Enzymatic conversion with steroid 3β-ol dehydrogenase. Pregnenolone and progesterone were separately incubated with a steroid 3β-ol dehydrogenase preparation from Pseudomonas testosteroni as described in Chapter 2.5.2. After extraction and TLC, all the radioactivity of pregnenolone was recovered in the ultra violet spot corresponding to authentic progesterone. Progesterone remained essentially unchanged.

2. Testosterone, Androstenedione, 17α-Hydroxyprogesterone

(a) Rechromatography

These 4-ene-3-oxo-steroids, which were not easily distinguished after TLC in system VII, were clearly separated by TLC in either solvent system II or VI. The accumulation of steroids in this group was found to vary depending on the age of the rats and conditions of incubation used (as discussed in Chapter 4). In most cases, testosterone and androstenedione (Ratio 3:1) were the major steroids isolated after incubation of mature testes with [4-^{14}C] cholesterol and either NADPH or isocitrate plus $NADP^+$. System VI also clearly separated 17α-hydroxypregnenolone from the above 4-ene-3-oxo-steroids. However, no significant radioactivity was observed in the region corresponding to authentic 17α-hydroxypregnenolone.

(b) Oxidation

After oxidation and TLC in system VI, it was observed that androstenedione remained unchanged, whereas testosterone was converted to androstenedione. Under the oxidation conditions described only 50% of both the authentic [4-^{14}C] 17α-hydroxyprogesterone (control) and isolated steroid were oxidized to testosterone.

(c) Reduction

After reduction and TLC, testosterone was found unchanged, whereas androstenedione was converted to testosterone. The radioactive compound exhibited the same mobilities as the authentic steroids in solvent systems II and VI.

(d) Acetylation

After acetylation and TLC in solvent system V and VI, testosterone acetate formed was well separated from the unreacted androstenedione and 17α-hydroxyprogesterone.

3.6 CHOLESTERYL ESTERS - TLC SEPARATION

Occasionally an additional radioactive peak was obtained from the mitochondrial incubations (using NADPH generating system. Rats 7-9 weeks) which migrated close to the solvent front in solvent system VII and corresponded to cholesterol esters (as represented by standard cholesterol-oleate or -palmitate or -linolenate). This fraction sometimes accounted for as much as 2% of the radioactive cholesterol added. The conditions which resulted in the esterification of cholesterol were not investigated. However, it was observed that when cholesterol esters were formed, cholesterol side-chain cleavage activities were low.

The cholesterol ester fraction from several plates were pooled, eluted and the extracts concentrated under nitrogen, as previously described, and aliquots taken for analysis.

(a) Hydrolysis

Aliquots of the ester fraction were hydrolysed with methanolic KOH, as described in Chapter 2.8.2, and the extracts submitted to TLC in solvent system VII and IV. 92% of the radioactivity migrated as a single peak corresponding to standard cholesterol, thus confirming this fast moving fraction as consisting mainly of cholesterol.

(b) Fractionation of cholesterol esters by TLC

Insight into the nature of the esters of cholesterol was achieved by TLC on silver nitrate-impregnated silica gel plates. This procedure separated the various cholesterol esters according to their degree of unsaturation. The method

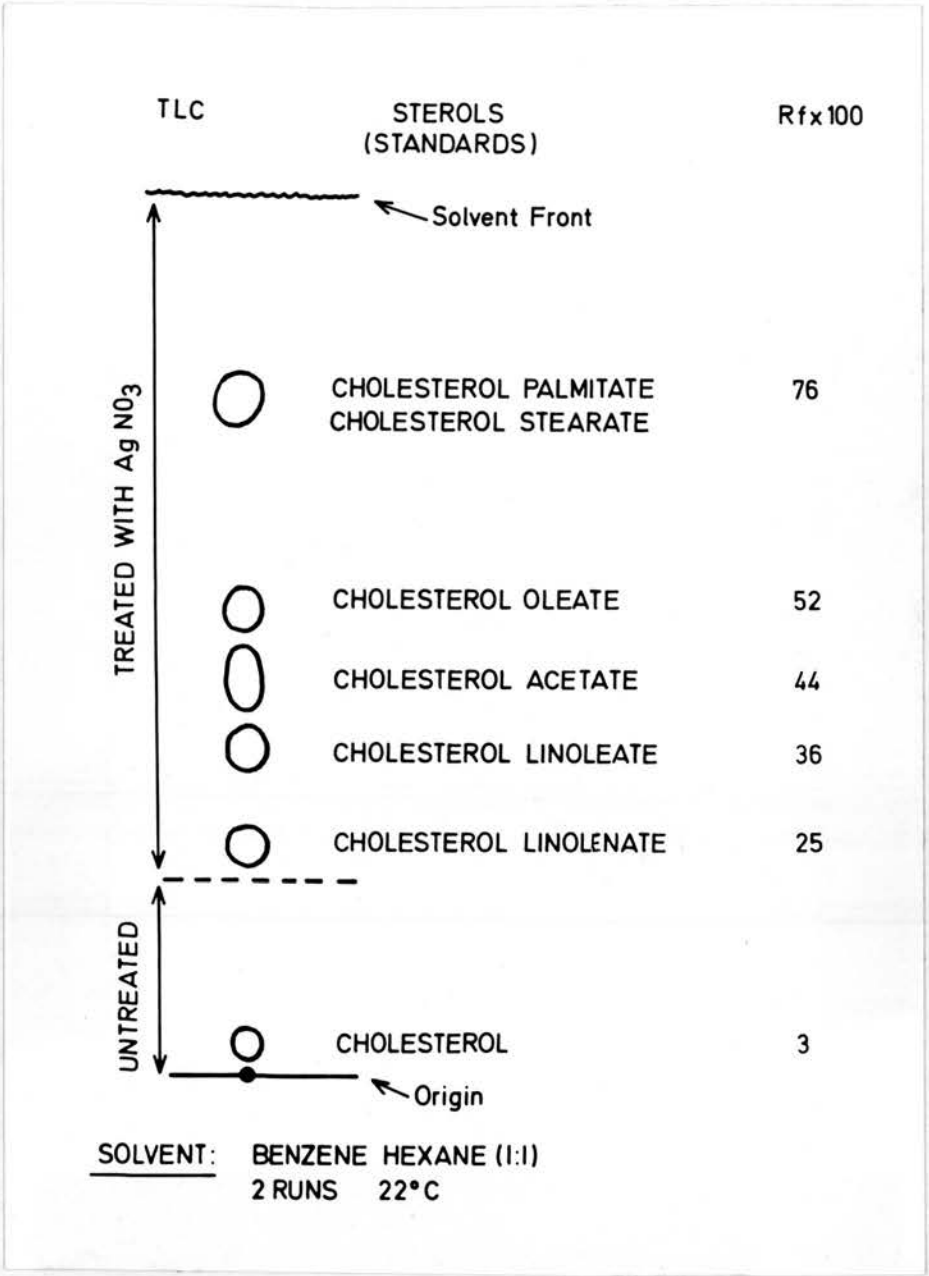


FIGURE 3.11 TRACE SHOWING A TYPICAL TLC RESOLUTION OF CHOLESTEROL-ESTERS

The upper 15 cm portion of preprepared silica-gel GF254 plates were impregnated with silver nitrate. Plates were developed twice in benzene: hexane (1:1). Cholesterol esters were located under u.v. after spraying with 0.05% rhodamine 6G in methanol.

employed is a modification of that described by Morris (1963) and Goodman and Shiratori (1964).

Pre-prepared silica-gel F254 plates, 0.25 mm layer thickness and size 5 x 20 cm were used. The upper 15 cm of the plates were impregnated with silver nitrate by dipping the plates in 90% methanol saturated with silver nitrate for 30 seconds (for better reproducibility) and air dried. The area of the plate 4 cm from the line of origin was left untreated. The isolated cholesterol ester extract and standard cholesterol esters representing different degrees of unsaturation (see Figure 3.11) were spotted and the plates developed twice at room temperature in benzene:hexane (1:1). The separated components were located under ultra violet light after spraying with 0.05% rhodamine 6G in methanol. The compounds appeared as orange spots against a greenish-yellow background. The results illustrated in Figure 3.11 show that the different classes of cholesterol fatty acid esters were clearly resolved from each other, cholesterol and cholesteryl acetate. The radioactivity was found to be fairly evenly distributed among the different classes of esters.

SUMMARY

1. The subcellular distribution of the cholesterol side-chain cleavage enzyme system was investigated. This enzyme system was localized entirely in the heavy mitochondrial fraction. Light mitochondria, microsomes and supernatant were completely devoid of any cholesterol side-chain scission activity.

2. Fractionation of the heavy mitochondrial preparation was achieved by discontinuous density gradient centrifugation using 0-10% ficoll. The heavy sedimenting fractions contained most of the cholesterol side-chain cleavage activity. This fractionation resulted in a 3-4-fold purification over the crude heavy mitochondria.

3. The phosphorylative and respiratory control capacities of the testicular heavy mitochondria were measured. They indicated that the mitochondria prepared were biochemically intact. Respiratory control ratios could be demonstrated only when potassium phosphate, EDTA and BSA were all present together. Mg^{++} uncoupled the mitochondria. The optimal requirements for these factors were investigated and are discussed.

4. A procedure employing multiple development, one-dimensional TLC in Di-isopropyl ether:petroleum ether:glacial acetic acid (80:35:2) solvent system and silica gel F254 preprepared glass plates, was developed for resolving the multiple mixture of intermediate steroids in testosterone biosynthesis from cholesterol. Other systems investigated were not suitable.

5. A modified TLC procedure for fractionating cholesterol esters according to their degree of unsaturation is presented.

Not only were the different classes of cholesterol fatty acid esters resolved from each other but also separated from cholesterol and cholesterol acetate.

6. Identification of the steroid products formed from cholesterol was based on the following criteria:-

(a) Identical mobilities of the metabolites with those of authentic steroid standards on TLC developed in different solvent mixtures.

(b) Identical chromatographic behaviour with those of authentic steroids after reactions such as

(1) acetylation with acetic anhydride in pyridine;

(2) oxidation with chromic acid in acetic acid;

(3) reduction with borohydride in methanol;

(4) enzymatic conversion using purified microbial enzyme preparations.

CHAPTER 4

SOME CHARACTERISTICS OF THE CHOLESTEROL SIDE-CHAIN CLEAVAGE REACTION OF RAT TESTIS

CONTENTS

SOME CHARACTERISTICS OF THE CHOLESTEROL SIDE-CHAIN CLEAVAGE REACTION OF RAT TESTIS

CHAPTER 4

CHAPTER 4 cont'd

CHAPTER 4

SOME CHARACTERISTICS OF THE CHOLESTEROL SIDE-CHAIN CLEAVAGE REACTION OF RAT TESTIS

In this chapter the studies on the effect of different nucleotides, Krebs cycle intermediates, metal ions, reducing agents and inhibitors, on the rate of cholesterol side-chain cleavage and the nature of steroid products are presented. Also discussed are the optimal conditions for cholesterol side-chain cleavage.

4.1 REQUIREMENT OF CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY FOR NADPH

Washed rat testes heavy mitochondria were incubated in medium A (see Chapter 2, Table 2.2) for 2 hours at 37°C in the presence or absence of different nucleotides as indicated in Figure 4.1. The results show that in the absence of a reducing source no side-chain cleavage of cholesterol occurred. NADPH (supplied by an extra mitochondrial generating system consisting of 2.5 mM $NADP^+$, 40 mM glucose-6-phosphate and glucose-6-phosphate dehydrogenase) was the most efficient electron donor. NADH was only one-tenth as effective as NADPH. NAD^+ was totally ineffective. Neither $NADP^+$ alone nor $NADP^+$ in the presence of glucose-6-phosphate could support cholesterol side-chain cleavage activity, suggesting that these mitochondria did not possess glucose-6-phosphate dehydrogenase activity. The 40-50% inhibition of the NADPH supported side-chain cleavage activity by NADH might be

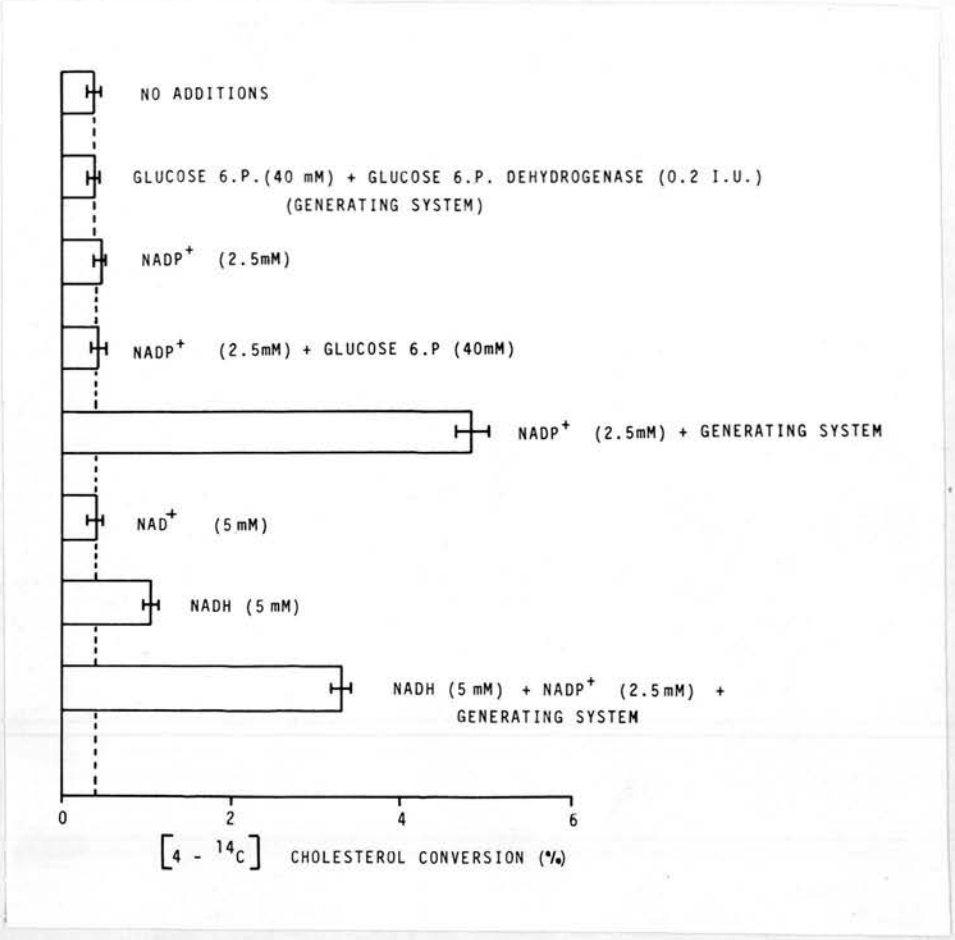


FIGURE 4.1 REQUIREMENT OF NADPH FOR CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY

Washed heavy mitochondria (0.45 mg protein/tube) were incubated with [4-^{14}C] cholesterol (200,000 c/m) in medium A for 2 hours with nucleotides and generators as shown.
The bars represent the mean and SEM of four separate experiments, each involving duplicate determinations. Rats used were of the same age group (10 weeks old). Generating system (extra-mitochondrial) consisted of 40 mM glucose-6-phosphate and 0.2 I.U. glucose-6-phosphate dehydrogenase.

explained if it was supposed that electrons were lost via the respiratory system when NAD(H) was available (see Figure 9.1). It might also be explained if testes heavy mitochondria were like the mitochondrial preparation from immature rat ovary, in possessing an NAD^+-dependent 5-ene-3β-hydroxysteroid dehydrogenase which converts pregnenolone to progesterone (Sulimovici & Boyd, 1969). The presence of NADH would therefore influence the equilibrium of the enzyme causing the accumulation of pregnenolone. This explanation seems unlikely as it was observed that in rat testis pregnenolone is removed by conversion to androgens via an $NADP^+$-dependent pathway (Shikita & Tamaoki, 1965; also see Chapter 8).

The results obtained do not agree in every respect with those of other workers in the field. Dorfman and co-workers (Menon et al., 1967; Drosdowsky et al., 1965; Toren et al., 1964) reported that their mitochondrial preparation from rat testis contained glucose-6-phosphate dehydrogenase activity: $NADP^+$ plus glucose-6-phosphate or $NADP^+$ alone supported the cholesterol side-chain cleavage reaction. NAD^+ was found to be half as effective as NADH. Glucose-6-phosphate dehydroenase, although primarily localized in the interstitial cells (Niemi & Ikonen, 1962; Ito, 1966; Blackshaw & Elkington, 1970) is a cytoplasmic enzyme. Therefore, it is possible that the mitochondrial preparations in the above mentioned reports were contaminated with cytoplasmic proteins.

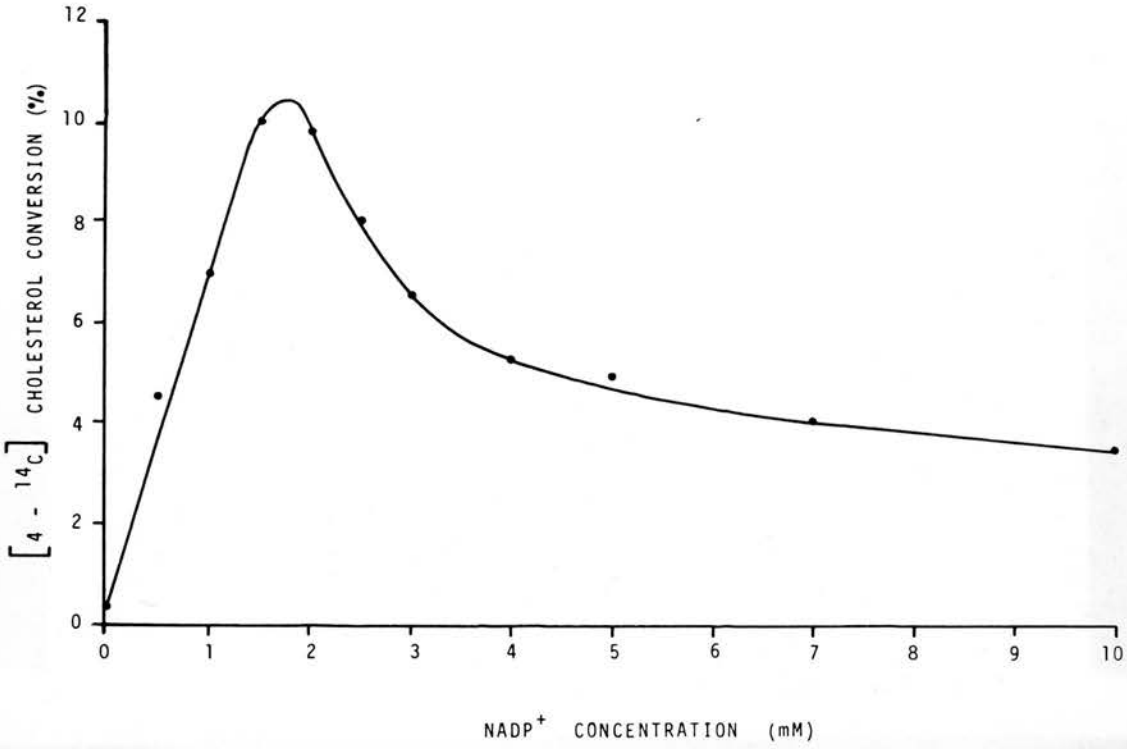


FIGURE 4.2 EFFECT OF INCREASING NADP(H) CONCENTRATIONS ON CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY

Washed heavy mitochondria ficoll fraction 5 (0.4 mg protein) was incubated at 37°C for 2 hours in medium A with [4-^{14}C] cholesterol, generating system (glucose-6-phosphate 40 mM and glucose-6-phosphate dehydrogenase 0.2 I.U.) and varying concentrations of $NADP^+$. Rats were 14 weeks old.

4.2 EFFECT OF INCREASING NADPH CONCENTRATIONS ON THE RATE OF CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY

Depending on the type of steroidogenic tissue increasing NADPH concentrations appears to have different effects on cholesterol side-chain cleavage. Thus Satoh et al. (1966) and Yago et al. (1967a) found that excess NADPH above 1 mM inhibited the side-chain cleavage in some adrenal cell preparations while high $NADP^+$ concentrations (5-10 mM) stimulated the reaction in bovine corpus luteum (Uzgiris et al., 1971).

The effect of varying concentrations of NADP(H) on cholesterol side-chain cleavage in washed heavy mitochondria ficoll fraction 5, prepared from rat testes are shown in Figure 4.2. It can be seen that cholesterol side-chain cleavage activity increased sharply until NADP(H) concentration was 1.8 mM. At higher concentrations NADP(H) became increasingly inhibitory. However, with unwashed heavy mitochondria as shown in Figure 4.3 the optimal concentration for NADP(H) was much higher (2.5 mM). NADP(H) at 5 mM was only slightly inhibitory. This difference in the inhibitory concentrations of NADP(H) between the crude and purified mitochondrial preparations could be due to the presence of phosphatases (and probably other hydrolytic enzymes) found to be present in the crude mitochondrial preparations. The presence of nicotinamide to protect the $NADP^+$ from hydrolytic activity or the titration of NADPH (i.e. addition of 0.8 mM aliquots of NADPH to the incubation medium at 20 minute intervals to keep the concentration constant) did not result in an increase in cholesterol side-chain cleavage activity

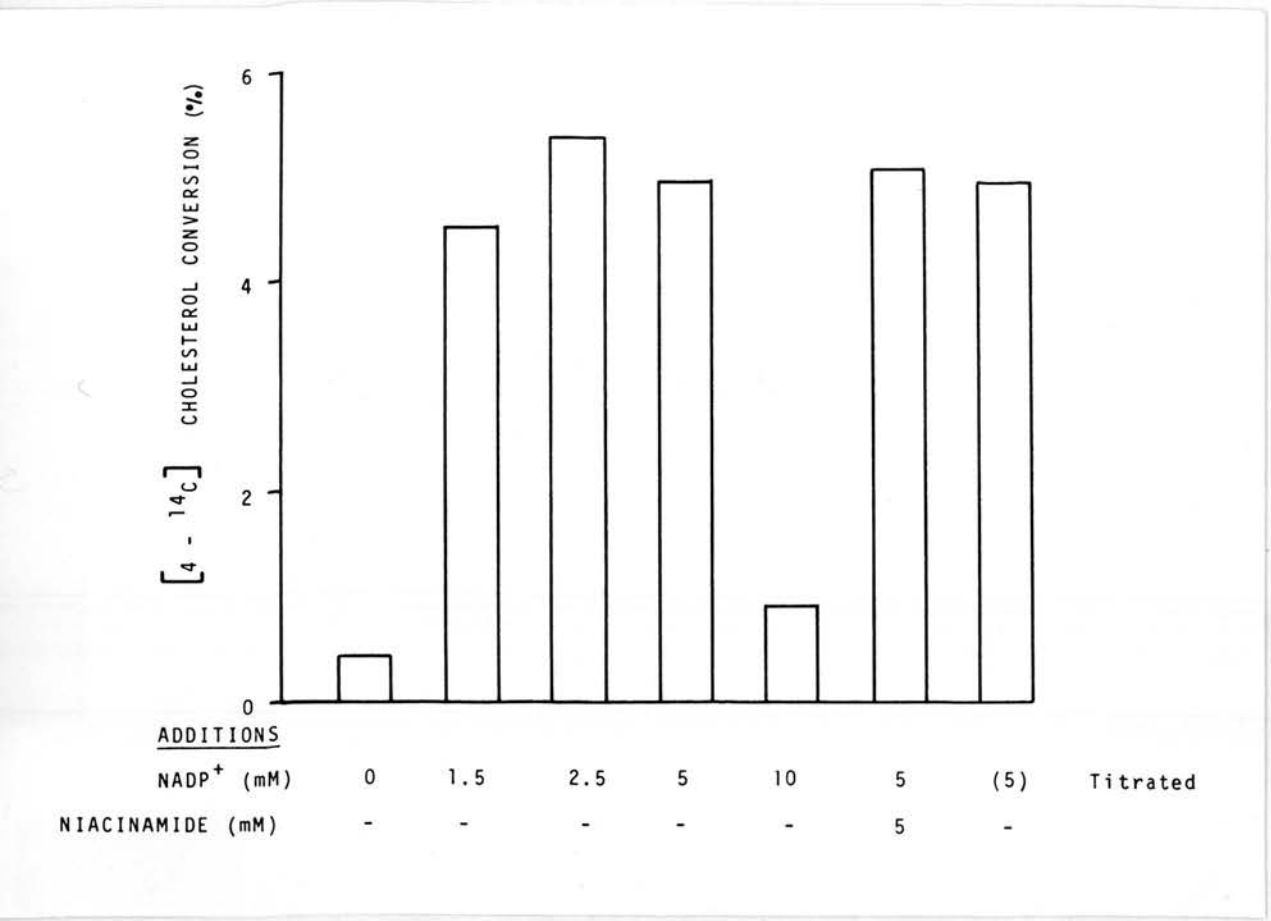


FIGURE 4.3 EFFECT OF UNWASHED MITOCHONDRIA ON OPTIMAL NADPH REQUIREMENTS FOR CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY

Unwashed heavy mitochondria (5,000 x g ppt) was incubated under similar conditions to that of Figure 4.2.with varying amounts of NADPH. In one tube marked "titrated" 0.8 mM aliquots of $NADP^+$ was added at 20 minute intervals. In another tube 5 mM niacinamide was included. Rats were 10 weeks old.

(see Figure 4.3). The concentrations of glucose-6-phosphate and glucose-6-phosphate dehydrogenase used were sufficient to completely reduce 10 mM $NADP^+$ in 3 minutes.

4.3. EFFECT OF VARYING THE AMOUNT OF RADIOACTIVE SUBSTRATE-[4-^{14}C] CHOLESTEROL ON CLEAVAGE ACTIVITY

Washed heavy mitochondria were incubated in medium A, in the presence of an NADPH generating system. The incubations were started by the addition of varying amounts of [4-^{14}C] cholesterol and continued for 1.5 hours at 37°C. Cholesterol side-chain cleavage activity was estimated as described in Chapter 2, section 2.4. The results in Figure 4.4 show that the amount of [4-^{14}C] steroid products formed was proportional to the amount of radioactive substrate added. The total percentage conversion of [4-^{14}C] cholesterol was constant under these experimental conditions. This relationship is probably a reflection of the abundant endogenous cholesterol present in these preparations. The total cholesterol content of heavy mitochondria, measured by GLC (see Chapter 2.8) was 33 ± 8 µg/mg protein, (85.27 ± 20.67 nmole per mg protein; mean ± standard deviation of 10 determinations; rats 10-15 weeks old). Of this total, esterified cholesterol accounted for less than 10%.

4.4 INFLUENCE OF VARIOUS COMPOUNDS ON THE NADPH SUPPORTED CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY

Washed testicular heavy mitochondria were incubated for 2 hours in medium A with [4-^{14}C] cholesterol, extra mitochondrial

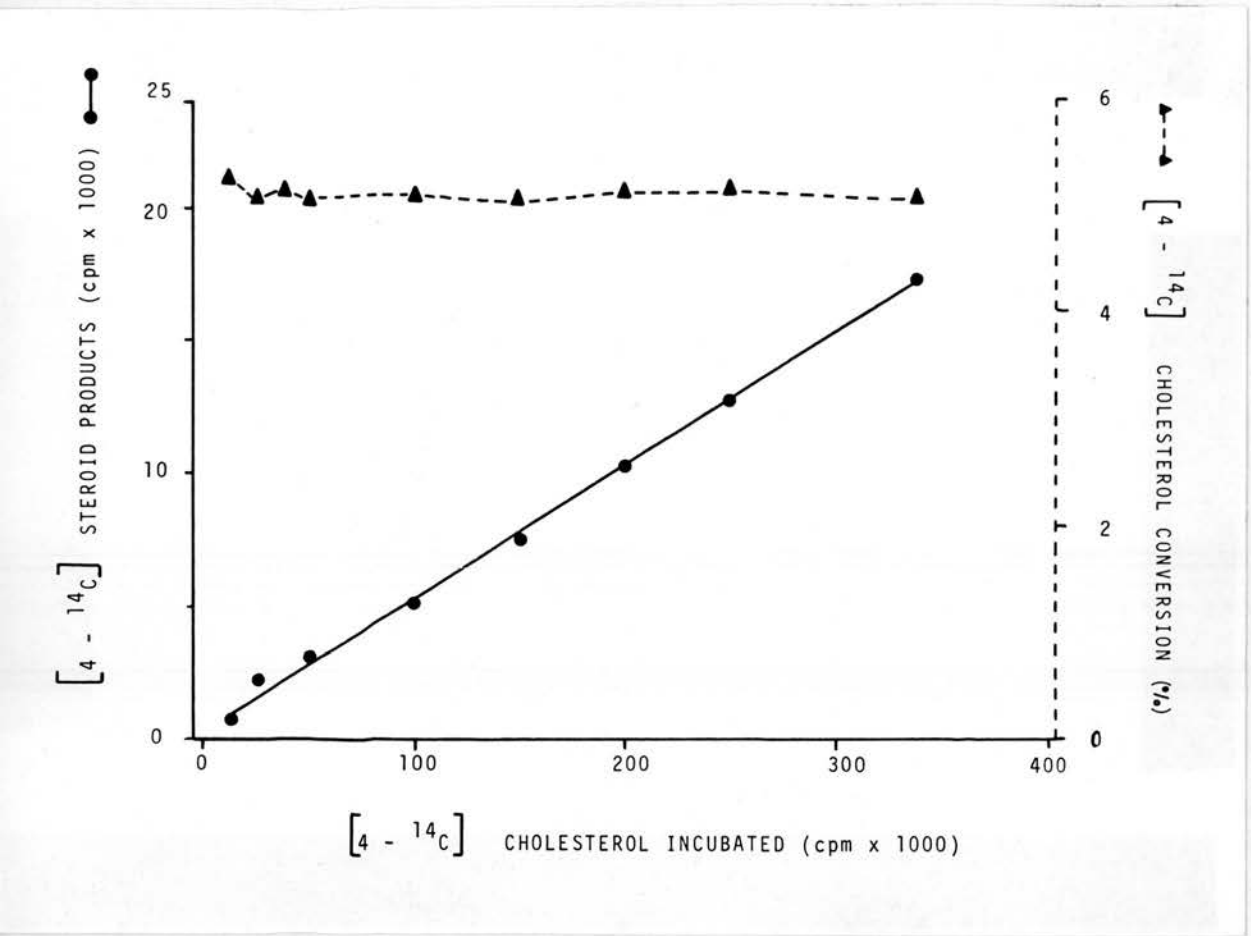


FIGURE 4.4 THE EFFECT OF INCREASING THE CONCENTRATION OF [4-^{14}C] CHOLESTEROL ON THE CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY

The solid line represents [4-^{14}C] steroid products formed, the dotted line is the activity expressed as the percent conversion of [4-^{14}C] cholesterol.

NADPH generating system (see Table 2.2) and different factors, Cholesterol side-chain cleavage was assayed in the usual manner (see section 2.4). The results are illustrated in Figure 4.5. Cyanide increased cholesterol side-chain cleavage activity (10%) probably by preventing NADPH removal via pyridine nucleotide transhydrogenase. ATP inhibited the side-chain cleavage activity by 30% and BSA (fatty acid free) by 65%. Ca^{++} in the presence of cyanide, almost doubled the enzymic activity. These effects of ATP, BSA and Ca^{++} can be explained on the basis of mitochondrial integrity and membrane permeability. ATP, at certain concentrations, prevents the cyclical low amplitude swelling and contraction characteristic of intact mitochondria. Mitochondria in such contracted states as well as intact mitochondria are known to be virtually impermeable to NADPH (Hirschfield & Koritz, 1964; Lehninger, 1965). Yago et al. (1967) reported that exogenous NADPH was a poor source of reducing equivalents for the cholesterol side-chain cleavage reaction in intact bovine corpora lutea. Bovine serum albumin (BSA) as demonstrated in Chapter 3.4.2.2 was found to have a protective effect on mitochondrial integrity (Peron & McCarthy, 1968; Sauer & Mulrow, 1969). Thus BSA could inhibit cholesterol side-chain cleavage activity by reducing the permeability of the mitochondrial membrane to externally generated NADPH. There are various theories for the effect of Ca^{++} ions. Ca^{++} causes swelling of the mitochondria (Lehninger, 1965) and thus allows exogenous NADPH access to the cholesterol side-

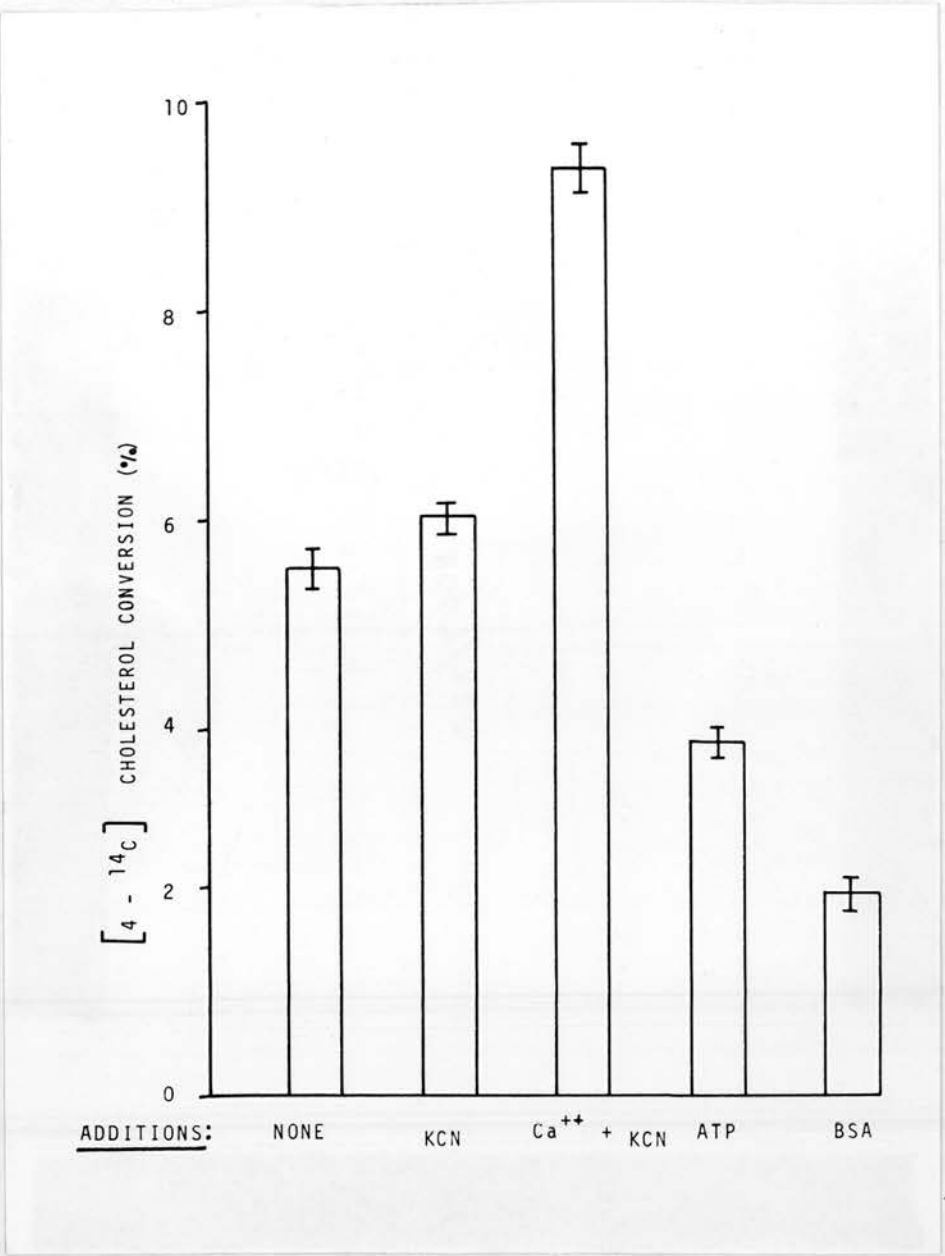


FIGURE 4.5 INFLUENCE OF VARIOUS COMPOUNDS ON NADPH SUPPORTED CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY

For each experiment the corresponding controls were included. Controls consisted of the complete incubation mixture with effectors but without generating system. Results presented are corrected values, i.e. experimental minus control values. The concentration of KCN was 2 mM, Ca^{2+} 2 mM, ATP 5 mM and BSA (fatty acid free) 1% (w/v).

chain cleavage enzymes. Ca^{++} ions could also enhance the cleavage of cholesterol to pregnenolone by activating the adenyl cyclase (Sayers et al., 1972); by stimulating the effect of cyclic AMP on steroidogenesis (Rubin et al., 1972; Haksar & Peron, 1972; Hermier & Jutisz, 1969) or by directly effecting the cholesterol side-chain cleavage enzyme system (Simpson et al., 1974). The observations in this present study appear to be compatible with the view that agents which induce mitochondrial swelling stimulate the conversion of cholesterol to pregnenolone, while those which decrease swelling inhibit this conversion.

The labelled steroid products formed from [4-^{14}C] cholesterol in the presence of extra mitochondrial NADPH generating system were testosterone, androstenedione, 17α-hydroxyprogesterone, progesterone and pregnenolone.

Under the incubation conditions described above no metal other than Ca^{++} could be shown to stimulate cholesterol side-chain cleavage activity. The addition of Mn^{++}, Mg^{++}, Co^{++}, EDTA and EGTA each at 10 mM concentrations, had no significant effect.

4.5 EFFICIENCY OF KREBS CYCLE INTERMEDIATES IN SUPPORTING CHOLESTEROL SIDE-CHAIN CLEAVAGE REACTION

In view of the possible relative impermeability of mitochondria to NADPH (Yago et al., 1967) and also because of the possible effect of phosphatases on $NADP^{+}$ and glucose-6-phosphate concentrations an alternative source of reducing equivalents was considered.

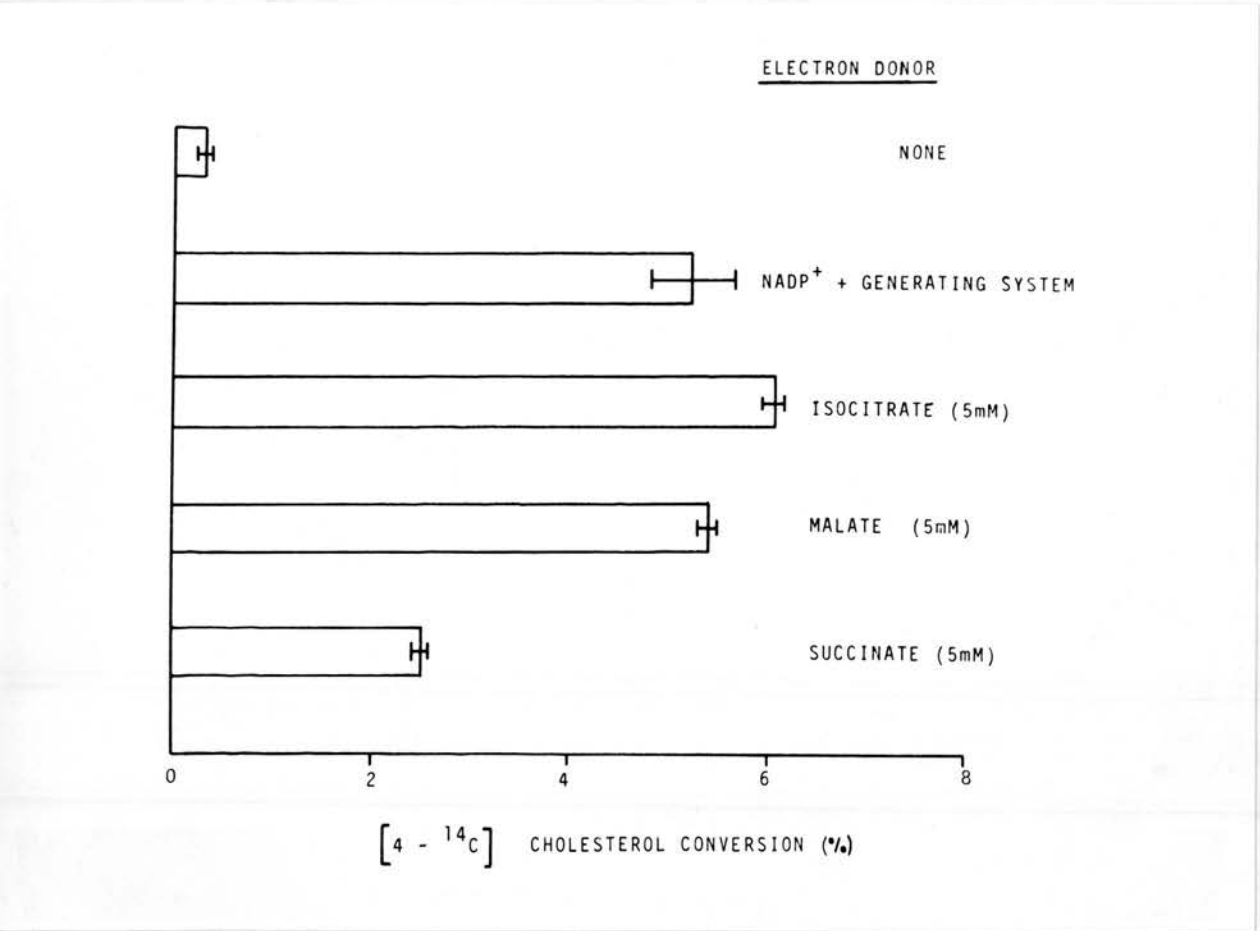


FIGURE 4.6 COMPARATIVE EFFICIENCY OF KREBS CYCLE INTERMEDIATES IN SUPPORTING CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY

Washed heavy mitochondria (0.45 mg protein) were incubated in medium A for 2 hours at 37°C in the presence of [4-^{14}C] cholesterol and the different electron donors. The NADPH generating system consisted of $NADP^+$ (1.5 mM); glucose-6-phosphate 40 mM and glucose-6-phosphate dehydrogenase 0.2 I.U. Krebs cycle intermediates were 5 mM each. Results are mean ± standard deviation of 8 duplicate determinations.

The ability of substrates other than extra mitochondrial NADPH, namely intermediates of the tricarboxylic acid cycle (TCA), to donate electrons and support steroids 11β-hydroxylation has been extensively documented in adrenal cortex mitochondria by several groups (Brownie & Grant, 1954; Sweat & Lipscomb, 1955; Harding et al., 1965; Cammer & Estabrook, 1967; Peron & McCarthy, 1968; Kahnt & Wettstein, 1951) but not so well studied for cholesterol side-chain cleavage.

The effect of three TCA intermediates - DL isocitrate L-malate and succinate were compared with the externally generated NADPH with regard to their ability to support the cholesterol side-chain cleavage reaction in the testis. The results are shown in Figure 4.6. All three TCA intermediates tested supported cholesterol side-chain cleavage. Isocitrate was a more effective electron donor than either malate or succinate. In comparison to the extramitochondrially generated NADPH, isocitrate was better and malate just as efficient in supporting cholesterol conversion, whereas succinate was only half as effective. Under the above assay conditions, with the Krebs cycle intermediates as electron donors, the products accumulating were mainly pregnenolone and progesterone. The 3-oxo -4-ene- steroid group — testosterone androstenedione and 17α-hydroxyprogesterone - accounted for less than 0.4% of the radioactivity of [4-^{14}C] cholesterol added.

These results are comparable with those concerning steroid hydroxylations in mitochondria of adrenal cortex (Peron & McCarthy, 1968) and corpus luteum (Robinson &

TABLE 4.1

RELATIVE EFFICIENCY OF TCA INTERMEDIATES AND NADPH IN SUPPORTING MITOCHONDRIAL CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY

Electron Donor	CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY							
	Adrenals			Corpus Luteum		Placenta	Testes	
	a	b	c	d	e	f	g	Present
Citrate	0	7	27	100	-	-	-	-
Isocitrate	0	13	25	96	78	43	78	100
α Ketoglutarate	0	64	36	41	-	-	2	-
Succinate	100	66	100	75	100	78	68	41
Fumarate	-	72	-	96	-	7	100	-
Malate	0	-	36	94	90	7	-	91
Oxaloacetate	-	-	-	30	-	-	-	-
NADPH	-	100	18	50	21	100	100	80

The relative activities are expressed as a percentage of that of the most effective electron donor studied in each series. Source of data:-

(a) Hall, P. F. (1967)
(b) Halkerston et al. (1961)
(c) Koritz, S. B. (1966)
(d) Robinson, J. (1971)
(e) Uzgires et al. (1971)
(f) Mason, I. (1970)
(g) Menon et al. (1967)

Stevenson, 1971) in that some TCA intermediates were apparently more efficient electron donors than the exogenous NADPH. In contrast, in the earlier work on testis (Menon et al., 1967) and placenta (Mason, 1970) it was reported that the TCA intermediates were less efficient than NADPH in supporting cholesterol side-chain cleavage activity.

The relative efficiency of the various TCA intermediates in supporting cholesterol side-chain cleavage activity, reported by other investigators, in different steroid producing tissues is presented in Table 4.1 and is compared with that obtained in this present study with rat testes.

Because isocitrate was found to be the most efficient of all electron donors studied and in particular because it yielded only pregnenolone and progesterone as steroid products, it was chosen as the electron source (i.e. intra mitochondrial NADPH generating system)in the assay for cholesterol side-chain cleavage.

4.6 FACTORS INFLUENCING THE ISOCITRATE SUPPORTED CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY

The influence of nucleotides, BSA, cations and anions on the isocitrate supported cholesterol side-chain cleavage reaction was investigated. The results presented in Figure 4.7 show that compared to isocitrate alone, the additional presence of $NADP^+$, stimulated the conversion of [4-^{14}C] cholesterol to labelled steroids, whereas NAD^+ had no appreciable effect, indicating the presence of an active $NADP^+$ dependent isocitrate dehydrogenase. Endogenous $NADP^+$ appears to be a limiting

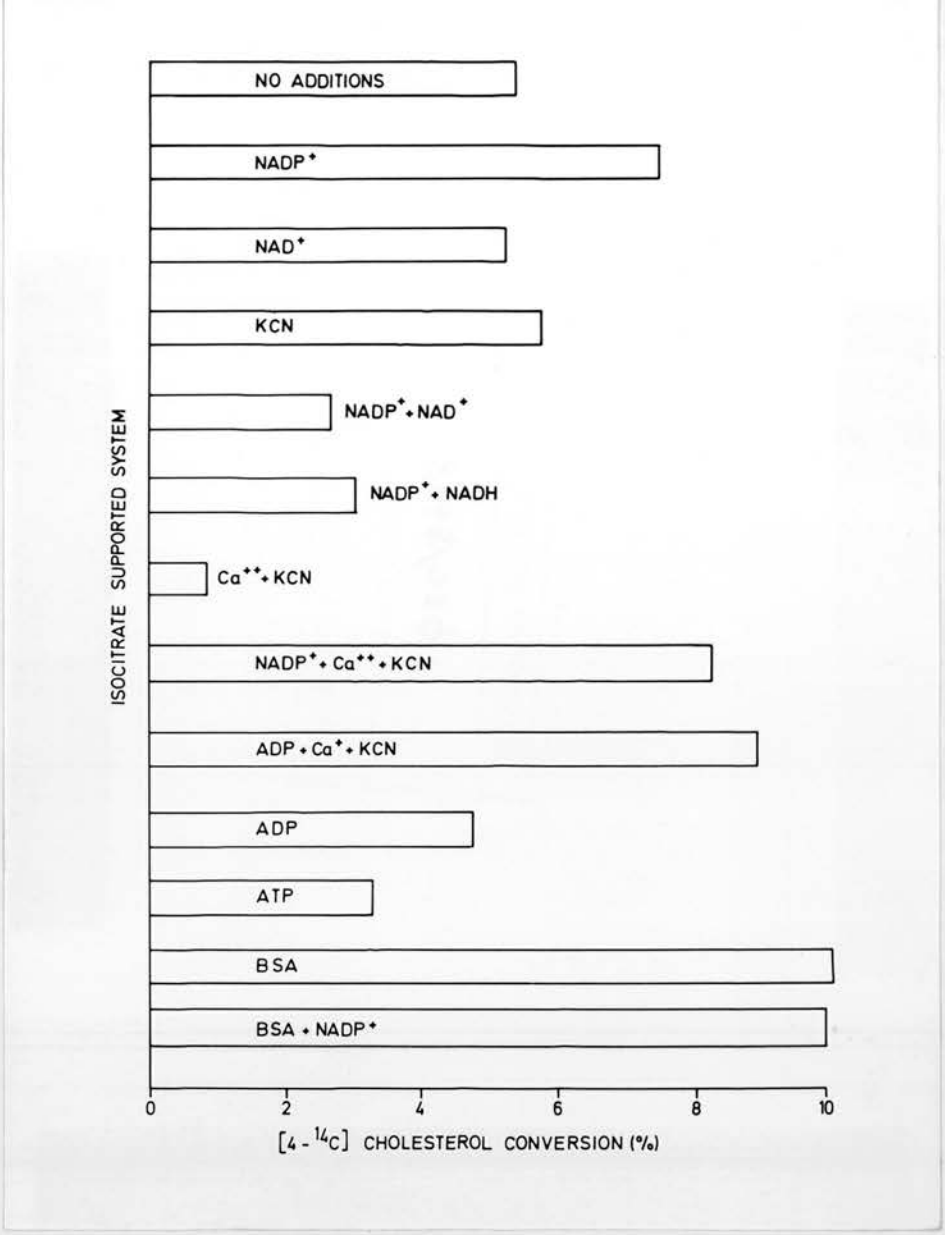


FIGURE 4.7 INFLUENCE OF DIFFERENT FACTORS ON CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY SUPPORTED BY ISOCITRATE

Washed heavy mitochondria (0.45 mg) were incubated in medium A with [4-^{14}C] cholesterol, isocitrate (5 mM) and different additional factors as indicated $NADP^+$ (5 mM); NAD^+ (5 mM), NADH (5 mM), KCN (2.0 mM), Ca^{2+} (2.0 mM), ATP (5.0 mM), ADP (5.0 mM) and BSA 1% (w/v). In each case controls were run which were identical to the experimental except that isocitrate was omitted. The control values obtained were subtracted from the experimental value.

factor in isocitrate supported side-chain cleavage activity in the washed heavy mitochondria. Cyanide - a respiratory inhibitor, slightly stimulated the cholesterol side-chain cleavage activity probably by conserving the intramitochondrially generated NADPH. When incubations were carried out in the presence of $NADP^+$ plus NAD^+ or $NADP^+$ plus NADH support for the cleavage activity was reduced by up to 50%. This inhibition could be explained as due to the activation of transhydrogenase by NAD^+.

In contrast to the stimulatory affect of Ca^{++} on the extramitochondrial NADPH supported system, with the isocitrate supported system the presence of Ca^{++} resulted in a pronounced inhibition of cholesterol side-chain cleavage activity. This inhibition could be attributed to the loss of intramitochondrial NADP(H) which could occur as a consequence of the Ca^{++} induced swelling and disruption of the structural integrity of the mitochondria. This view was supported by the observation that when exogenous $NADP^+$ was added to this system consisting of isocitrate, Ca^{++} and cyanide, the reaction was now greatly enhanced. The rate of cholesterol side-chain cleavage was much higher than that obtained with isocitrate plus $NADP^+$. A similar marked stimulation of the cholesterol side-chain cleavage reaction was also observed when ADP was included in the incubations, together with Ca^{++} and cyanide. When ATP or ADP alone were added to the isocitrate supported cholesterol side-chain cleavage system they were found to be slightly inhibitory. Although the magnitude of the stimulation of NADP and ADP in the presence of Ca^{++} were similar, the important difference was in the products formed (see Figure 4.8 and section 4.7).

An explanation for these results is possible if it is assumed that NADH:NADPH transhydrogenation is occurring in these mitochondrial preparations. Kaplan et al. (1952, 1953) and Kaufman and Kaplan (1961) have described such a mitochondrial pyridine nucleotide transhydrogenase which, when isolated from various mammalian tissues, had an equilibrium constant for the reaction close to unity (Kaplan et al., 1953). If such a reaction were occurring in the testis mitochondrial preparations, then the addition of inhibitors of NADH oxidation might be expected to displace the NADH:NADPH equilibrium in favour of NADPH. Furthermore, if one assumes that under normal conditions in steroidogenic mitochondria, most of the electron flux from NADPH is to the NADPH-cytochrome P450 reductase system, rather than to the transhydrogenase reaction, and that the greater part of the electron flux from NADH is to the NADH-cytochrome oxidase system, then some of the results shown in Figure 4.7 and also in Figure 4.5 can be explained:

(i) exogenous NADPH would be expected to be a better electron donor for cholesterol side-chain cleavage than exogenous NADH.

(ii) Inhibitors of NADH-cytochrome oxidase electron transport (e.g. CN^-) would cause diversion of electrons from NADH to NADPH, so stimulating cholesterol side-chain cleavage.

(iii) Agents which stimulate NADH-cytochrome oxidase electron flux (e.g. ADP and uncoupling agents) would displace the equilibrium of the transhydrogenase in favour of NADH and so tend to inhibit cholesterol side-chain cleavage.

The effect of bovine serum albumin on the isocitrate supported cholesterol side-chain cleavage reaction is shown in Figure 4.7. BSA (fatty acid free) had a marked stimulatory effect (approximately a 2-fold increase). The percentage conversions at the different BSA concentrations (0.5 to 2.5%) were the same. This stimulatory effect was probably due to the "protective effect" of BSA on the mitochondria; free fatty acids have been reported to effect the permeability of the mitochondrial membrane (Van den Bergh, 1967). BSA protects against this effect by binding the free fatty acids and thus prevents the leakage of endogenous cofactors essential for cholesterol side-chain cleavage. The failure of $NADP^+$ to exert any significant stimulatory influence on the isocitrate supported side-chain cleavage system in the presence of BSA (see Figure 4.7) is consistent with this view. In rat adrenal mitochondria, Peron and McCarthy (1968) also observed a stimulatory effect of BSA on 11β-hydroxylation of deoxycorticosterone supported by Krebs cycle intermediates. Robinson (1971) showed that the fatty acids in undefatted albumin could support cholesterol side-chain cleavage in porcine corpus luteum mitochondria.

4.7 INFLUENCE OF SUBSTRATES AND COFACTORS UPON THE PRODUCTS OF CHOLESTEROL METABOLISM BY RAT TESTICULAR HEAVY MITOCHONDRIA

It is evident from studies presented in this section that the total yield, the nature and ratio of products of cholesterol metabolism could be greatly influenced, depending

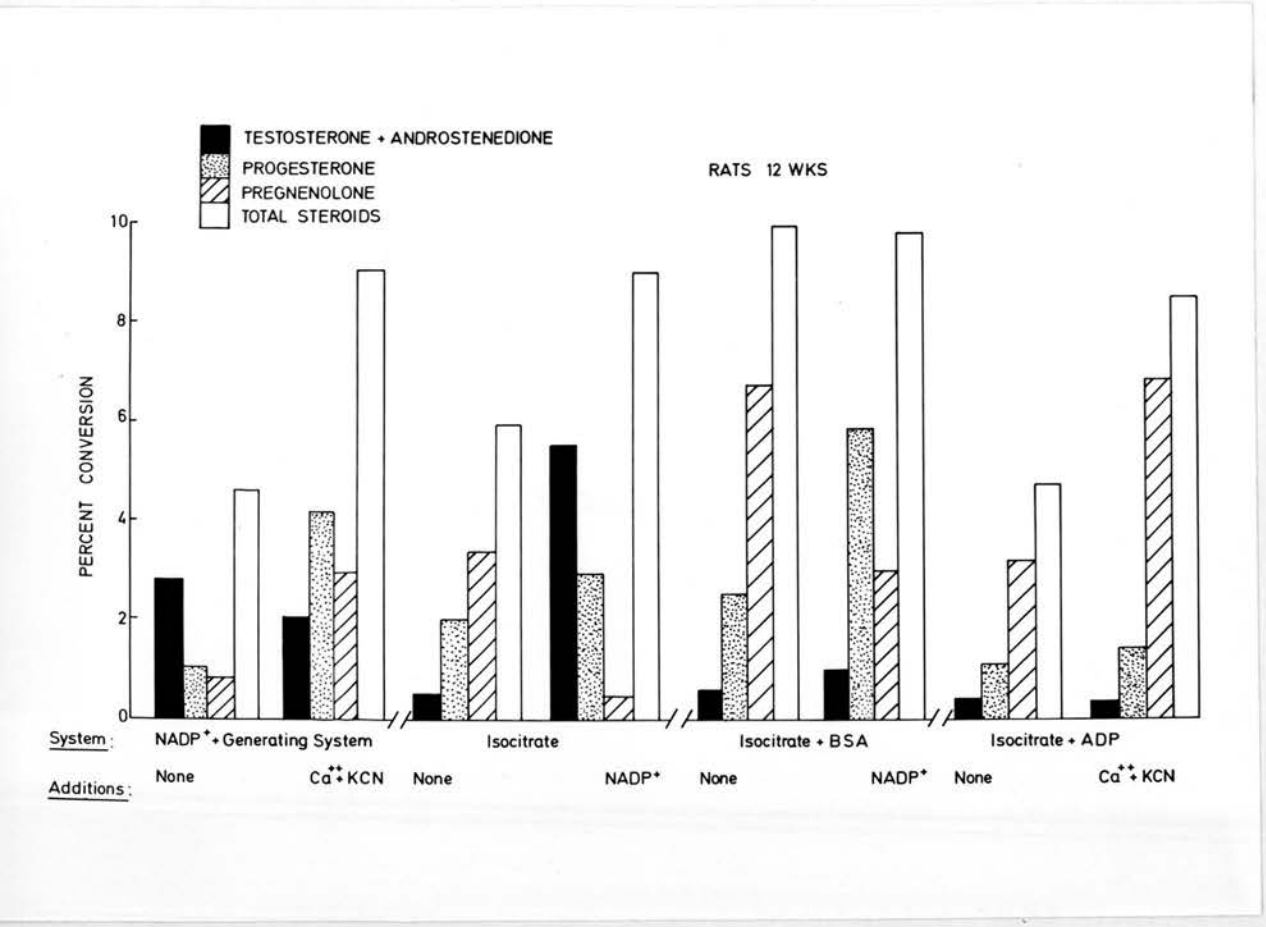


FIGURE 4.8 INFLUENCE OF DIFFERENT ELECTRON DONORS AND COFACTORS ON THE NATURE OF STEROIDS FORMED FROM CHOLESTEROL

Washed heavy mitochondria were incubated with [4-^{14}C] cholesterol and either extramitochondrial NADPH-generating system or DL isocitrate. In addition the following factors were added as indicated $NADP^+$ (5 mM), ADP (5 mM), Ca^{2+} (2 mM), KCN (2 mM) and BSA 1% (w/v).

on whether the electron source employed during incubations, was the extra mitochondrial NADPH generating system or isocitrate. The nature of steroid products could be further altered by the addition of certain factors such as $NADP^+$, Ca^{++}, BSA, ADP, and cyanide to the incubation. These variations in steroid products formed are summarized in Figure 4.8.

When the extramitochondrial NADPH was used as an electron source for cholesterol side-chain cleavage, testosterone, androstenedione and 17α-hydroxyprogesterone (i.e. 3-oxo-4-ene steroids) were the main products together with small amounts of progesterone and pregnenolone. The accumulation of pregnenolone was generally smaller than the other steroids formed. Under the experimental conditions used the amounts of pregnenolone and progesterone together did not exceed 70% of the total 3-oxo-4-ene steroids present at the end of the reaction. The ratio of the three 3-oxo-4-ene steroid products often varied with different mitochondrial preparations and age of the rats. In immature rats (7-8 weeks old) the predominant steroids found were androstenedione and 17α-hydroxyprogesterone and in older rats (9 weeks plus) testosterone and androstenedione. It would appear therefore that rat testes heavy mitochondria contain all the enzymes necessary for androgen biosynthesis from cholesterol, namely cholesterol side-chain cleavage system, 3β-hydroxysteroid dehydrogenase, 17α-hydroxylase, C-17,20 lyase and 17β-hydroxysteroid dehydrogenase. These findings are consistent with the earlier reports of Menon et al. (1965) and Tamaoki and

Shikita (1966) who observed that rat testis mitochondrial preparations possessed significant quantities of enzyme activities involved in the bio-transformation of pregnenolone to testosterone (but believed them to be of microsomal origin). In other steroid producing tissues, namely adrenals (Simpson & Boyd, 1967a), ovaries (Sulimovici & Boyd, 1968), corpus luteum (Robinson & Stevenson, 1971; Hall & Koritz, 1964) and placenta (Morrison, 1965; Mason, 1970), pregnenolone and/or progesterone were the main products formed by the mitochondria in the presence of NADPH.

In the presence of Ca^{++} plus extramitochondrial NADPH generating system, relatively more progesterone and pregnenolone accumulated in the testicular incubations than the 3-oxo-4-ene-androgens.

When isocitrate was used as the electron donor, the pattern of steroid products formed was different to that obtained with extramitochondrial NADPH. Pregnenolone was the major product with a smaller quantity of progesterone. (Ratio of progesterone/pregnenolone = 0.6.) The 3-oxo-4-*ene*-steroids formed rarely exceeded 0.5% of the total [4-^{14}C] cholesterol incubated. $NADP^{+}$ in addition to stimulating the isocitrate supported cholesterol side-chain cleavage reaction altered the pattern of steroids formed - mimicing that obtained with extramitochondrial NADPH. Testosterone was the main product together with progesterone (Ratio 2:1). The pregnenolone accumulation was very small and accounted for less than 5% of the total steroid products. These observations lend support

to the earlier suggestion that intramitochondrially generated NADPH, (in the presence of isocitrate) although readily available to the cholesterol side-chain cleavage enzyme complex, is not available to the enzymes responsible for the further metabolism of progesterone to testosterone.

When either BSA or ADP or ATP were added to the isocitrate supported cholesterol side-chain cleavage system, the end products isolated were essentially the same as with isocitrate alone, but the amount of pregnenolone accumulated was greater. The ratio of progesterone:pregnenolone was approximately 0.37. When Ca^{++} plus cyanide were present in addition to isocitrate and ADP, pregnenolone was the major product formed. Of the total labelled products formed pregnenolone accounted for 81%, progesterone 16% and the 3-oxo-4*ene*-steroids only 4%. When ADP was replaced by $NADP^{+}$ in the above experiment, the steroid pattern was again altered - testosterone was now the major product with little pregnenolone and progesterone (similar to $NADP^{+}$ plus isocitrate).

In the presence of BSA the stimulatory influence of $NADP^{+}$ on the isocitrate supported cleavage activity was prevented. The main products accumulating were progesterone and pregnenolone. Of significance was the observation that only a small amount of testosterone (1%) accumulated compared to 5.6% in the absence of BSA.

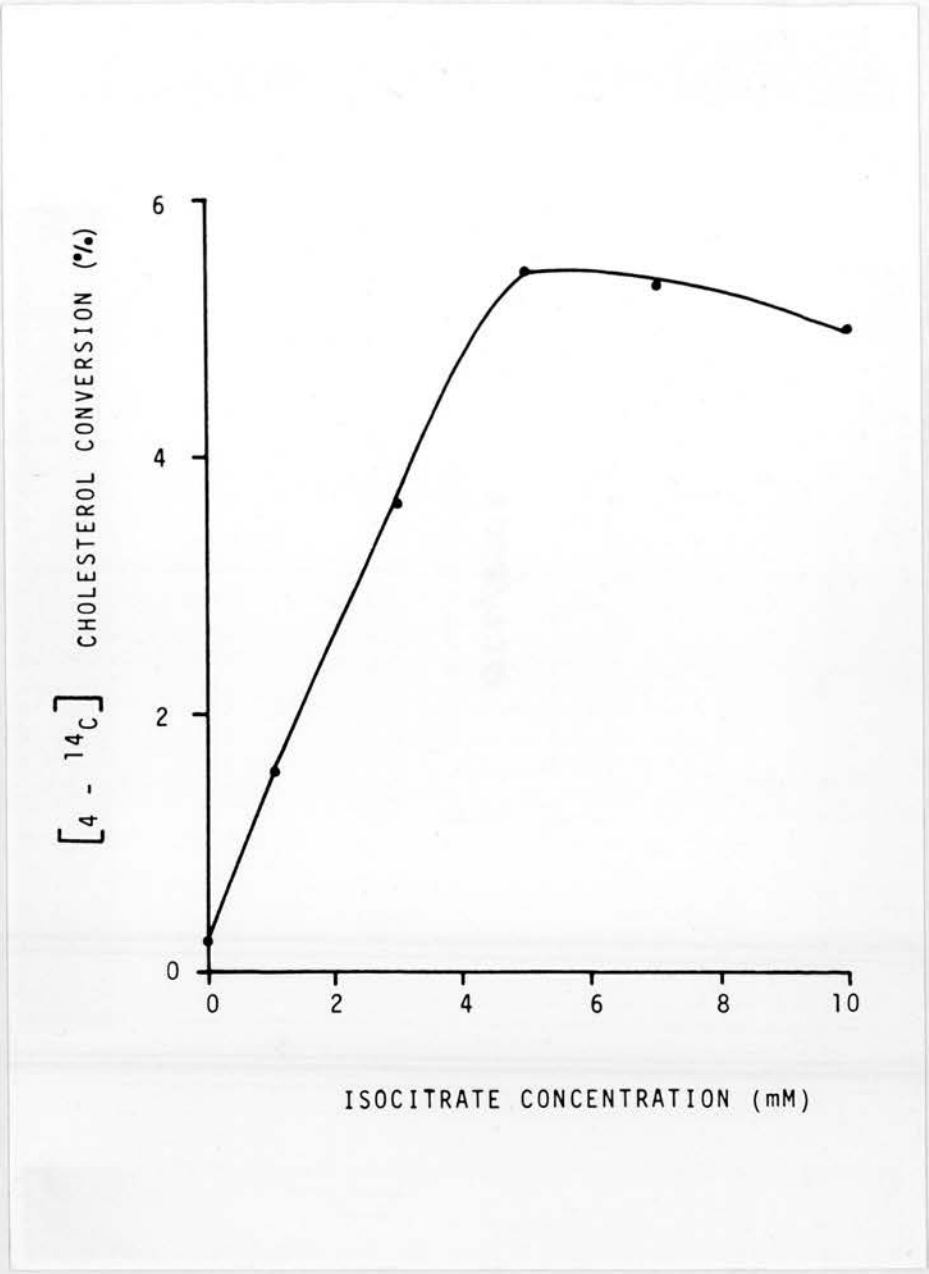


FIGURE 4.9 THE EFFECT OF VARYING CONCENTRATIONS OF ISOCITRATE ON CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY

Washed heavy mitochondria (0.45 mg protein) were incubated in medium A with [4-^{14}C] cholesterol and different amounts of DL isocitrate for 90 minutes at 37°C. Cholesterol side-chain cleavage activity was estimated as described in Chapter 2.

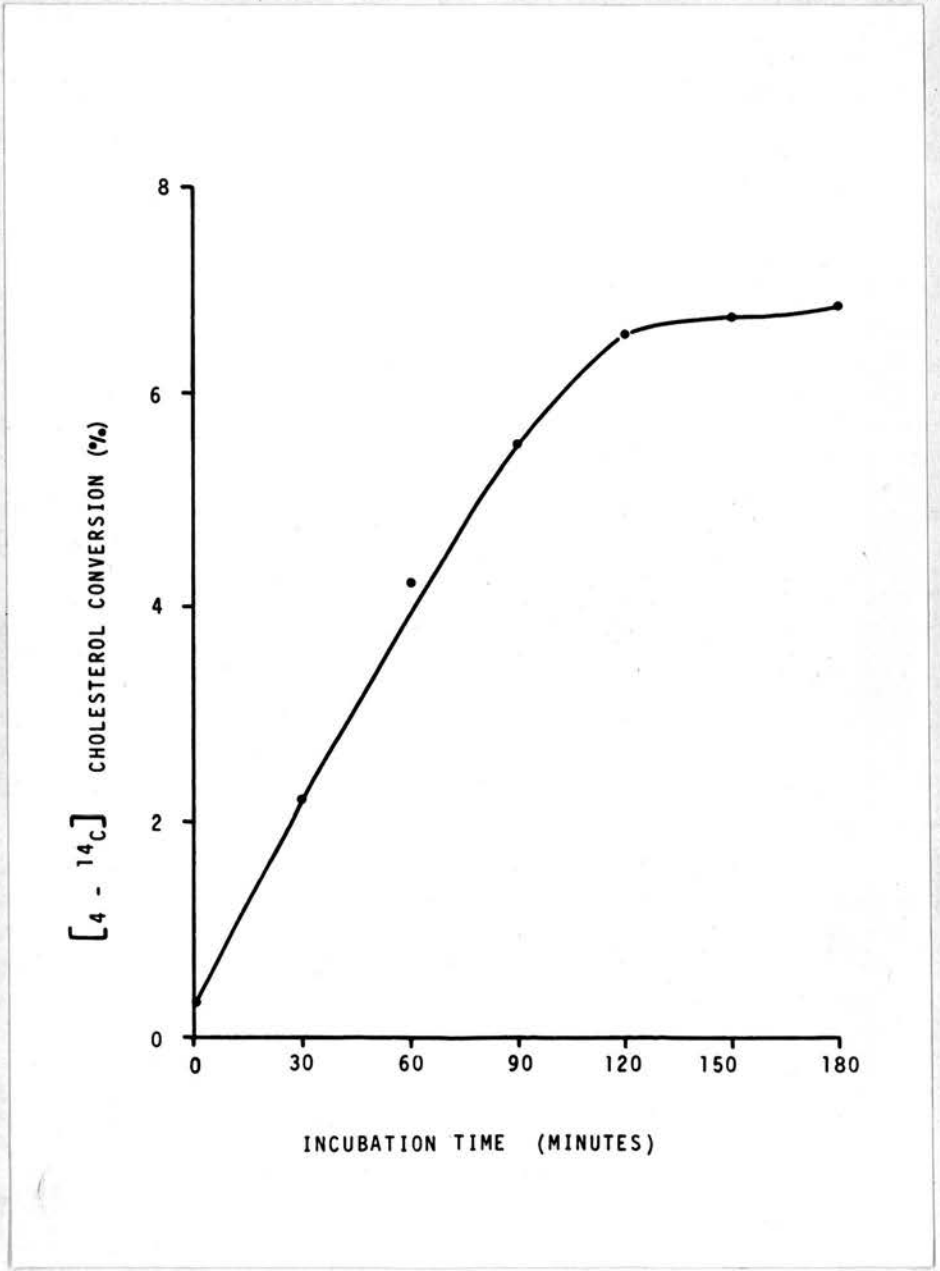


FIGURE 4.10 TIME COURSE OF THE REACTION

Washed heavy mitochondria were incubated in medium H with [4-^{14}C] cholesterol and isocitrate (5 mM) for varying periods up to 2 hours.

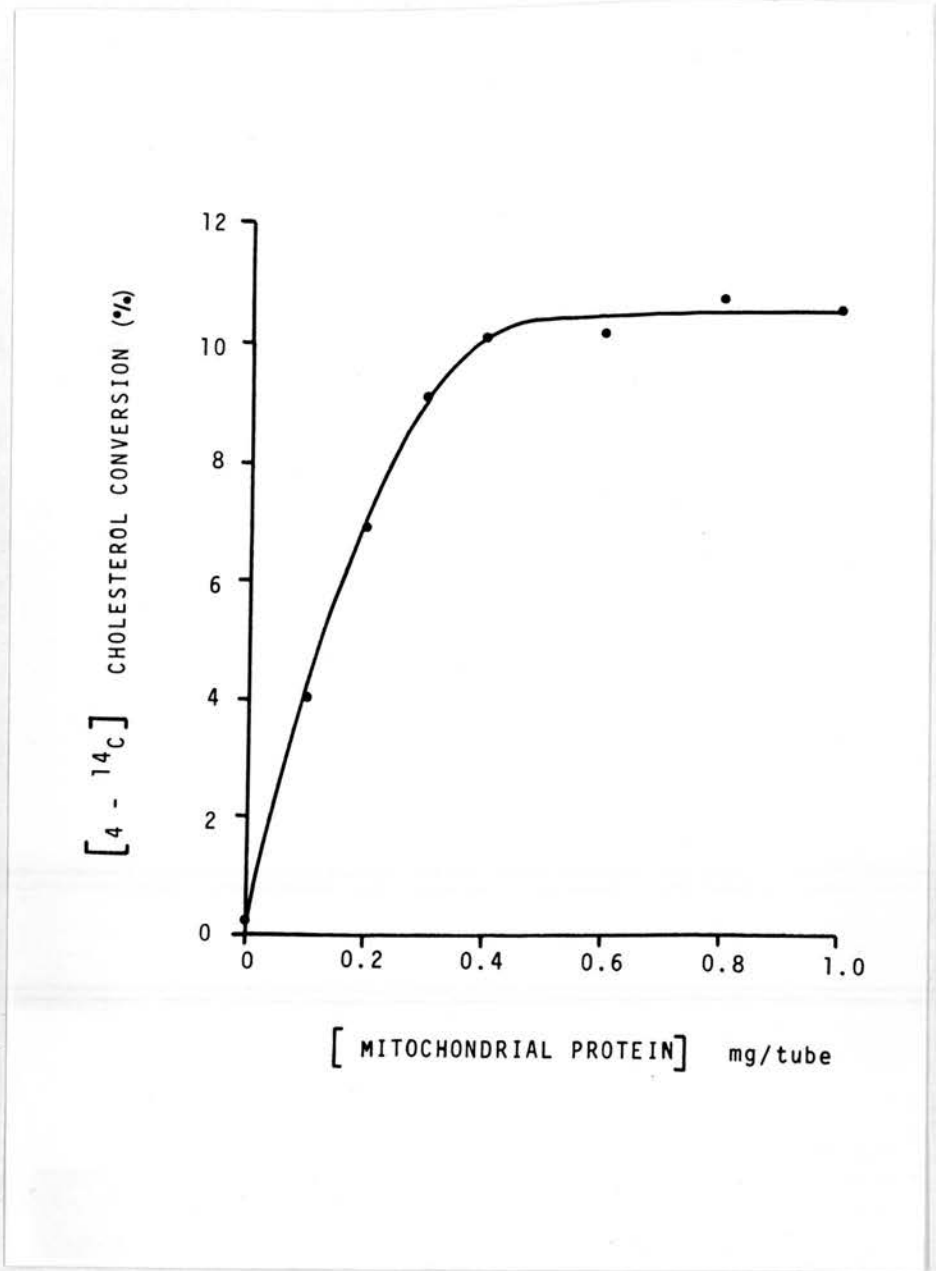


FIGURE 4.11 RELATIONSHIP BETWEEN CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY AND CONCENTRATION OF MITOCHONDRIAL PROTEIN

Incubation system consisted of [4-^{14}C] cholesterol in medium A; DL isocitrate (5 mM) and varying amounts of washed heavy mitochondria. Incubations were carried out at 37^{o}C for 90 minutes.

4.8 OPTIMAL CONDITIONS FOR ISOCITRATE SUPPORTED CHOLESTEROL SIDE-CHAIN CLEAVAGE REACTION

4.8.1 Effect of Varying Isocitrate Concentrations

Washed heavy mitochondria were incubated in medium A with [4-^{14}C] cholesterol and in the presence of different amounts of isocitrate for 90 minutes at 37°C, and cholesterol side-chain cleavage activity was estimated as described in Chapter 2, section 2. The results of a typical experiment are shown in Figure 4.9. The optimal concentration of isocitrate was 5 mM. A further increase up to 10 mM had little additional effect on the cleavage activity.

4.8.2 Time Course of the Reaction

This is shown in Figure 4.10. The rate of side-chain cleavage of cholesterol increased linearly for about 90 minutes. Similar results were obtained with extramitochondrial NADPH generating system.

4.8.3 Relationship Between Rate of Cholesterol Side-Chain Cleavage and Concentration of the Enzyme

Figure 4.11 shows the effect of varying amounts of mitochondrial enzyme on the rate of side-chain cleavage. Different amounts of washed heavy mitochondria were incubated with isocitrate (5 mM) and [4-^{14}C] cholesterol for 90 minutes. The rate of cleavage was linear up to 0.5 mg mitochondrial protein per tube.

4.8.4 Influence of Different Buffer Systems

For this experiment mitochondria were isolated in 0.25 M sucrose and incubated with isocitrate (5 mM), Mg^{++} (10 mM -

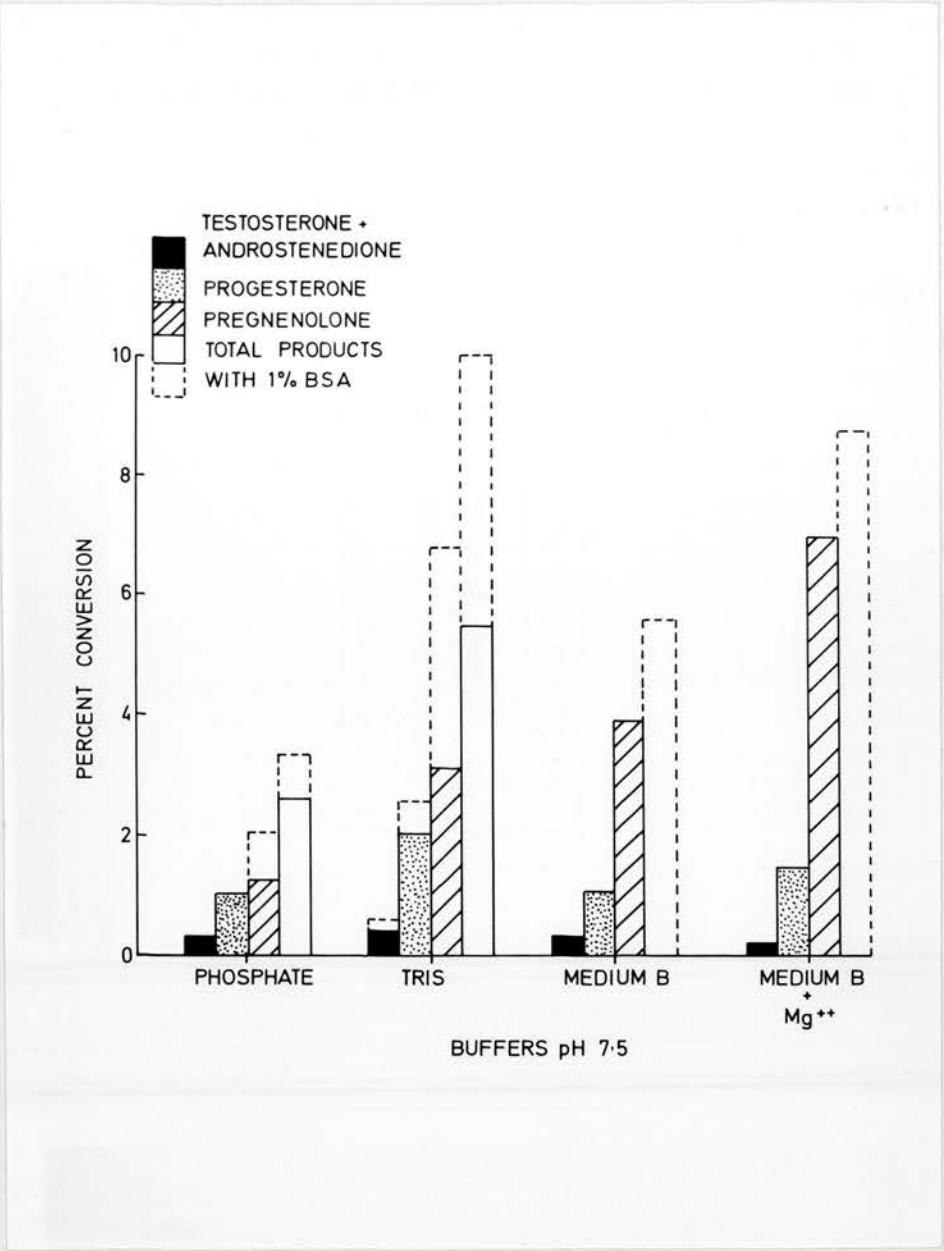


FIGURE 4.12 INFLUENCE OF DIFFERENT BUFFER MEDIA ON CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY

Washed heavy mitochondria isolated in 0.25 M sucrose were incubated at 37°C for 90 minutes with [4-^{14}C] cholesterol and DL isocitrate (5 mM) in different media at pH 7.5 with and without BSA (1% w/v). The final buffer concentrations were 60 mM. Mg^{2+} (10 mM) was present except in buffer medium B.
Rats were 11 weeks old.

except in medium B) and [4-^{14}C] cholesterol. Different buffer media (pH 7.5) with or without BSA - 1% (w/v) were used. The results are illustrated in Figure 4.12. It can be seen that phosphate buffer, with or without BSA, was a poor medium. Compared to other buffer media, phosphate buffer appeared to inhibit the cholesterol side-chain cleavage reaction. When Tris buffer was substituted for phosphate buffer the conversion of cholesterol to steroids doubled. The addition of BSA (1%) to the Tris buffer resulted in a further marked two-fold stimulation of the cleavage reaction. Medium B was more effective than phosphate buffer (with or without BSA) in supporting cholesterol oxygenase activity. This rate of cleavage was however comparable to that obtained with Tris buffer (in the absence of BSA) but very much less (40%) than that with Tris buffer plus BSA. Medium B contained BSA (0.15%) but no Mg^{++}. However, with the addition of 10 mM Mg^{++} to incubations containing medium B, a 50% stimulation of cholesterol side-chain cleavage was observed (Figure 4.12). As discussed earlier in Chapter 3, mitochondria exhibited respiratory coupling only in the presence of medium B. The presence of Mg^{++} completely uncoupled the mitochondria. From the above observations it appears that uncoupling conditions favour cholesterol side-chain cleavage.

The steroid products formed in each case were mainly pregnenolone and progesterone. The amount of 3-oxo-4-ene-steroids formed was very small - less than 0.5%. The ratio of progesterone:pregnenolone formed in both Tris and phosphate buffers without BSA was approximately equal (0.8), but

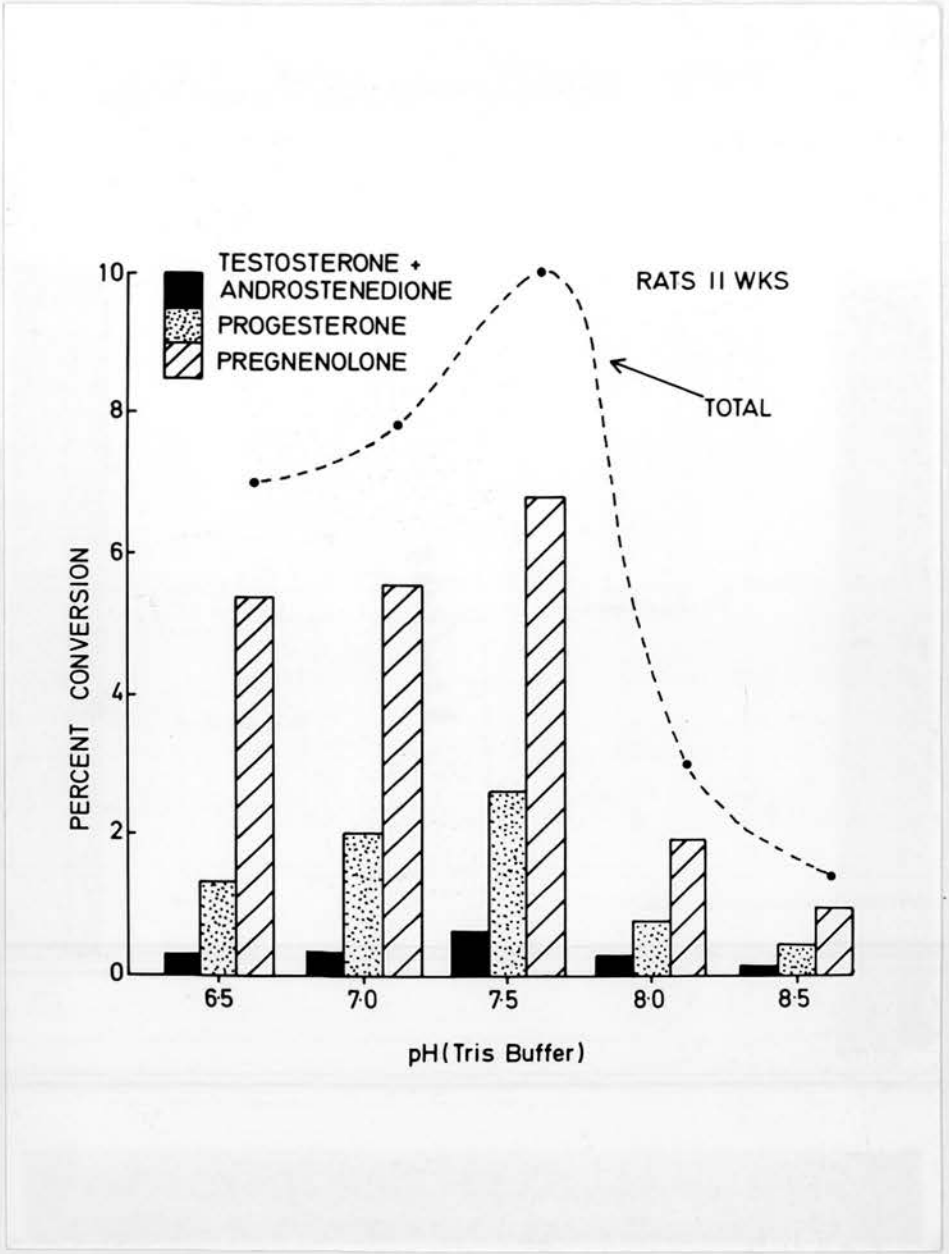


FIGURE 4.13 EFFECT OF VARYING pH ON ISOCITRATE SUPPORTED CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY

The pH of Tris buffer (60 mM) used varied between 6.5 and 8.5 (measured at room temperature 23^{o}C). 1% BSA was included in the incubation medium.

when BSA was present there was a marked accumulation of pregnenolone with comparatively little or no increase in progesterone or 3-oxo-4-ene-steroids. In the case of medium B, pregnenolone accounted for 63% of the total steroid formed and progesterone 23%.

4.8.5 Effect of Varying pH

Cholesterol side-chain cleavage estimations were carried out in medium A as described previously. The pH of the Tris buffer in the incubation medium was varied between 6.5 and 8.5 (measured at room temperature 23°C). In addition, incubation media contained 1% BSA. The activity profile over this range, is presented in Figure 4.13 and shows that the optimal pH was gradually reached near pH 7.5. The rate of cholesterol side-chain cleavage fell off very rapidly as the pH increased stepwise to pH 8.5. The data also show that the ratio of the products formed (progesterone and pregnenolone) were not significantly altered at different pHs. For the same reaction sequence in rat adrenal mitochondria Koritz (1962) found the pH optimum to be 6.2.

From the studies presented in this section the optimal conditions for the isocitrate-supported cholesterol side-chain cleavage reaction could be summarized as isocitrate 5 mM, Tris buffer pH 7.5 incubation time 90 minutes, and mitochondrial protein 0.5 mg/tube. These conditions were therefore used for most of the studies on cholesterol side-chain cleavage.

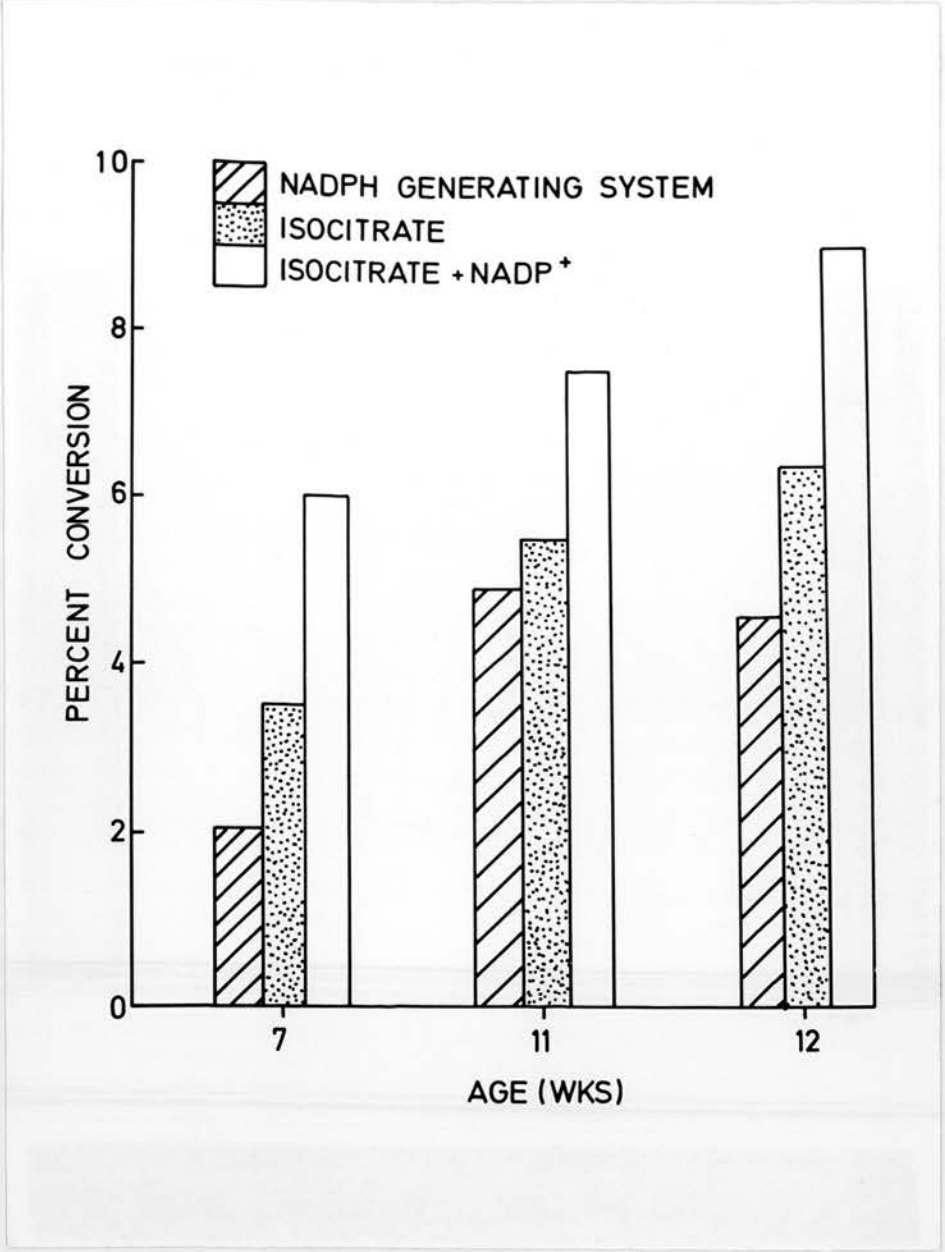


FIGURE 4.14 CORRELATION BETWEEN THE NADPH AND ISOCITRATE IN SUPPORTING CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY IN RAT TESTES OF DIFFERENT AGES

Washed heavy mitochondria (0.45 mg) from rats of different age groups were incubated for 90 mins. in medium A with [4-^{14}C] cholesterol and the following electron donor systems: extramitochondrial NADPH-generating system (see Table 2.3); DL isocitrate (5 mM) and isocitrate (5 mM) plus $NADP^+$ (1.5 mM). Each group consisted of 12 animals.

4.9 CORRELATION BETWEEN ISOCITRATE AND EXTRAMITOCHONDRIAL NADPH IN SUPPORTING CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY IN RAT TESTES MITOCHONDRIA AT DIFFERENT AGES

Washed heavy mitochondria from rats of different age groups were incubated for 1.5 hours in medium A with [4-^{14}C] cholesterol. The electron sources were extramitochondrial NADPH generating system or isocitrate or isocitrate plus $NADP^+$. The results in Figure 4.14 confirm that with isocitrate there is a greater conversion of cholesterol to steroids than with extramitochondrial NADPH generating system. This difference was more pronounced in mitochondrial preparations from younger rats (8 to 10 weeks). With both electron sources an increase in cholesterol side-chain cleavage activity with increasing age of the animals was observed but the correlation was much clearer with the isocitrate system. With isocitrate plus $NADP^+$ the rate of conversion, although higher, paralleled those with isocitrate alone at the different ages. From such studies it appears that the isocitrate system is more reliable than the NADPH generating system for the study of cholesterol side-chain cleavage in rat testicular mitochondria.

4.10 EFFICACY OF EXTRAMITOCHONDRIAL NADPH AND ISOCITRATE AS ELECTRON SOURCE FOR THE MEASUREMENT OF CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY

4.10.1 In Different Subcellular Fraction

Testicular homogenate (350 x g supernatant), heavy mitochondria and heavy mitochondria ficoll fraction 5 (HMF_5) were assayed for cholesterol side-chain cleavage activity, using

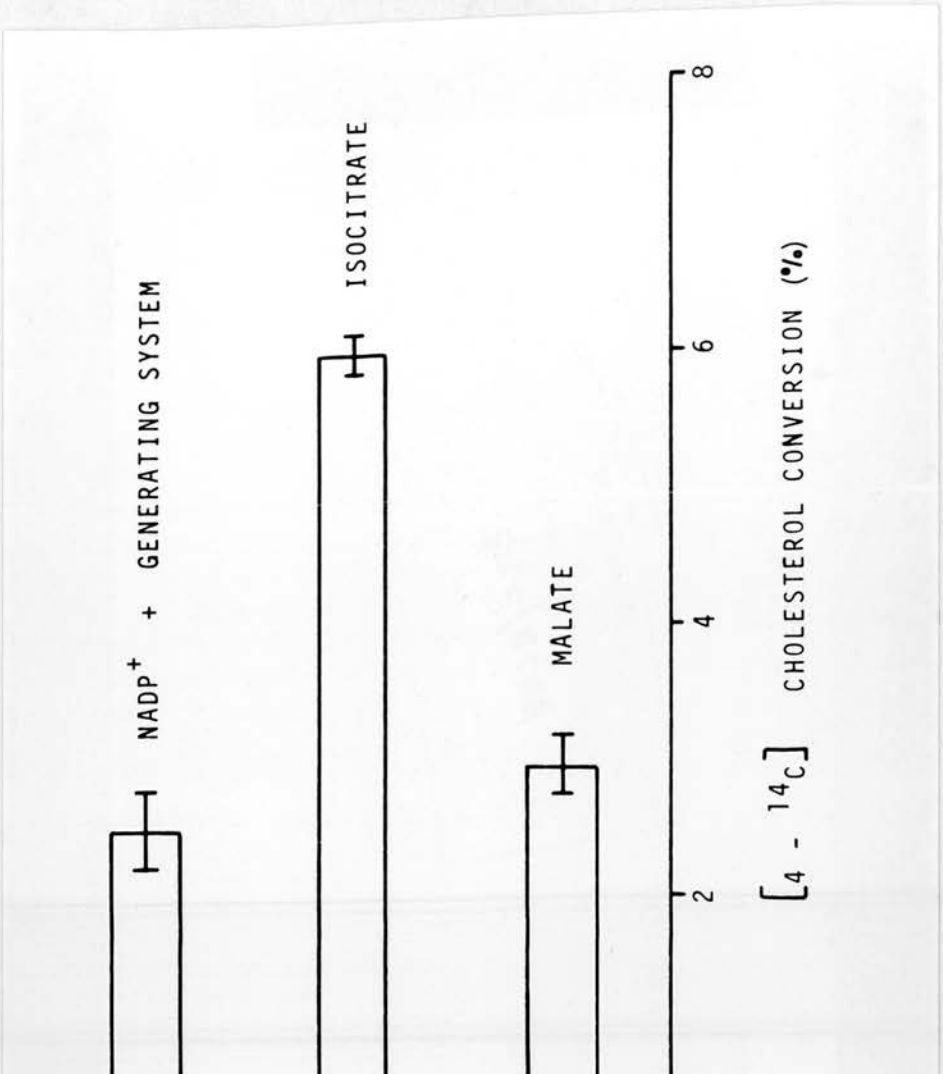


FIGURE 4.15 EFFICACY OF DIFFERENT ELECTRON DONORS IN SUPPORTING CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY IN COUPLED MITOCHONDRIA

Washed heavy mitochondria ficoll fraction 5 (0.5 mg) were incubated in medium B for 90 minutes with [4-^{14}C] cholesterol and an electron source: extramitochondrial NADPH-generating system or isocitrate (5 mM) or malate (5 mM). Results are mean ± SD of 5 different determinations run in duplicate. Rats were of the same age. Mitochondria were prepared in 0.25 M sucrose-10 mM Tris-0.1 mM EDTA (pH 7.5) and fractionated through a ficoll gradient. Respiratory control ratios = 1.8 (with succinate).

the two electron donor systems. The results in Table 4.2 show that with crude testicular preparations the exogenous NADPH supported activity was very low, probably due to the presence of hydrolytic enzymes. With purer preparations (HMF_5) the values approached those obtained with isocitrate. Compared with homogenate, heavy mitochondria ficoll subfraction 5 in the presence of isocitrate showed a four-fold increase in activity and with extramitochondrial NADPH generating system a ten-fold increase.

4.10.2 With Coupled Mitochondria

Since most of the investigations presented were carried out under conditions in which the mitochondria were observed to be in the uncoupled state it was of interest to study cholesterol side-chain cleavage activity under coupled conditions. For this study heavy mitochondria ficoll subfraction 5 was prepared as previously described using 0.25 M sucrose - 10 mM Tris, 0.1 mM EDTA as the homogenizing medium These mitochondria were coupled and showed good respiratory control (RCR = 1.8 with succinate). Cholesterol side-chain cleavage activity was measured in these heavy mitochondria incubated in medium B with either isocitrate (5 mM) malate or NADPH generating system. The results presented in Figure 4.15 show that extramitochondrial NADPH was less than half as effective as isocitrate confirming the relative impermeability of the pyridine nucleotide. Compared to isocitrate malate too was a comparatively inefficient donor (only 50% of isocitrate supported activity). However, the overall pattern was essentially similar to that previously described.

TABLE 4.2

ASSAY OF CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY IN THE DIFFERENT SUBCELLULAR FRACTIONS

SUBCELLULAR FRACTIONS	% [4-^{14}C] CHOLESTEROL CONVERTED		
	ELECTRON SOURCE		RATIO
	(A) NADPH	(B) ISOCITRATE	A/B
Crude Homogenate (350 x g supernatant)	0.4	1.3	0.3
Heavy Mitochondria (6000 x g ppt-washed)	1.2	4.0	0.3
Ficoll Fractionated Heavy Mitochondria (HMF_5 - washed)	4.0	6.1	0.7

Subcellular fractions were prepared as described in Chapter 2.

Each subcellular fraction (0.46 mg) was incubated for 90 minutes in medium A with [4-^{14}C] cholesterol and an electron source - either isocitrate (5 mM) or extramitochondrial NADPH-generating system. Cholesterol side-chain cleavage activity was determined as previously described.

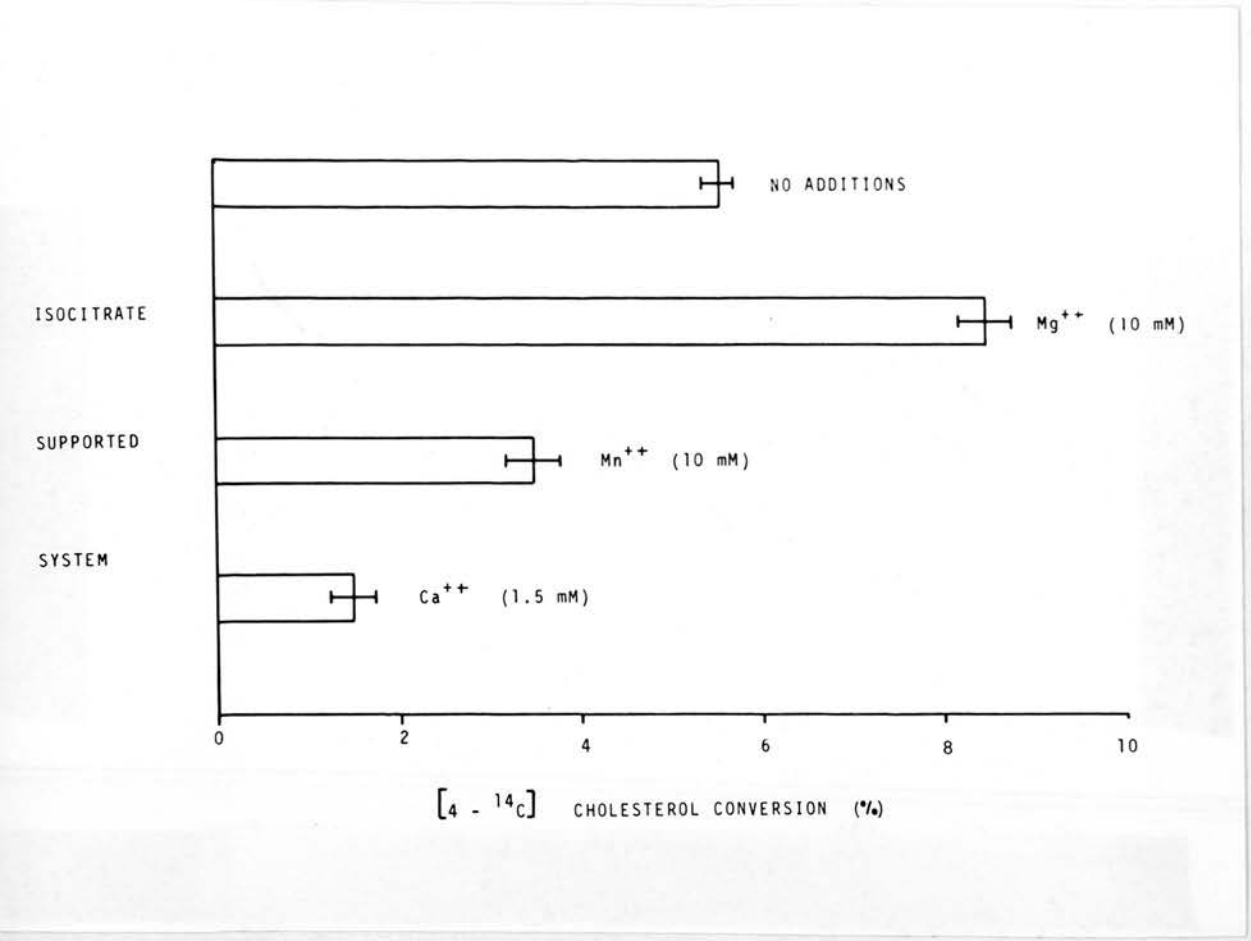


FIGURE 4.16 INFLUENCE OF METAL IONS ON CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY SUPPORTED BY ISOCITRATE

Washed heavy mitochondria ficoll fraction 5 were incubated in medium B at 37°C for 90 minutes. The final concentration of metal ions were Mg^{++} (10 mM), Mn^{++} (10 mM) and Ca^{++} (1.5 mM). Results are mean ± SD of 4 experiments of duplicate determinations.

4.11 INFLUENCE OF METAL IONS ON CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY

The requirement for Mg^{++} for cholesterol side-chain cleavage activity could only be clearly demonstrated in intact mitochondria, prepared and incubated as described above. The results in Figure 4.16 show that Mg^{++} stimulates cholesterol oxygenase activity (also see Figure 4.12). The failure to demonstrate such an effect with earlier preparations could be attributed to the presence of adequate amounts of the endogenous metallic ion. Mn^{++} was observed to have an inhibitory effect. This difference may be linked to the specific requirement of $NADP^{+}$ dependent isocitrate dehydrogenase for Mg^{++}. Ca^{++}, as previously observed, was a potent inhibitor of the isocitrate supported cleavage activity.

4.12 EFFECT OF REDUCING AGENTS ON CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY

It has previously been reported that in ovarian tissue (Goldzieher, 1953; Lazarow, 1954) and adrenal gland (Harding, 1961), there is a relationship between glutathione and ascorbic acid. Sulimovici (1968) demonstrated that cholesterol side-chain cleavage activities in immature rat ovaries were influenced by reducing agents - glutathione, ascorbic acid, cyteine and cysteamine. Since ascorbic acid - a normal cytoplasmic constituent is also found in high concentrations in the interstitium of the testis, the effect of glutathione, ascorbic acid and cysteine on cholesterol side-chain cleavage

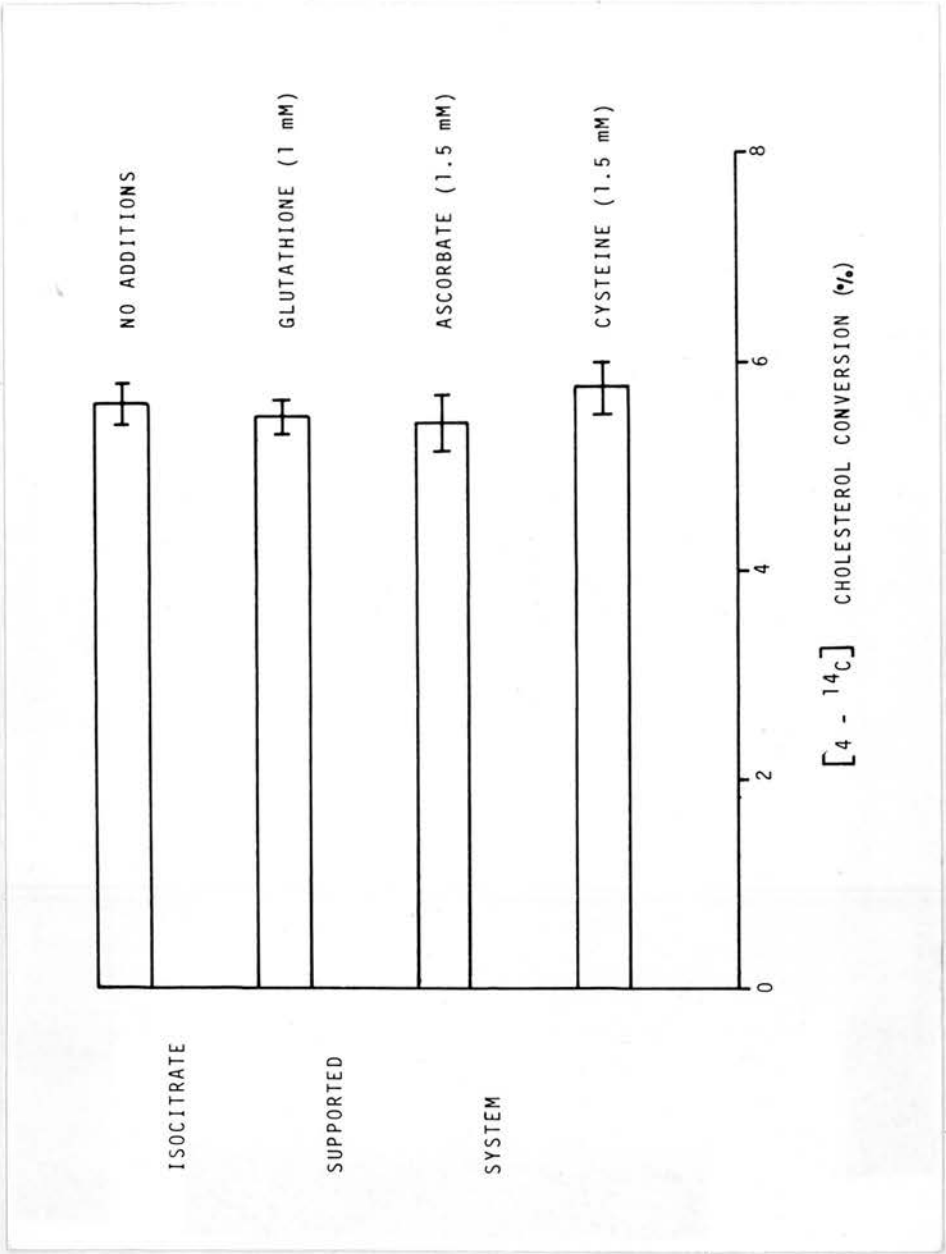


FIGURE 4.17 EFFECT OF REDUCING AGENTS ON CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY IN INTACT MITOCHONDRIA

Washed heavy mitochondria ficoll fraction 5 were incubated in medium B for 90 minutes at 37°C. Isocitrate 5 mM was the electron donor. Reducing agents tested were glutathione (1 mM), ascorbic acid (1.5 mM) and cysteine (1.5 mM). Results are mean ± SD of three experiments each of quadruplet determinations. Rats were 13 weeks old.

in intact mitochondria from rat testis, was studied. Figure 4.17 shows that all three reducing agents tested had no significant effect on cleavage activity supported by isocitrate. The concentrations of glutathione and cysteine used in this study were shown to produce a maximal effect in immature rat ovaries (Sulimovici, 1968).

4.13 EFFECT OF CYANOKETONE ON CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY IN TESTIS MITOCHONDRIA

In the course of the above studies it became evident that the rat testicular mitochondria possess the enzymes involved in testosterone synthesis. Since the main interest of this study was on the cholesterol side-chain cleavage reaction it was felt that, if the further metabolism of pregnenolone could be prevented, it would be an advantage in routine analysis. In this case pregnenolone would be the only product formed from cholesterol. This in turn would reflect, in the main, Leydig cell mitochondrial activity, since it is likely that a significant portion of the enzymes involved in pregnenolone metabolism to testosterone may be of tubular origin (Christensen & Mason, 1965). To this end the effect of cyanoketone - a synthetic steroid and an inhibitor of 5-ene-3β-hydroxysteroid dehydrogenase, was tested.

Cyanoketone causes a marked inhibition of 5-ene-3β-hydroxysteroid dehydrogenase in extracts of Pseudomonas testosteroni (Ferrari & Arnold, 1963; Goldman et al., 1965; Neville & Engel, 1968) and in the ovary and adrenals of rats in vitro (Goldman et al., 1965; McCarthy et al., 1966).

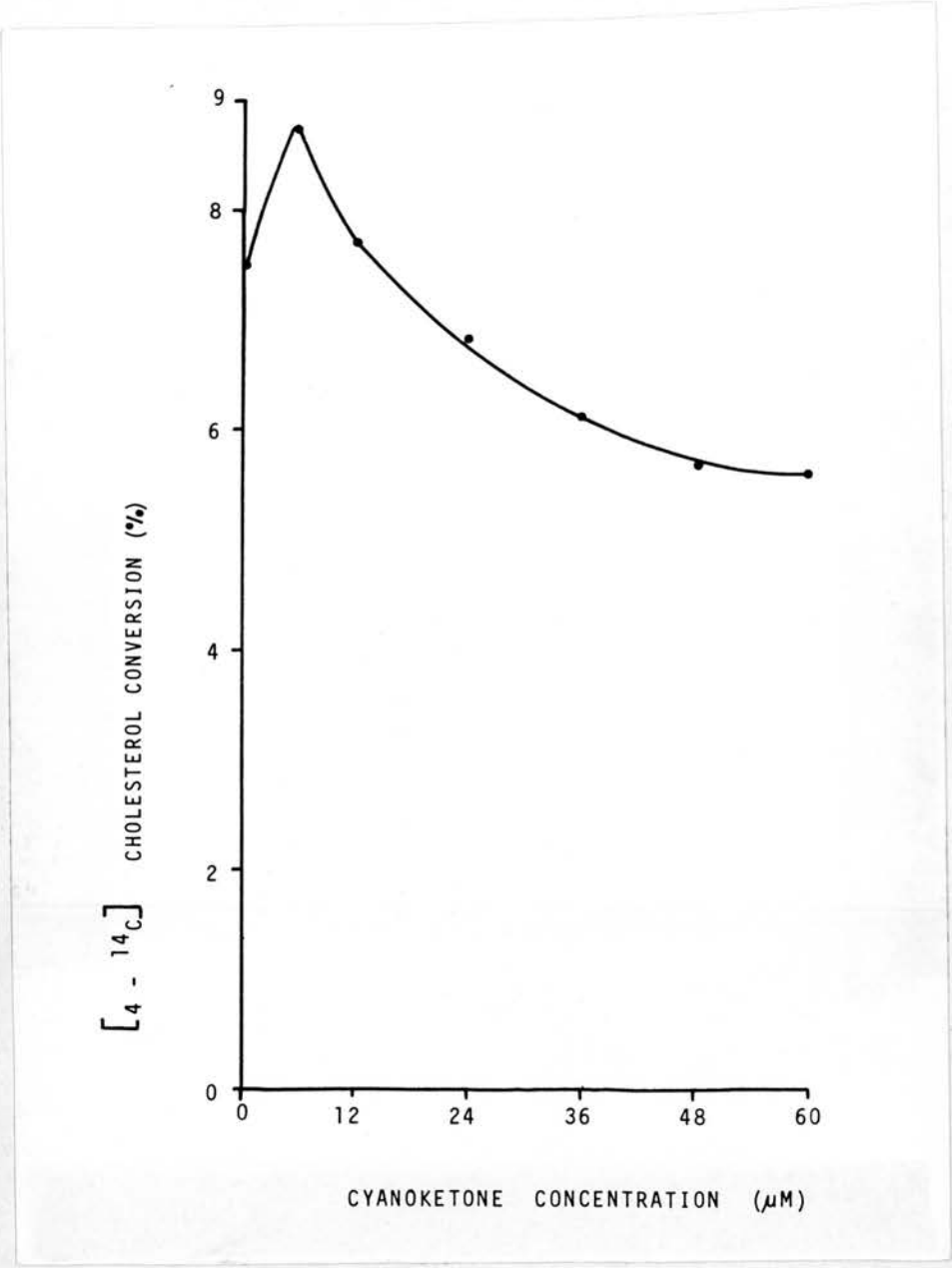


FIGURE 4.18 EFFECT OF VARYING CONCENTRATIONS OF CYANOKETONE ON CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY

Incubations consisted of [4-^{14}C] cholesterol, isocitrate (5 mM), medium B, heavy mitochondria ficoll fraction 5 (0.4 mg) and varying amounts of cyanoketone (in ethanol). The amount of ethanol in the final incubation did not exceed 2% (v/v). Incubation time 90 minutes. Incubated controls containing equivalent amounts of ethanol were also run. Results are corrected for zero time control values. Results of the ethanolic controls were identical to those not containing the alcohol. Results in figure are typical of those observed repeatedly.

The effect of cyanoketone on the rate of rat testicular cholesterol side-chain cleavage and the nature of steroids formed from cholesterol was investigated.

Figure 4.18 shows the effect of different concentrations of cyanoketone on the rate of cholesterol side-chain cleavage activity. It can be seen that at concentration of 6-12 μM, cyanoketone did not inhibit the cleavage reaction but was in fact stimulatory. 6 μM cyanoketone resulted in a 13-20% increase in pregnenolone formed. This stimulatory effect was consistently observed as shown in Table 4.3. At higher concentrations, cyanoketone appeared to inhibit the cholesterol side-chain cleavage reaction. At 60 μM inhibition was approximately 27%. Farese (1970) found that cyanoketone at high concentrations (1 mM) inhibited rat adrenal cholesterol side-chain cleavage activity. This inhibition was suggested to be a direct effect of cyanoketone on the cleavage enzyme complex.

In the absence of cyanoketone but with isocitrate as the electron donor, pregnenolone and progesterone were the products formed from labelled cholesterol. In the presence of cyanoketone pregnenolone was the only labelled steroid formed, indicating that 3β-hydroxysteroid dehydrogenase was inhibited. This was confirmed by the results shown in Figure 4.19. As demonstrated earlier when testicular mitochondria were incubated with isocitrate, the presence of $NADP^+$ stimulated overall, the cholesterol side-chain cleavage reaction and the 3β-hydroxy steroid dehydrogenase activity, resulting in the formation of

TABLE 4.3

EFFECT OF CYANOKETONE ON CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY

EXPT. No.	% [4-^{14}C] CHOLESTEROL CONVERSION			% STIMULATION By CYANOKETONE	RATIO (a)/(c)
	ADDITIONS TO ISOCITRATE SYSTEM				
	(a) None	(b) Ethanol	(c) Cyanoketone		
1	5.0	5.1	5.9	18	0.85
2	5.6	5.6	6.4	14	0.87
3	7.2	7.1	8.3	15	0.87
4	4.3	4.4	5.2	17	0.84

Ficoll purified heavy mitochondria (HMF_5 0.4-0.5 mg) were incubated at 37°C for 90 minutes with [4-^{14}C] cholesterol and DL isocitrate (5 mM). Ethanol (1 µl) or cyanoketone (6 µM in 1 µl ethanol) was added as indicated. Each experiment consisted of a different batch of mitochondria from testis of rats of different ages (11-20 weeks). Rats in each group consisted of 8-12 animals of the same age.

Results shown are the means of duplicate determinations. The zero time values from unincubated samples were subtracted from values of the incubated samples.

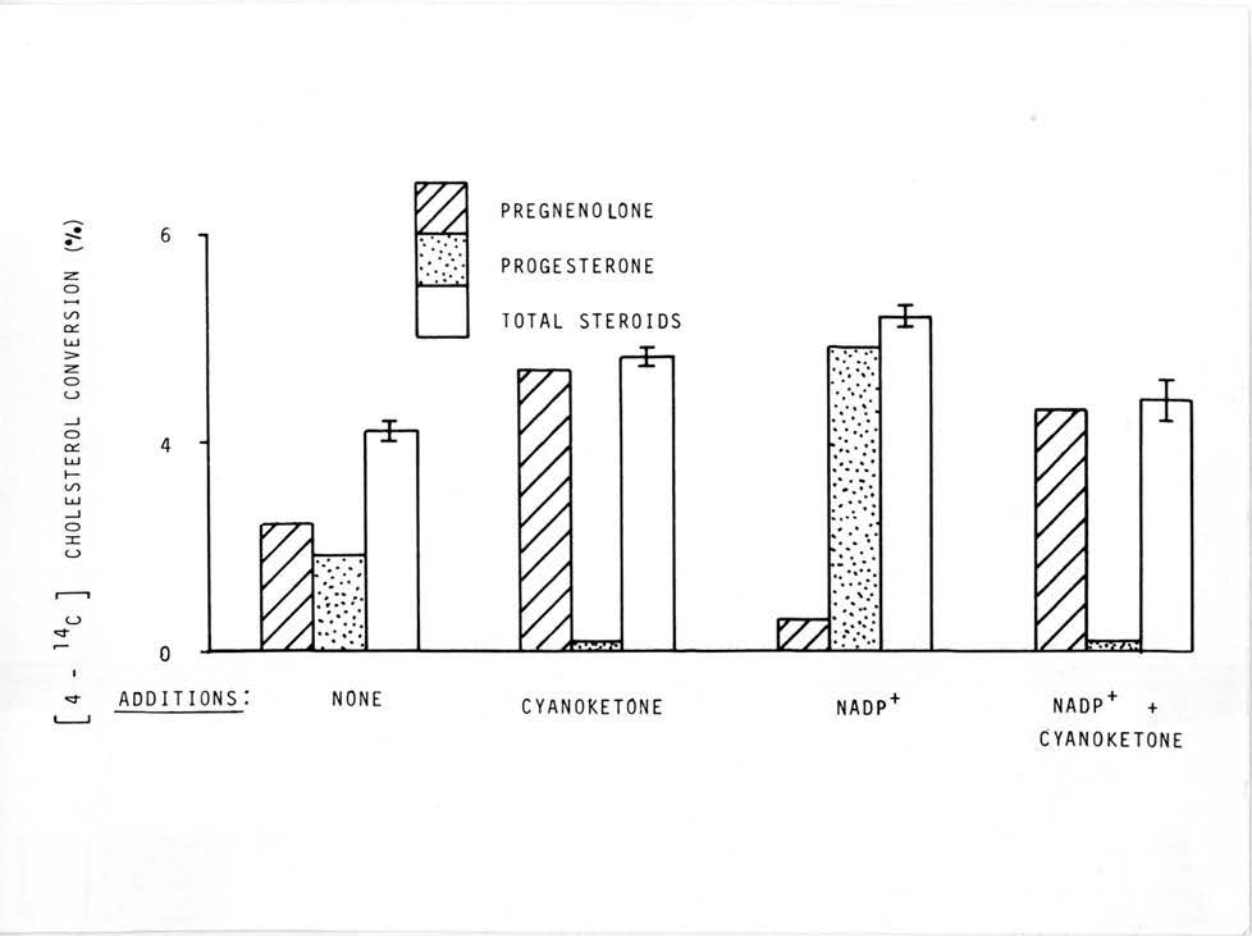


FIGURE 4.19 EFFECT OF CYANOKETONE ON CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY IN INTACT TESTICULAR MITOCHONDRIA AND NATURE OF STEROID PRODUCTS FORMED

Ficoll fractionated heavy mitochondria (HMF_5 0.4-0.5 mg protein) were incubated in medium A at 37°C for 90 minutes with [4-^{14}C] cholesterol and either isocitrate (5 mM) or isocitrate and $NADP^+$ (1.5 mM). Cyanoketone (6 µM in ethanol) was added where indicated. Incubated controls containing an equivalent amount of ethanol were also included. Results were corrected for zero time control values. The amount of ethanol added was always less than 2% of the final incubation volume.

testosterone as the major product. However, when cyanoketone (6 μM) was included in the incubations with $NADP^+$ and isocitrate, the 3β-hydroxysteroid dehydrogenase was completely inhibited, since now pregnenolone was the only product formed. The total percentage conversion of cholesterol was however reduced by 25%.

The reasons for the stimulatory effect of cyanoketone on the isocitrate supported side-chain cleavage system and its inhibitory influence on the $NADP^+$-isocitrate system is not clear, but it does not appear to be related to the pregnenolone feed-back inhibition of the desmolase complex as reported by Koritz and Hall (1964a).

The data with testis show that the presence of cyanoketone produces a large rise of pregnenolone concentration in the mitochondria, yet the rate of cholesterol side-chain cleavage was not inhibited but was similar to that obtained in the absence of cyanoketone, when pregnenolone levels were lower due to further metabolism.

In bovine adrenal cortex and corpus luteum mitochondria pregnenolone has been reported to inhibit cholesterol side-chain cleavage activity (Koritz & Hall, 1964a; Ichii et al., 1963). On the basis of such observations it was postulated that the rate limiting step in steroidogenesis was the efflux of pregnenolone for the mitochondria. The action of ACTH was considered to result in the increased efflux of pregnenolone from the mitochondria thus releasing the inhibition. From the results obtained with testicular mitochondria, such a control of steroidogenesis may not exist in testes.

SUMMARY

1. The requirement of NADPH for cholesterol side-chain cleavage activity in rat testis was confirmed. NADH could not be substituted for NADPH. NAD^+ and $NADP^+$ were completely ineffective, whereas NADPH-generating system and NADH together were inhibitory. The results also indicate the absence of glucose-6-phosphate dehydrogenase in these mitochondrial preparations.

2. The effect of varying [4-^{14}C] cholesterol and NADPH concentrations on the cleavage reaction were investigated. With purified mitochondrial preparations, the concentrations of NADP(H) required for maximal activity was about 1.8 mM. Higher concentrations were inhibitory. With crude preparations higher concentrations of the nucleotide (2.5-5 mM) were required.

3. Studies on the influence of various factors (KCN, Ca^{++}; ATP and BSA) on cholesterol side-chain cleavage activity supported by extramitochondrial NADPH-generating system indicated that, in general, compounds such as Ca^{++} which induce mitochondrial swelling enhanced the activity, whereas those that decrease swelling (like ATP) and protect the mitochondrial integrity (e.g. BSA), inhibited the conversion.

4. Of the Krebs cycle intermediates examined as alternative electron sources, isocitrate was more effective than malate, and both were better than NADPH in supporting cholesterol side-chain cleavage activity. Succinate was least efficient.

5. The optimum conditions for cholesterol side-chain cleavage activity supported by isocitrate were investigated. These included the effect of substrate concentrations, increasing

amounts of mitochondrial protein and varying pH. The optimum pH in Tris buffer was pH 7.5. Studies with different buffered media showed that Tris buffer was the most efficient medium whereas phosphate buffer was inhibitory. Medium B, although better than phosphate buffer, was not as favourable as Tris. However, the inclusion of Mg^{++} in medium B greatly enhanced cleavage activity - implying that uncoupling conditions favour the reaction.

6. The presence of various cations and anions, nucleotides and BSA in the incubation medium with isocitrate greatly influenced the rate of the cholesterol side-chain cleavage reaction and the nature of steroid products accumulating. Reducing agents such as glutathione and ascorbate had no effect on the reaction. Evidence suggest that testis mitochondria contain an $NADP^{+}$ dependent isocitrate dehydrogenase.

7. From a consideration of the nature of steroid products, the studies show that the total yield, ratio and type of products formed from [4-^{14}C] cholesterol could be greatly influenced by the electron donor used, (whether extramitochondrial NADPH generating system or isocitrate) and the picture further altered by the presence of cations, anions, nucleotides and BSA. These testes mitochondria appear to contain the enzymes involved in testosterone biosynthesis.

8. Cholesterol side-chain cleavage activity was found to be slightly stimulated by cyanoketone at low concentrations (6 μM) and inhibited a very high concentration (50 μM). Pregnenolone was the only product formed indicating that the 3β hydroxysteroid dehydrogenase activity in these mitochondria was inhibited.

CHAPTER 5

EVIDENCE FOR THE PRESENCE OF CYTOCHROME P450 AND CYTOCHROMES OF THE NORMAL RESPIRATORY CHAIN IN RAT TESTES MITOCHONDRIA

CONTENTS

EVIDENCE FOR THE PRESENCE OF CYTOCHROME P450 AND CYTOCHROMES OF THE NORMAL RESPIRATORY CHAIN IN RAT TESTES MITOCHONDRIA

CHAPTER 5

EVIDENCE FOR THE PRESENCE OF CYTOCHROME P450 AND CYTOCHROMES OF THE NORMAL RESPIRATORY CHAIN IN RAT TESTES MITOCHONDRIA

5.1 INTRODUCTION

In the course of steroid biosynthesis from cholesterol a number of hydroxylation reactions occur. Of these the cholesterol side-chain cleavage enzyme system of all steroidogenic tissues has been located in the mitochondria (see Sulimovici and Boyd, 1969) as has the 11β-and 18-hydroxylases of the adrenal cortex (Halkerston et al., 1961). By contrast the mixed-function oxidases involved in 17α- (Plager & Samuels, 1954) and 21-hydroxylations (Ryan & Engel, 1957) have been found to be associated with the endoplasmic reticulum (microsomes).

It is fairly well established for most steroid producing tissues that cytochrome P450 is involved as the terminal oxidase in these hydroxylase reactions (Harding et al., 1964, 1965; Kinoshita et al., 1966; Horie et al., 1966; Simpson & Boyd, 1967; Yohro & Horie, 1967; Meigs & Ryan, 1968; Mason & Boyd, 1971; Ryan & Engel, 1957; Estabrook et al., 1963; Greengard et al., 1967; Omura et al., 1965, 1966; Kimura & Suzuki, 1967; Wilson & Harding, 1970; Jefcoate et al., 1970) and is responsible for oxygen activation in both mitochondrial and microsomal mixed function oxidases.

To date there has been no evidence for the presence of cytochrome P450 in testicular mitochondria from normal rats. Employing the conventional carbon monoxide difference spectra technique of Omura and Sato (1964a,b), significant amounts of

cytochrome P450 were detected only in the microsomal fraction from rat testis but none in the mitochondrial fractions, even of x-irradiated (Machino et al., 1969) and gonadotrophin treated rats (Machino et al., 1969; Mason et al., 1973). Hence studies were undertaken to assess the presence or absence of cytochrome P450 in the mitochondria of rat testis. In addition, the present study examines for comparison the presence and distribution of the normal respiratory chain cytochromes.

5.2 PRESENCE AND SUBCELLULAR DISTRIBUTION OF THE CYTOCHROMES OF THE NORMAL RESPIRATORY CHAIN

For these investigations "heavy mitochondria", "light mitochondria" and microsomal fractions were prepared from homogenates of rat testes by differential centrifugation and the heavy mitochondria further fractionated on 3-10% ficoll as previously described in Chapter 2, section 2.2 and 2.3. (Heavy mitochondria ficoll fraction 5 was used.) These particulate fractions were suspended in medium A and the difference spectrum measured at room temperature (approximately 22°C) in an Aminco-Chance dual wavelength/split beam recording spectrophotometer set to scan wavelengths. Two silica cuvettes of 3.0 ml. capacity and 1 cm light path were used and are referred to as reference and experimental cuvettes. The spectral data presented are replots made by substracting the appropriate base-line at intervals of 2 nm from the spectrum under consideration.

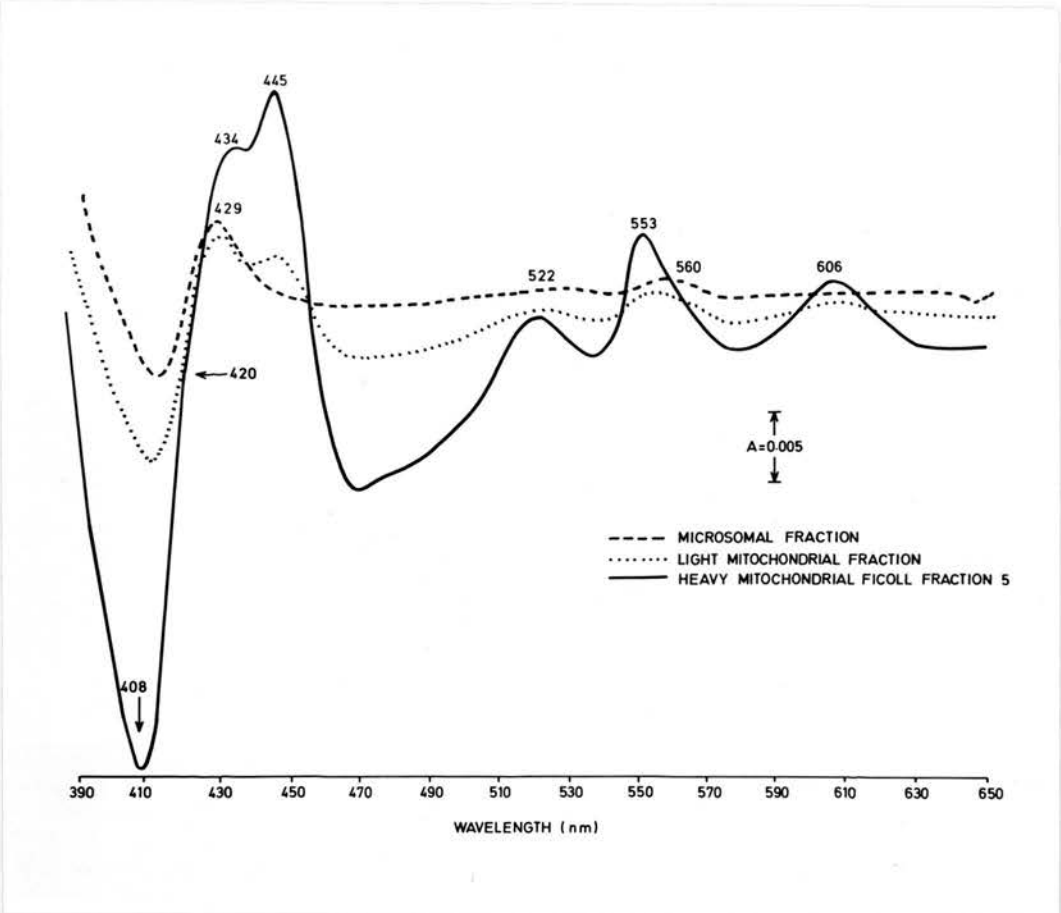


FIG. 5.1 SUBCELLULAR DISTRIBUTION OF THE NORMAL RESPIRATORY CHAIN CYTOCHROMES OF RAT TESTES

Reduced minus oxidized difference spectra of testicular heavy mitochondria (ficoll fraction 5), light mitochondria and microsomal fractions. Each subcellular fraction was divided equally into two cuvettes and a base-line of equal light absorption established. A few crystals of solid sodium dithionite were added to the contents of the experimental cuvette with a stirring rod and the difference spectrum of reduced minus oxidized cytochrome recorded. The final protein concentrations were: heavy mitochondria 1.6 mg/ml, light mitochondria 2.6 mg/ml and microsomes 3.3 mg/ml.

5.2.1 Dithionite reduced minus oxidized difference spectra of testicular heavy mitochondria, light mitochondria and microsomal fractions

To obtain this spectrum, each subcellular fraction was divided equally (3 ml. each) into the two cuvettes. After a base-line of equal light absorbance was established between the oxidized suspensions in the reference and experimental cuvettes, a few grains of solid sodium dithionite were added to the contents of the experimental cuvette with a stirring rod and the difference spectrum of reduced minus oxidized cytochromes recorded. The results are presented in Figure 5.1. The heavy mitochondria exhibited the typical absorption bands of the classical respiratory chain cytochromes characteristic of mitochondria isolated from other tissues (Harding & Nelson, 1966, Yohro & Horie, 1967). The α absorption band of cytochrome a(+ a_3) was at 606 nm and that of cytochrome C + C_1 at 553 nm with a shoulder at about 560 nm produced by the α band of cytochrome b. The combined β bands of the cytochromes appeared at approximately 520 nm and the oxidized flavo-protein trough at 470 nm. In the Soret region the prominent γ bands of cytochromes a_3(+ a) and b were seen at 445 nm and 434 nm respectively with a slight shoulder at about 420 nm representing probably the γ band of cytochrome C + C_1.

The reduced versus oxidized difference spectrum of light mitochondrial fraction was essentially the same as that of the heavy mitochondria except that the levels of the cytochromes were lower. This presence of respiratory chain cytochromes confirmed the mitochondrial nature of this fraction.

TABLE 5.1

CONCENTRATION OF MITOCHONDRIAL RESPIRATORY CHAIN

CYTOCHROMES AND MICROSOMAL CYTOCHROME P450 IN RAT TESTES

COMPONENT	WAVELENGTH PAIRS (nm) (λmeasure$\rightarrow\lambda$ref)	Δ E (cm^{-1} mM^{-1})	CYTOCHROME CONTENT (nmoles/mg protein) Mean ± SD
HEAVY MITOCHONDRIAL FRACTION (HMF_5)			
Cytochrome a (+ a_3)	605-630	16	0.19 ± .02
a_3(+ a)	445-451	91	0.04 ± .01
b	562-575	20	0.15 ± .01
C + C_1	551-540	19	0.31 ± .02
MICROSOMAL FRACTION			
Cytochrome P450:CO	450-490	91	0.08 ± .01

The concentration of the respiratory chain cytochromes in the heavy mitochondrial fraction 5 was determined from the dithionite reduced minus oxidized difference spectra using the molar extinction coefficients indicated by Estabrook and Holowinsky (1961) and Chance and Williams (1955). Microsomal cytochrome P450 was determined from the carbonmonoxide reduced minus reduced difference spectra and the molar extinction coefficient determined by Omura and Sato (1964). Results are expressed as mean and standard deviation of 5 experiments.

The difference spectrum produced with the microsomal fraction showed the presence of α absorption band at 560 nm and a Soret band with maxima at 429 nm, similar to that of cytochrome b. No other absorption peaks characteristic of cytochromes a + a_3 or C + C_1 were detectable.

From spectral studies of this type the concentrations of the normal respiratory chain cytochromes in testes mitochondria were determined using the molar extinction coefficients indicated by Estabrook and Holowinsky (1961). The results are summarized in Table 5.1. These values compare well with those of other steroidogenic tissue like the adrenal cortex (Cammer & Estabrook, 1967; Harding & Nelson, 1966) and corpus luteum (McIntosh et al., 1971). However, compared with testes mitochondria of other species (Mason et al., 1973) rat testes contains an unusually high concentration of cytochrome oxidase (a + a_3).

5.3 PRESENCE AND SUBCELLULAR DISTRIBUTION OF CYTOCHROME P450

5.3.1 Reduced-carbon monoxide difference spectra of the subcellular particulate fractions from rat testis homogenates

To obtain such a reduced-carbon monoxide versus reduced difference spectrum, sodium dithionite was added to both cuvettes containing the oxidized cytochromes. After the base-line was established, carbon monoxide was bubbled into the experimental cuvette for a few seconds and the difference spectrum recorded. Figure 5.2 shows the typical carbon monoxide-reduced minus reduced spectra obtained with each

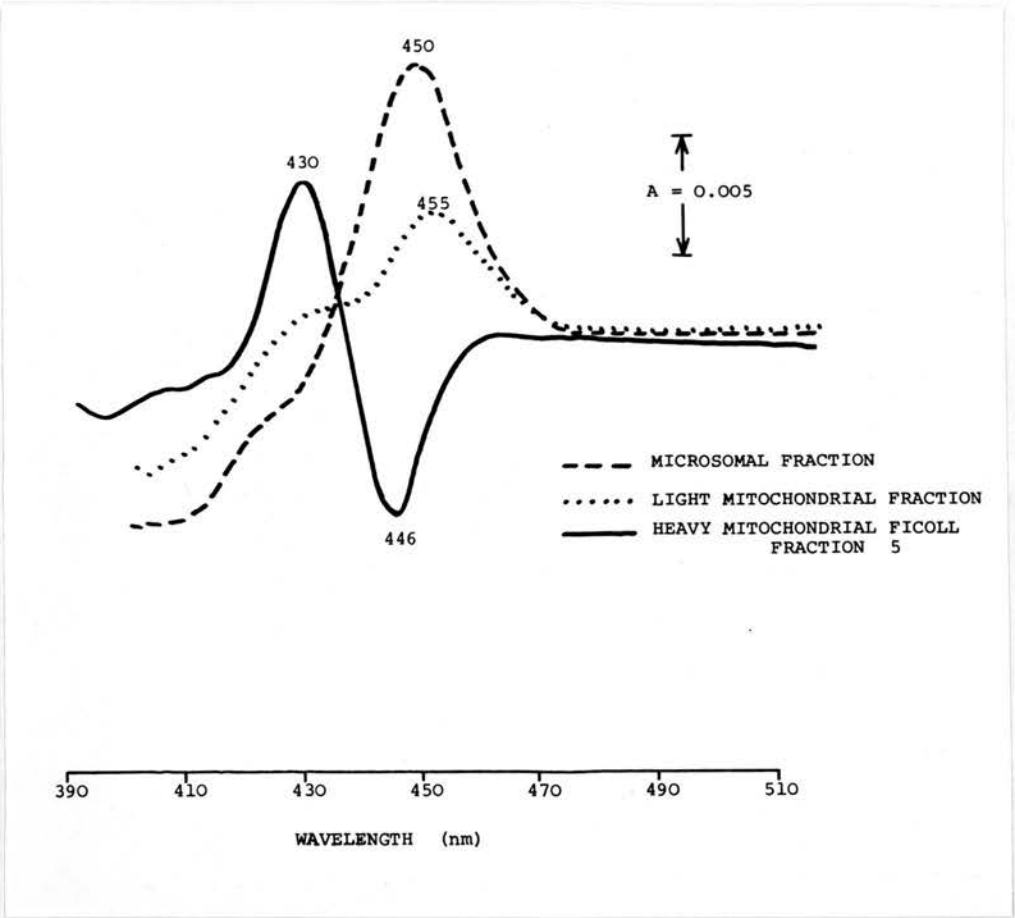


FIG. 5.2 PRESENCE AND SUBCELLULAR DISTRIBUTION OF CYTOCHROME P450

Carbon monoxide reduced minus reduced difference spectra of heavy mitochondria, light mitochondria and microsomes.

Both cuvettes containing equal amounts of a subcellular suspension were treated with sodium dithionite and the base-line recorded. The experimental cuvette was then saturated by bubbling carbon monoxide through the suspension for 30 secs. and the difference spectrum recorded. The final protein concentrations of the particulate suspensions were: heavy mitochondria (ficoll fraction 5) 1.6 mg/ml, light mitochondria 2.6 mg/ml and microsomal fraction 3.3 mg/ml

subcellular fraction. The addition of CO to the reduced microsomal preparation produced an intense absorption band at 450 nm which completely dominated the spectrum and thus confirmed the presence of cytochrome P450 in this fraction. However, the heavy mitochondrial fraction did not show the 450 nm peak of cytochrome P450:CO complex but instead gave rise to a large broad trough with a minimum at 446 nm and a prominent absorption peak at 430 nm associated with the formation of the CO complex of reduced cytochrome oxidase (cytochrome a_3 ++:CO). No discernible cytochrome P450 peak was detectable even at high mitochondrial protein concentrations of 5 mg/ml. The 450 nm minus 490 nm absorbance difference was negative. Kowal et al. (1970) reported that cytochrome P450 could be determined by the 460 nm minus 490 nm absorbance difference contribution since the difference spectrum of the reduced CO complex of cytochrome oxidase had only a small spectral contribution in this region.

The CO- difference spectrum of the light mitochondria appeared to be of a mixed type as indicated by the absorption bands at about 430 nm and 453 nm and thus could be attributable to the presence of both mitochondrial (cytochrome a + a_3) and microsomal (cytochrome P450) pigments respectively, in this fraction. The slight shift of the absorbance maximum of cytochrome P450:CO complex could be due to pertubation by cytochrome oxidase present in this fraction (also see Figure 5.3 curve of microsome and heavy mitochondria).

It has been reported that upon aging of the particulate preparations (Harding & Nelson, 1966) or exposure to various agents (Omura & Sato, 1964a,b) cytochrome P450 is converted

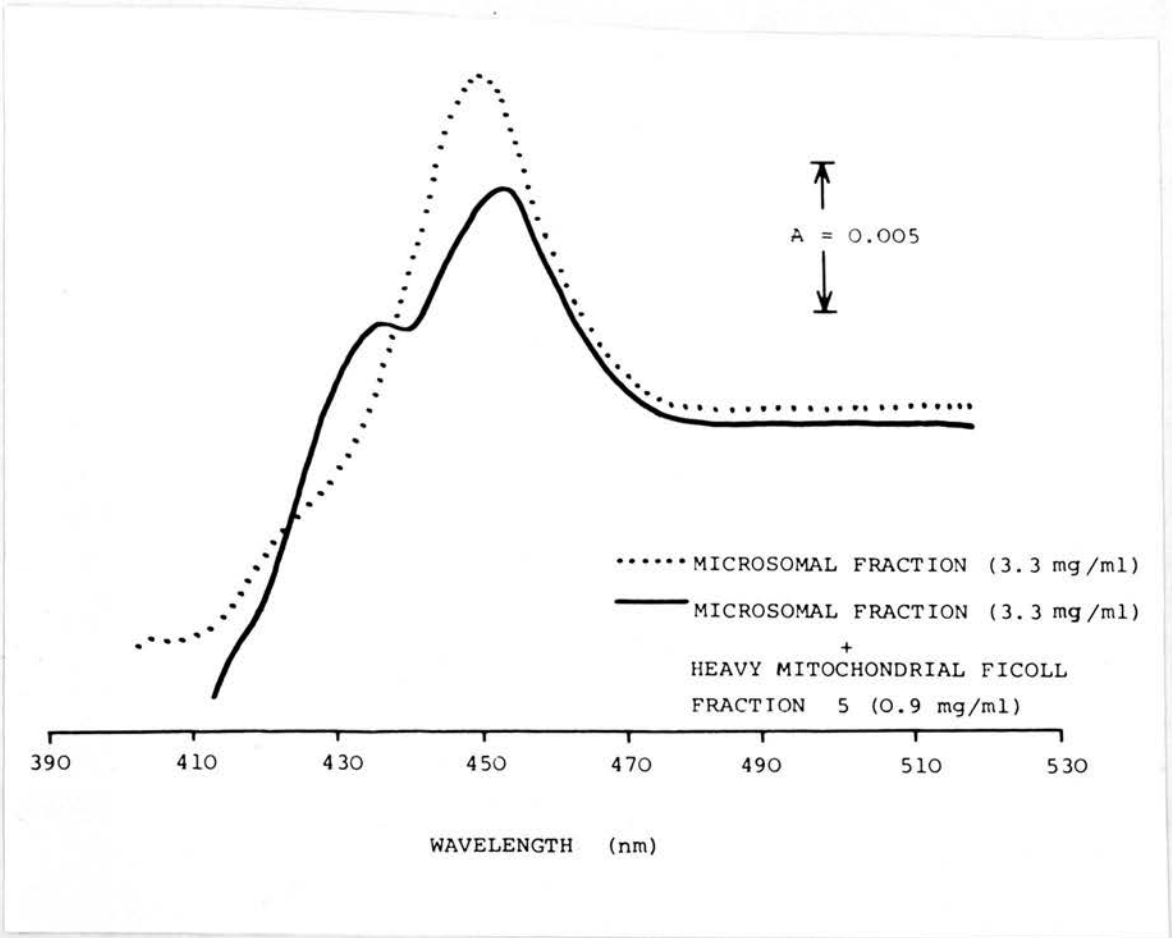


FIG. 5.3 EFFECT OF TESTICULAR MITOCHONDRIAL CYTOCHROMES ON MICROSOMAL CYTOCHROME P450:CO COMPLEX

The curves represent the carbon monoxide-reduced minus reduced difference spectrum produced by saturating the experimental cuvette which contained the reduced cytochromes with carbon-monoxide

..... Microsomes 3.3 mg/ml

——— Mixture of microsomes 3.3 mg/ml plus heavy mitochondria (ficoll fraction 5) 0.9 mg/ml (equivalent to 0.15 nmoles $a + a_3$)
Ratio: cytochrome oxidase/microsomal cytochrome P450 = 0.57.

to a complex absorbing at 420 nm. This cytochrome P420 was shown to be the enzymatically inactive form of cytochrome P450 (Ichikawa & Yamano, 1967b; Mason et al., 1965). In this study, with all three testicular subcellular fractions, the CO:reduced minus reduced difference spectra did not show the presence of such a P420 cytochrome.

The cholesterol side-chain cleavage enzyme complex of other steroidogenic tissues such as the adrenals (Simpson & Boyd, 1967), corpora luteum (Yohro & Horie, 1967; McIntosh et al., 1971) and placenta (Mason & Boyd, 1971) all contain readily detectable levels of cytochrome P450 localized to the mitochondria. Therefore, by analogy, the corresponding hydroxylating system of rat testes heavy mitochondria might be expected to involve cytochrome P450 and to function in a similar manner.

Since Leydig cells, which are the principal source of androgens, constitute only 3-6% of the total mass of the adult testis (Christensen & Mason, 1965) it is likely that small amounts of cytochrome P450, if present in the mitochondria could be masked by the relatively large content of other cytochromes such as cytochrome oxidase or haemoglobin and thus escape detection as the carbon monoxide reduced complex. Experiments were therefore performed to determine the effects of cytochrome oxidase and haemoglobin on the detection of cytochrome P450.

5.3.2 Effect of cytochrome oxidase on the determination of cytochrome P450:CO complex

To determine this effect, the experiments were carried out using a mixed subcellular preparation consisting of

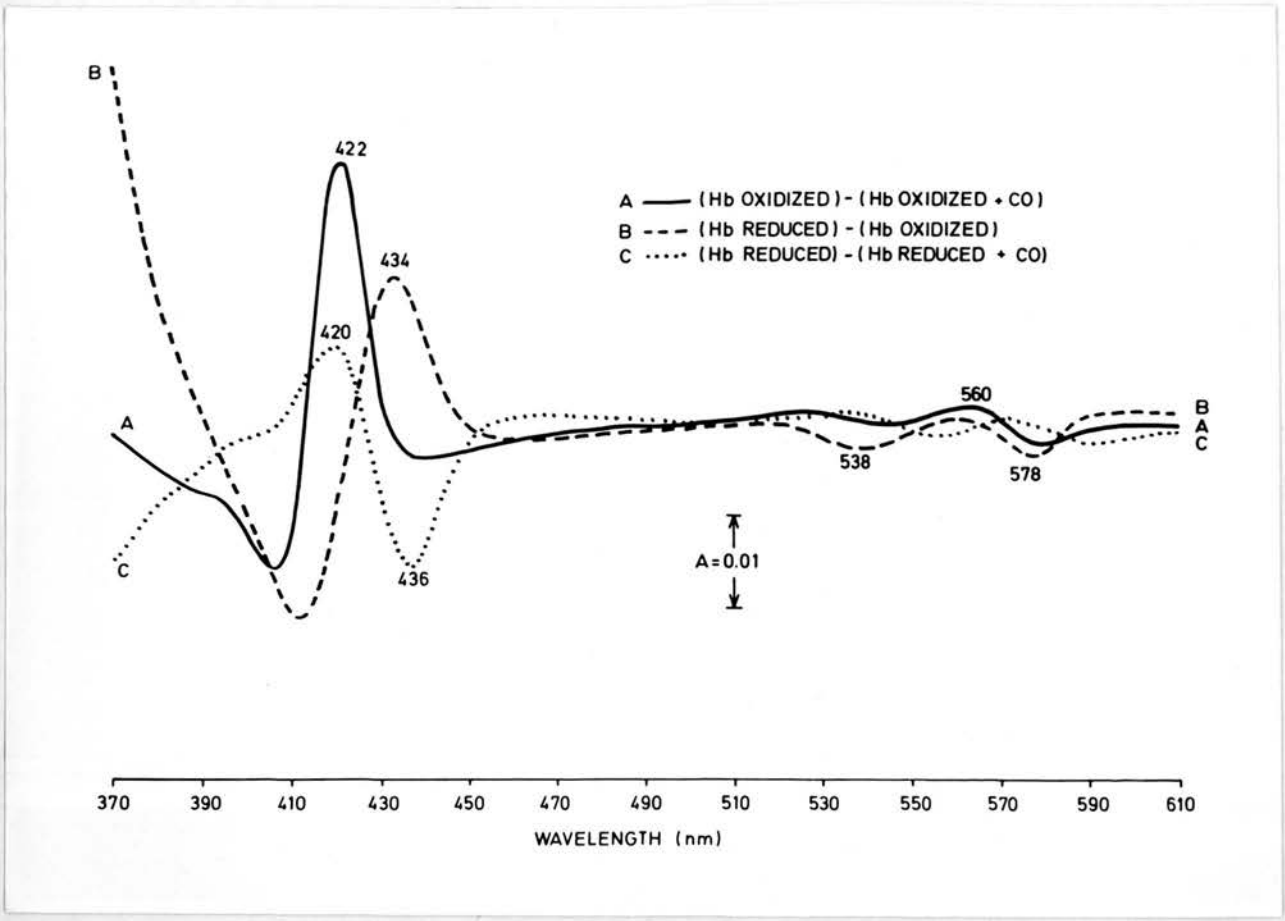


FIG. 5.4 HAEMOGLOBIN DIFFERENCE SPECTRA

A haemoglobin solution prepared from heparinized rat blood was divided equally into two cuvettes and a base-line of equal light was recorded. Carbon monoxide was bubbled into the experimental cuvette and the oxidized carbonmonoxide minus oxidized difference spectrum recorded - curve A ——— .
Curve B ▪▪▪▪▪ is the dithionite reduced minus oxyhaemoglobin difference spectrum.
Curve C ····· is the reduced carbonmonoxide minus reduced haemoglobin difference spectrum.

testicular heavy mitochondria as a rich source of cytochrome oxidase and testicular microsomes a source of cytochrome P450. Different ratios of the two subcellular preparations were mixed in medium A and the CO-reduced minus reduced difference spectrum determined as described above (section 5.2.2). The results presented in Figure 5.3 show that the CO-complex of cytochrome oxidase interfered with cytochrome P450 absorption by decreasing not only the amount of cytochrome P450 measurable by about 40%, but also by causing an apparent shift in the absorption maximum of the CO-complex of cytochrome P450 to 455 nm.

5.3.3 Effect of haemoglobin on the absorption spectra of cytochrome P450

Because the absorption bands of haemoglobin and the respiratory chain cytochromes tend to overlap, haemoglobin contamination of the mitochondrial fraction, if present, would distort the appearance of the absorption spectra and make quantitation of the cytochromes difficult. The absorption spectra of haemoglobin depicted in Figure 5.4 shows the difference spectrum of (a) carbonmonoxy-haemoglobin versus oxyhaemoglobin (curve A), (b) the dithionite reduced versus oxidized difference spectrum of haemoglobin (curve B) and (c) reduced carbon monoxide minus reduced difference spectrum of haemoglobin (curve C). In the latter a peak absorption band at 420 nm and a trough with a minimum at 436 nm was obtained. This negative absorbance could contribute to the masking of the cytochrome P450 absorption band.

To determine the effect of haemoglobin on cytochrome P450:CO reduced spectrum, the reduced carbon monoxide minus reduced

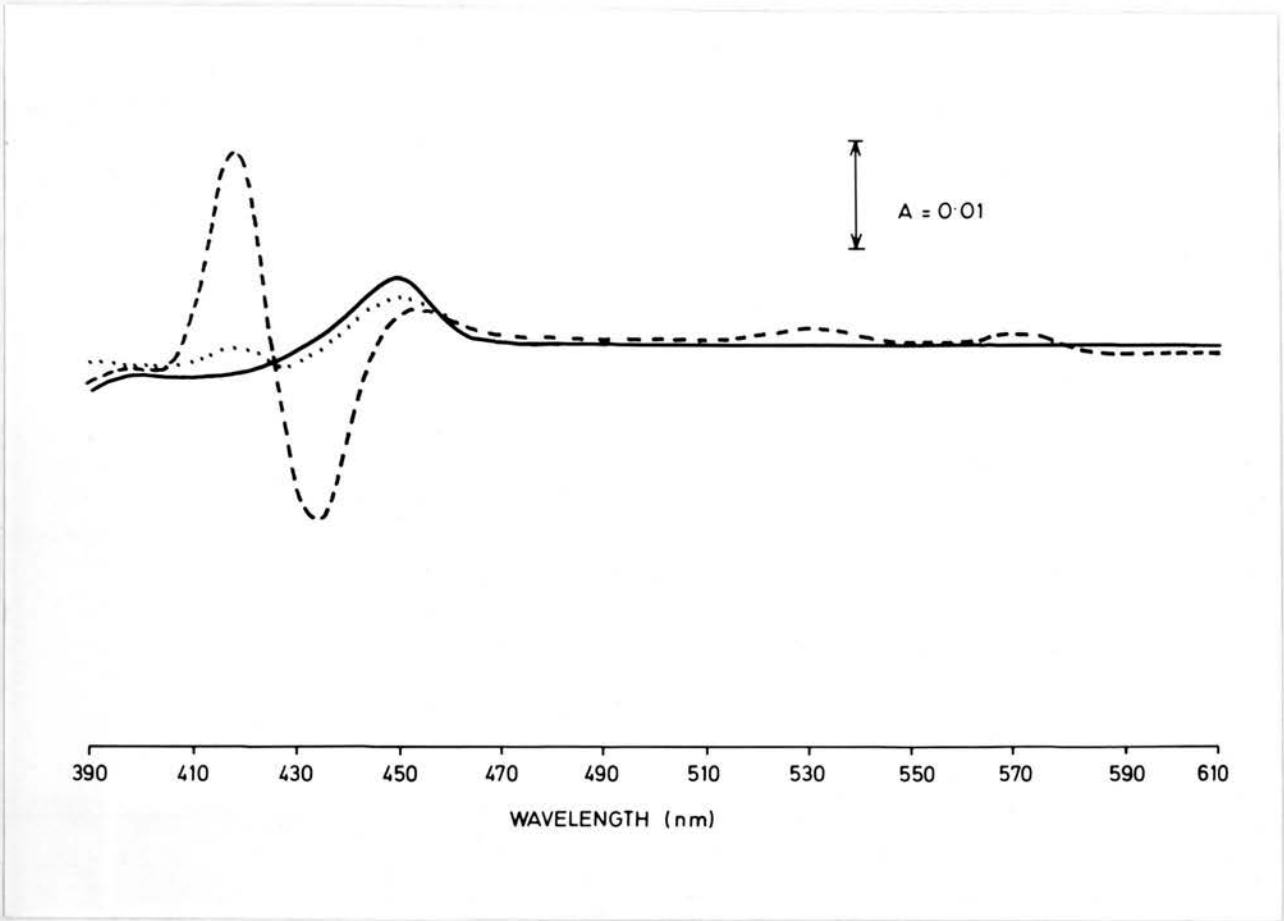


FIG. 5.5 EFFECT OF HAEMOGLOBIN ON CYTOCHROME P450:CO COMPLEX

The difference spectra were obtained by reducing the sample in both cuvettes with sodium dithionite and after recording the base-line the experimental cuvette was saturated with carbon monoxide and the reduced carbonmonoxide minus reduced difference spectrum recorded.

——— Microsomes 1.1 mg/ml

····· Microsomes 1.1 mg/ml plus haemoglobin 10 µl (= 0.07 nmoles Hb)

▪▪▪▪▪ Microsomes 1.1 mg/ml plus haemoglobin 50 µl (= 0.33 nmoles Hb).

difference spectrum of the microsomal fraction (used as source of cytochrome P450) was first determined, as described above. Aliquots of haemoglobin solution were added into the experimental cuvette and CO bubbled again. Into the reference cuvette an equivalent amount of water was added. The difference spectra obtained are shown in Figure 5.5. It can be seen that increasing concentrations of haemoglobin perturbed and markedly reduced the 450 nm-490 nm optical difference of the cytochrome P450:CO complex similar to the effect of cytochrome oxidase.

From these studies it can be inferred that haemoglobin also could interfere with the detection of cytochrome P450 in the testis mitochondria by contributing to the negative absorption at 446 nm. However, the absence of the haemoglobin peak at 420 nm and the symmetry of the absorption band at 430 in the reduced carbon monoxide difference spectrum of heavy mitochondria suggest that haemoglobin contamination, if present in these preparations, was not significant.

5.3.4 Presence of haemoglobin contamination in the heavy mitochondrial fraction

In order to detect the presence of haemoglobin contamination the aerobic carbon monoxide minus aerobic difference spectrum of the heavy mitochondrial fraction was examined as described above for carbon monoxy haemoglobin versus oxyhaemoglobin difference spectrum. As shown in Figure 5.6 curve A, a small absorption band at 420 nm was produced indicating that the degree of haemoglobin contamination in the heavy mitochondrial fraction was slight.

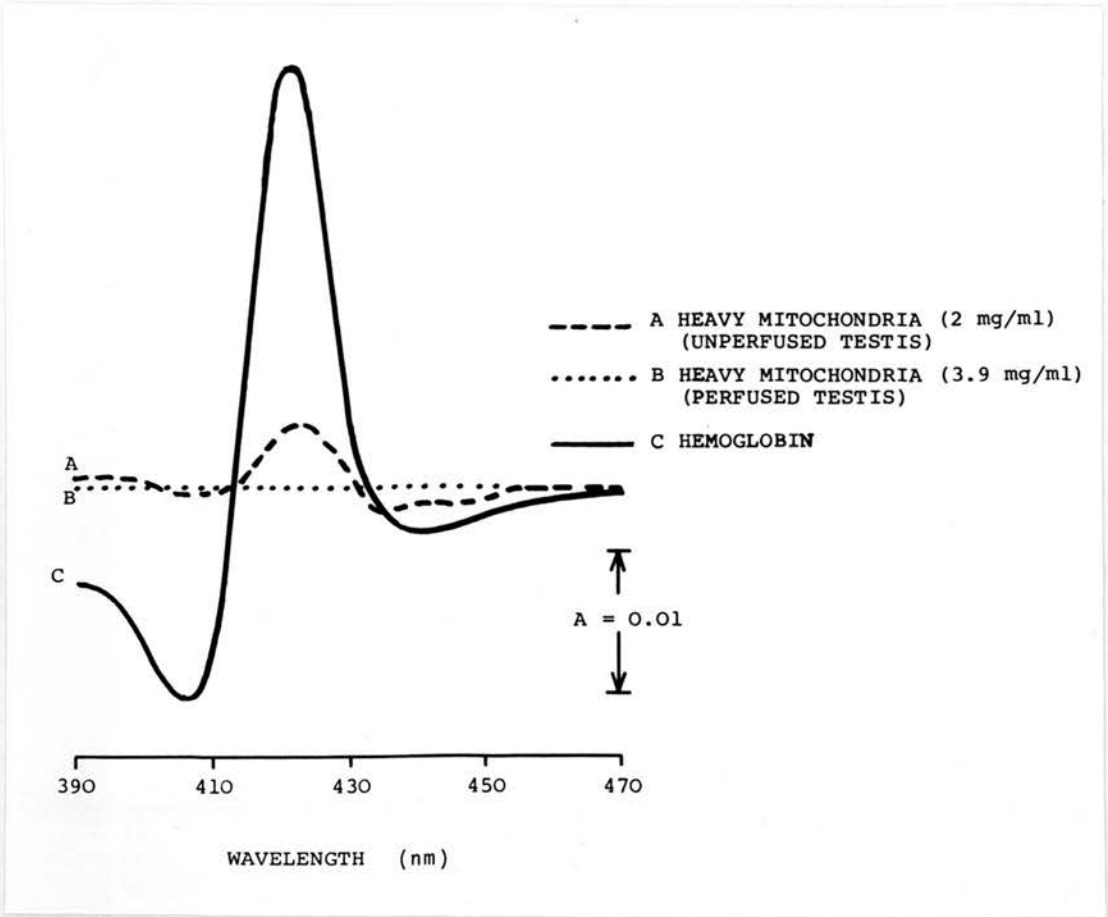


FIG. 5.6 DETECTION OF HAEMOGLOBIN CONTAMINATION OF HEAVY MITOCHONDRIA

A suspension of rat testis heavy mitochondria ficoll fraction 5 was divided equally into two cuvettes and a base-line of equal light absorption was recorded. The contents of the experimental cuvette was then saturated with carbonmonoxide and the carbonmonoxide oxidized minus oxidized difference spectrum recorded.

Curve A ----- is the difference spectra of heavy mitochondria (HMF_5) 2.0 mg/ml prepared from unperfused rat testis.

Curve B ..... difference spectra of heavy mitochondria (HMF_5) 3.9 mg/ml prepared from perfused animals.

Curve C _____ haemoglobin difference spectra (for comparison).

5.3.5 Attempt to detect cytochrome P450 in mitochondria from perfused rat testes

5.3.5.1 Perfusion of rat testes

In order to eliminate all possible interference of haemoglobin with the absorption spectrum of the P450:CO complex, attempts were made to remove the haemoglobin contamination in the heavy mitochondria rat testis. A perfusion technique was developed for washing out the blood from the testes in situ. Since the spermatic arteries in the rat were small, direct perfusion was not feasible. Furthermore, since the spermatic arteries originate directly from the aorta in the region of the kidneys, successful perfusion was achieved via the aorta just above the diaphragm (i.e. through the thoracic cavity). The ether-anaesthetized rats were perfused with Lock's perfusion medium (consisting of 0.92% NaCl, 0.042% Kcl, 0.02% $CaCl_2$, 0.015% $NaHCO_2$ and 0.2% glucose and 50,000 IU heparin/litre) until both testis were visually free of blood (i.e. about 10-15 minutes).

From such perfused testes heavy mitochondria were prepared as previously described. Perfusion usually decreased the haemoglobin contamination in the isolated heavy mitochondrial fractions to a spectrophotometrically undetectable level (see curve B in Figure 5.6).

5.3.5.2 Spectral studies

With these mitochondria the following spectral studies were conducted in a further attempt to detect cytochrome P450.

a. The reduced CO versus reduced difference spectrum was prepared and as shown in Figure 5.7 was identical with that produced with heavy mitochondria from unperfused testes. No cytochrome P450 was detectable.

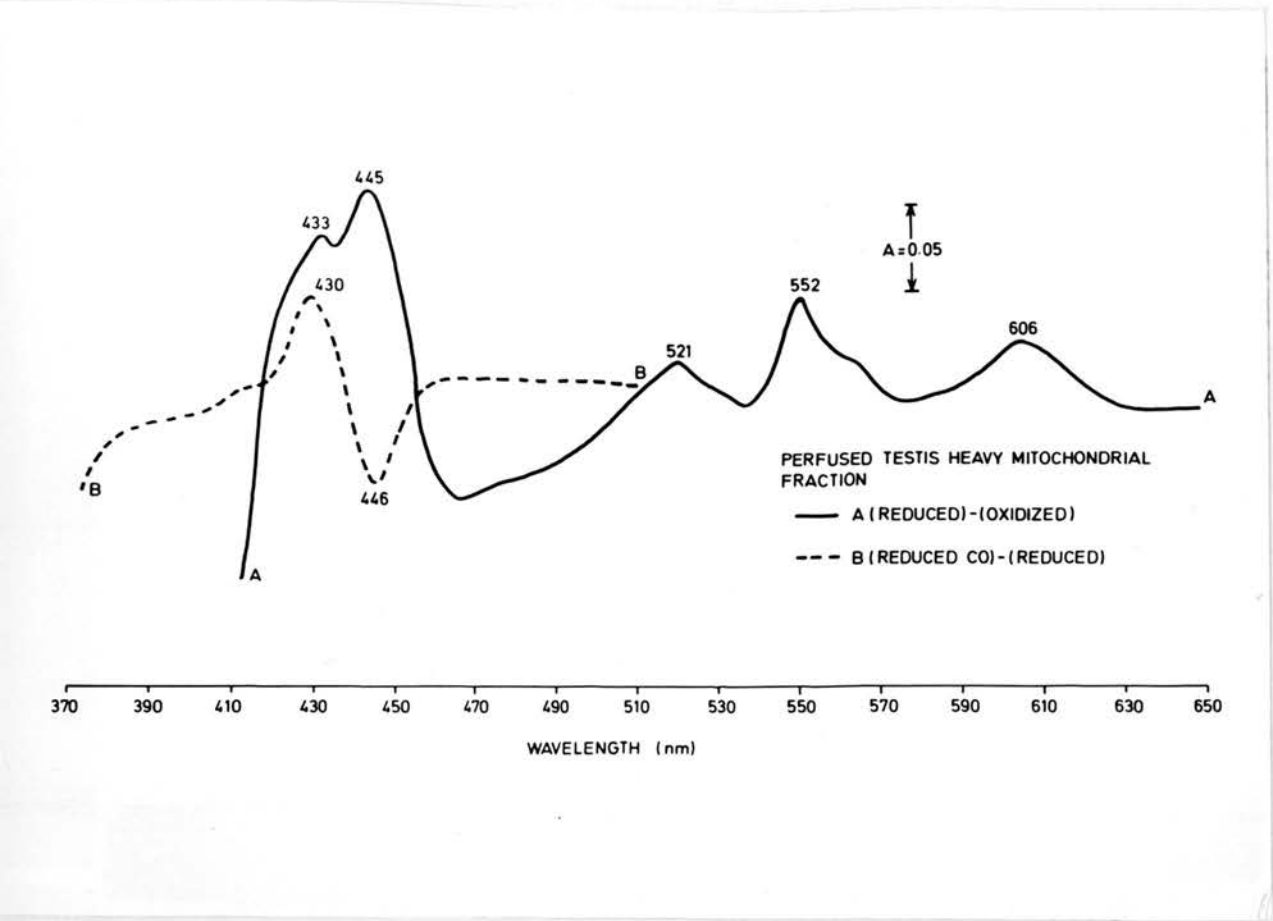


FIG. 5.7 DIFFERENCE SPECTRA OF HEAVY MITOCHONDRIA FROM PERFUSED RAT TESTES

Both experimental and reference cuvettes contained equal amounts of heavy mitochondria ficoll fraction 5 (1.6 mg/ml) prepared from perfused rats. After establishing a base-line a few crystals of solid sodium dithionite was added to the contents of the experimental cuvette and the reduced minus oxidized difference spectrum recorded - Curve A ——— . The reference cuvette was then also reduced with dithionite and after determining the reduced minus reduced base-line, the experimental cuvette was saturated with carbonmonoxide and the carbonmonoxide reduced minus reduced difference spectrum recorded (curve B ----)..

b. Reduction of cytochrome by succinate.

Cammer and Estabrook (1967), using bovine adrenal mitochondria, demonstrated that the addition of succinate caused a reduction not only of the respiratory cytochromes but also of those cytochromes concerned with steroid hydroxylation in these mitochondria. Furthermore, when succinate was used as an electron donor, the presence of inhibitors of mitochondrial respiration such as cyanide prevented the reduction of cytochrome P450 (i.e. only cytochromes of the normal respiratory chain were reduced).

In an attempt to obtain such a difference spectra a suspension of heavy mitochondria (from perfused testes) were divided equally into two cuvettes and the base-line established. After the addition of cyanide (1 mM final concentration) and succinate (5 mM) to the experimental cuvette curve A (Figure 5.8) was recorded and shows the characteristic absorption bands of reduced cytochromes of the classical mitochondrial respiratory chain similar to that observed with dithionite (see section 5.2). Curve B was obtained when succinate was added to the other (reference) cuvette. The spectrum shows the splitting of the broad Soret absorption band into two with peak maxima at 428 nm and 446 nm representing the bands of cytochromes b and ($a + a_3$) respectively. The optical increment at 446 nm was slightly decreased. No new absorption bands were detected at 432 or 557 nm as expected if cytochrome P450 were present (Cammer & Estabrook, 1967; Robinson, 1971). This spectrum would have represented the difference spectrum of reduced cytochrome P450 minus oxidized cytochrome P450.

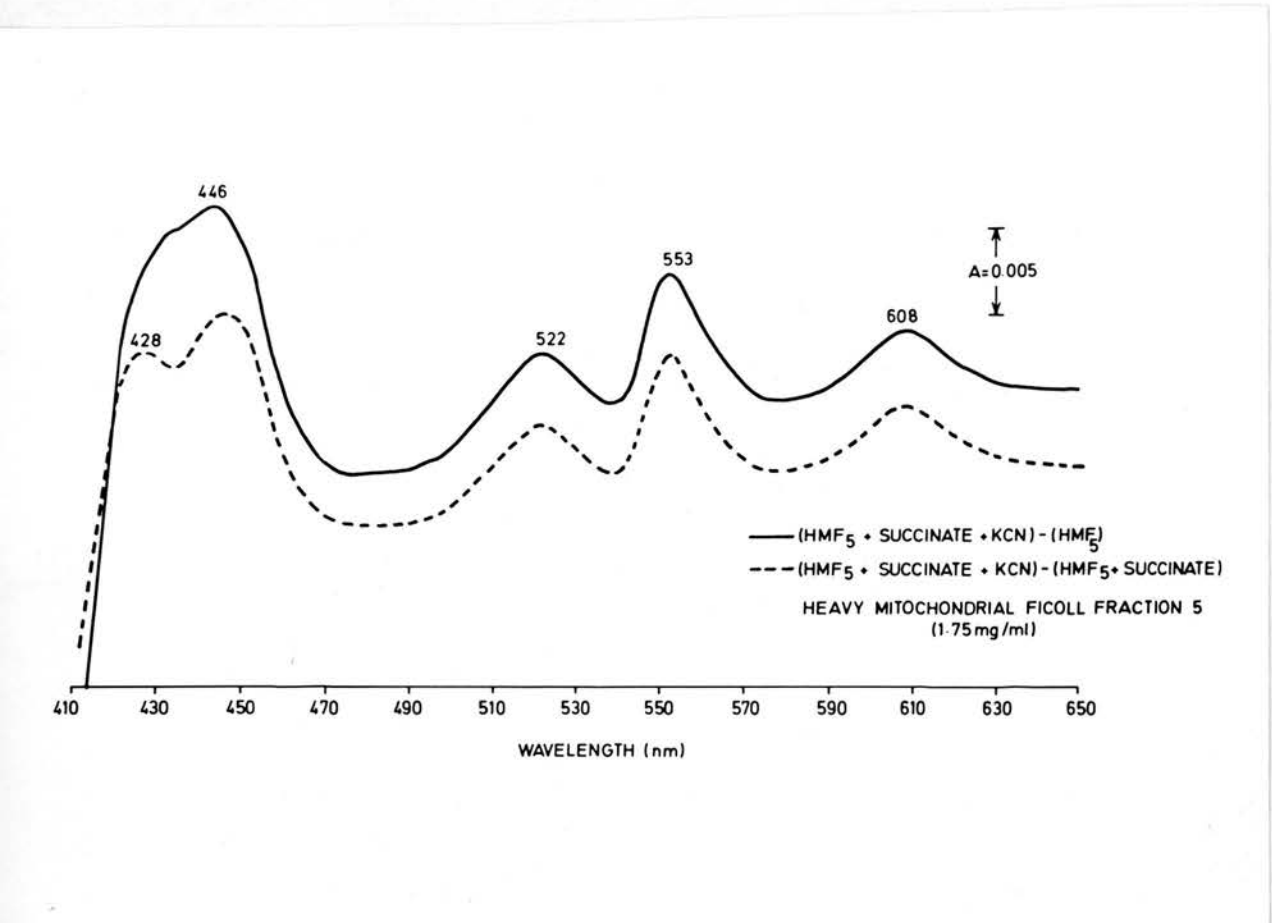


FIG. 5.8 SUCCINATE REDUCTION OF CYTOCHROMES

A suspension of heavy mitochondria (1.8 mg/ml) from perfused rat testes were divided equally into two cuvettes and the base-line established. Curve A ——— was recorded after the addition of potassium cyanide (1 mM final concentration) and succinate (5 mM) to the experimental cuvette (reduced minus oxidized cytochromes). Curve B - - - - was obtained when succinate was added to the reference cuvette (reduced cytochrome P450 minus oxidized cytochrome P450).

The results obtained so far show that rat testis mitochondria contain a compliment of cytochromes b, C + C_1 and a large amount of a + a_3. These mitochondria unlike those of other steroidogenic tissues do not appear to contain the second chain involving cytochrome P450. From the above investigations it was apparent that the ability to detect mitochondrial cytochrome P450 was dependent upon at least three factors (a) the absolute level of the pigment in the Leydig cell mitochondria, (b) the relative amount of interstitial cells and (c) the concentration of the mitochondrial respiratory chain cytochromes. Since it was likely that the spectral changes associated with cytochrome oxidase could obscure cytochrome P450 present in low concentrations, an alternative method was soughtto detect and measure this pigment in the heavy mitochondria without the interference from absorption bands of the normal respiratory chain cytochromes.

5.4 DETECTION OF CYTOCHROME P450 IN RAT TESTIS MITOCHONDRIA

It has been demonstrated with adrenal preparations and liver microsomes that various substrates (Cheng & Hardy, 1971; Schenkman et al., 1967) and inhibitors (Remmer et al., 1966; Imai & Sato, 1966; Wilson et al., 1969; Jefcoate et al., 1969) combine with the oxidized forms of cytochrome P450 so as to produce characteristic changes in the absorption spectrum. In general three types of difference spectra have been observed. (1) A type I spectral change with λ max 390 nm and λmin 420 nm. This spectra characterizes the change from oxidized free enzyme to the enzyme-substrate complex observed in the presence

of certain substrates such as cholesterol (Cheng & Harding, 1973) and deoxy corticosterone (Cooper et al., 1965). Whysner et al. (1970) demonstrated using E.P.R. spectroscopy, that such changes were due to the steroid converting low spin cytochrome P450 to a high spin form.

(2) An inverted type I spectral change with λ max at 420 nm and λ min at 390 nm (Mitani & Horie, 1969 ; Jefcoate & Boyd, 1971). E.P.R. spectroscopy has shown that this change is probably due to a high to low spin transition of the cytochrome P450 in the presence of certain steroids such as pregnenolone (Whysner et al., 1970). Inverted type I changes have also been termed type II spectral changes (see Jefcoate et al., 1974).

(3) The term type II spectral change has usually been applied to the binding of lipophilic amines such as aniline, pyridine, amphenone metyrapone octylamine or aminoglutethimide to cytochrome P450. These amines bind both high and low spin P450 to give a low spin amine-cytochrome P450 complex having a difference spectra with λmax 425 nm, λmin 390 nm and λmax 432 nm, λmin 410 nm respectively in the soret region of the spectrum (Jefcoate & Gaylor, 1970; Jefcoate & Boyd, 1971; Jefcoate et al., 1974). Studies with such spectrally identifiable species have indicated that the mitochondria of steroid producing organs possess a type of cytochrome P450 apparently different from that present in the microsomal fractions of the adrenal cortex (Sweat et al., 1970) and liver (Harding & Nelson, 1966).

Of the nitrogenous base inhibitors, it has been observed that metyrapone is a general ligand for cytochrome P450 (both

oxidized and reduced) of various sources such as adrenal mitochondria and microsomes (Sweat et al., 1970) liver microsomes (Hildebrandt et al., 1969) and Pseudomonas putida (Peterson et al., 1971) whereas aminoglutethimide has been reported to interact with all mitochondrial cytochrome P450 in the adrenal cortex (Jefcoate et al., 1973). Metyrapone and aminoglutethimide have been widely used in clinical practice, the former as a diagnostic and therapeutic tool in hypothalamic-pituitary-adrenal cortical function (Chart & Sheppard, 1959; Gaunt et al., 1968) and the latter in the treatment of adrenal hyperplasia (Cash et al., 1967; Smilo et al., 1967). These drugs block certain steps in adrenal steroid biosynthesis. Metyrapone competively inhibits the 11β-hydroxylase reaction (Liddle et al., 1958; Sanzari & Peron, 1966; Harding et al., 1969) while aminoglutethimide inhibits the cholesterol side-chain cleavage reaction in adrenal mitochondria (Kahnt & Neher, 1966; Dexter et al., 1967; Cash et al., 1967; Kowal, 1969). This apparent selective binding property of these lipophilic amines was investigated and employed to detect, characterize and estimate the cytochrome P450 in rat testis mitochondria.

5.4.1 Methodology

For these studies subcellular fractions of rat testes were prepared by differential centrifugation and the heavy mitochondrial preparation further fractionated on a ficoll gradient as previously described (Chapter 2, section 2.2 and 2.3). Spectral changes in these testicular preparations were measured using an Aminco-Chance dual wavelength/split beam recording spectrophotometer in the dual wavelength mode and at 1% transmission. The wavelength pairs (390-410)nm; (410-425)nm or

(406-448)nm were used for measuring the type II amine induced spectral changes and the (390-420)nm for the steroid inverted type I or substrate (sterol) type I difference spectra. These changes in percentage of transmittance were converted to absorbance. In most cases the spectral change ΔA (448-406)nm was used to estimate cytochrome P450 since the absorbance change between these wavelengths is not dependent on the initial spin state of the cytochrome P450, but solely on the concentrations of the haemoprotein. The subcellular fractions were suspended in medium B in a final volume of 3.0 ml per cuvette (1 cm light path). After establishing a base-line sufficient amine (approx. 35 µM aminoglutethimide or metyrapone in 5 µl aqueous solution), steroid or sterol (10 µM in 5 µl ethanol) was added to give a maximal spectral change. Controls with the same amount of solvent (water or ethanol) did not produce any spectral change. Aminoglutethimide (Ellipten) and metyrapone (SU4885) were purchased from CIBA. Because the amount of testicular preparation available was limited, the spectral changes with aminoglutethimide and metyrapone were often measured on the same sample. First the optical change induced with aminoglutethimide was recorded (since metyrapone binds to both mitochondrial and microsomal species of cytochrome P450). Metyrapone was then added to the same sample and a further spectral change, if any, measured. All determinations were made in duplicate. Figure 5.10 shows an example of this type of spectral recording.

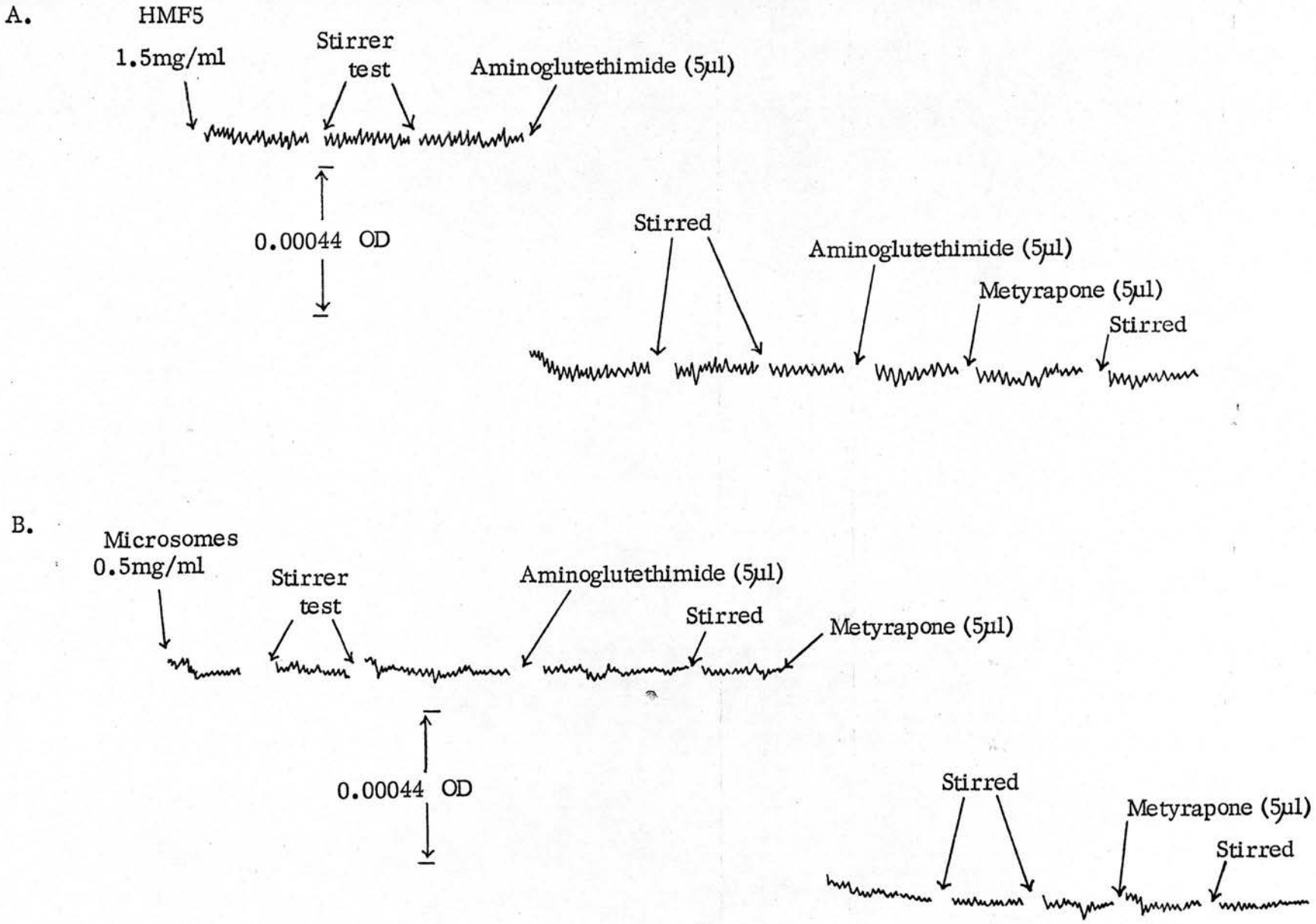

A.
HMF5
1.5mg/ml
Stirrer
test
Aminoglutethimide (5µl)
0.00044 OD
Stirred
Aminoglutethimide (5µl)
Metyrapone (5µl)
Stirred
B.
Microsomes
0.5mg/ml
Stirrer
test
Aminoglutethimide (5µl)
Stirred
Metyrapone (5µl)
0.00044 OD
Stirred
Metyrapone (5µl)
Stirred

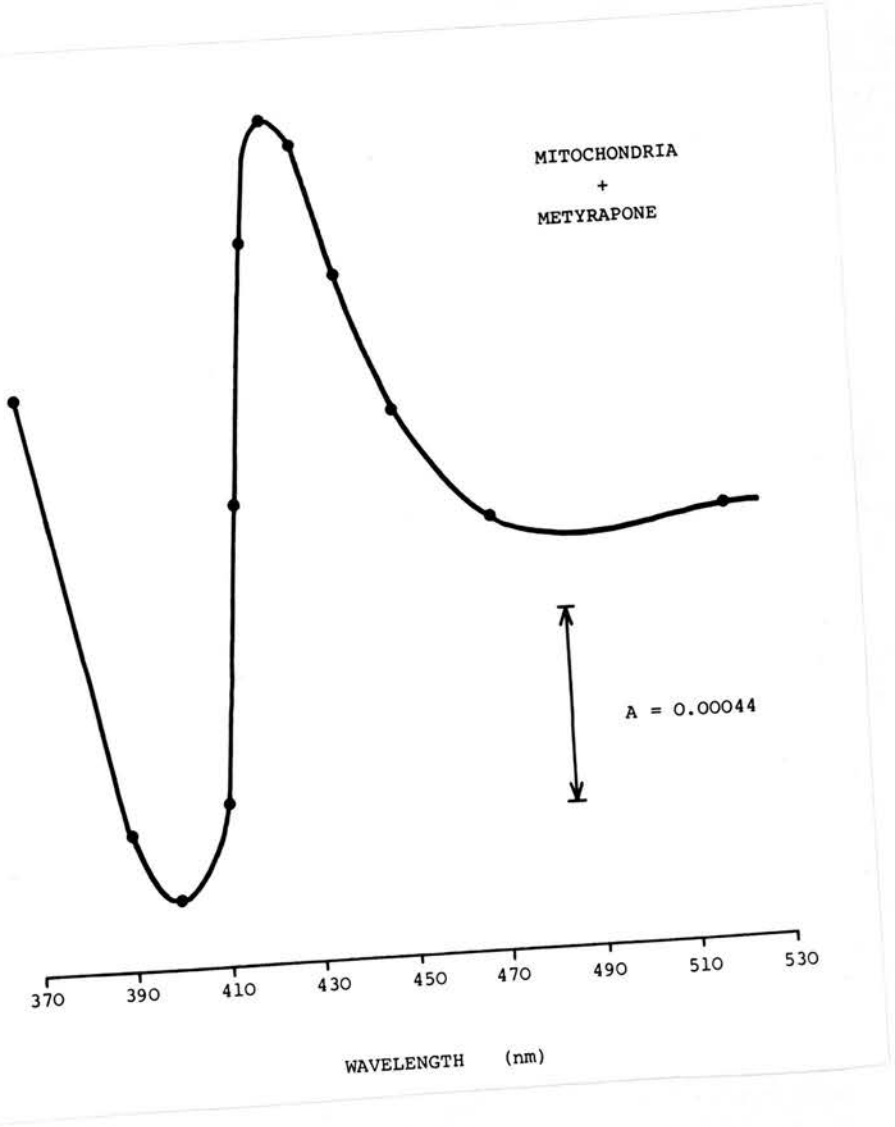
FIG. 5.10 ACTUAL RECORDING TRACE OF AN AMINE-INDUCED ABSORBANCE CHANGE

Optical measurements were made in an Aminco-Chance recording spectrophotometer in the dual wavelength mode and at 1% T. Wavelength pair (406nm-448nm). A 3.0 ml cuvette of 1 cm light path was used. With occasional stirring of the subcellular fraction in the cuvette, a base-line was established. Aliquots of amines were added as indicated with intermittant stirring and spectral changes recorded.

5.4.2 Aminoglutethimide and metyrapone induced binding spectra with heavy mitochondria and microsomal fractions from rat testes.

The induced difference spectra obtained are shown in Figure 5.11 A,B,C. Because of the low cytochrome P450 content and the difficulty in attenuating samples of high protein concentration, the spectra shown were constructed from the wavelength pairs taken at intervals of approximately 10 nm in the spectral range of 360 to 520 nm, in the dual wavelength mode as described above. Aminoglutethimide and metyrapone were added to separate samples of the same preparation.

It was observed that aminoglutethimide preferentially interacted with the heavy mitochondrial fraction (curve A) and not with the microsomes producing a difference spectrum with λ max 425 nm and λ min 400 nm whereas metyrapone produced similar spectra with both the heavy mitochondrial (curve B) and microsomal fractions (curve C). These induced difference spectra are characteristic of the modified low spin type II cytochrome P450 complex produced with a number of other nitrogenous base compounds such as amphenone, RS3504, 1-o-nitro-p-tolyl imidazole (Wilson et al., 1969) and octylamine (Jefcoate et al., 1969; Jefcoate & Gaylor, 1970). The difference spectra induced by aminoglutethimide and metyrapone with heavy mitochondria (HMF_5) were quantitatively similar. These spectra provide strong evidence for the presence of cytochrome P450 in the mitochondria of rat testis.


MITOCHONDRIA
+
METYRAPONE
A = 0.00044
370
390
410
430
450
470
490
510
530
WAVELENGTH (nm)

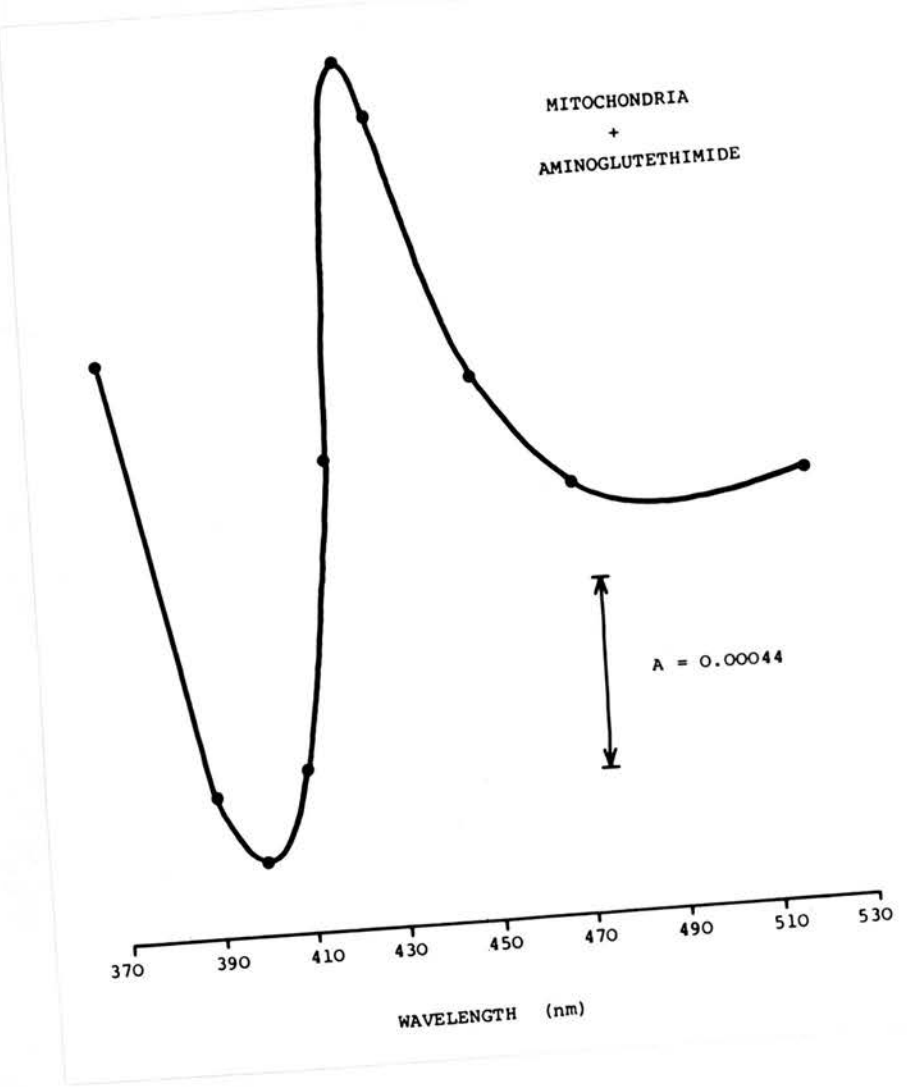

MITOCHONDRIA
+
AMINOGLUTETHIMIDE
A = 0.00044
370
390
410
430
450
470
490
510
530
WAVELENGTH (nm)

FIG. 5.11 AMINE INDUCED DIFFERENCE SPECTRA

Optical measurements were made in an Aminco-Chance recording spectrophotometer in the dual wavelength mode and at 1% T. Spectra were constructed from optical measurements taken at intervals of approximately 10 nm with 520 nm as the isobestic point.

(a) AMINOGLUTETHIMIDE INDUCED DIFFERENCE SPECTRUM IN HEAVY MITOCHONDRIA (FICOLL SUBFRACTION HMF_5)

The HMF_5 protein was 4.4 mg/ml and aminoglutethimide 30 μM.

(b) METYRAPONE INDUCED DIFFERENCE SPECTRUM IN HEAVY MITOCHONDRIA (HMF_5)

HMF_5 protein 4.4 mg/ml and metyrapone 30 μM.

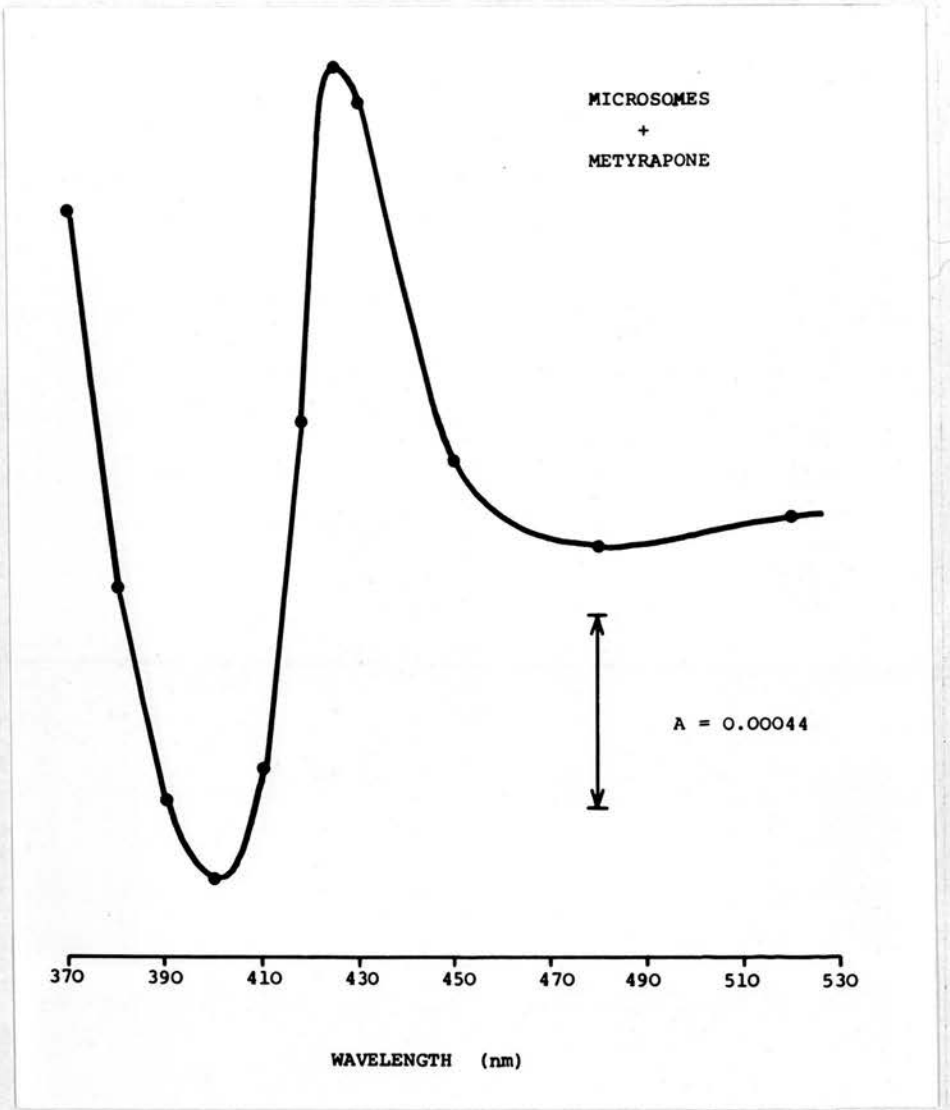


FIG. 5.11(c) METYRAPONE INDUCED DIFFERENCE SPECTRUM IN MICROSOMAL FRACTION

Microsomal protein used 3.0 mg/ml and metyrapone 30 μM.

5.4.3.1 Specificity and distribution of aminoglutethimide and metyrapone detectable cytochrome P450

The amines were added to a suspension of subcellular fractions of rat testes and the induced spectral change measured using the wavelength pair (406-425)nm as described above under "methodology". The results are presented in Table 5.2. In Experiment 1, aminoglutethimide was added first. After recording the resulting optical change metyrapone was then added to the same sample and any further optical change recorded. In Experiment 2 the order of the addition of amines was reversed - metyrapone was added first.

The results of Experiment 1 confirm that aminoglutethimide specifically complexes with the cytochrome P450 of the heavy mitochondrial fraction of the testis (rich in cholesterol side-chain cleavage activity) but not with the cytochrome P450 of the microsomes. When metyrapone was subsequently added to the sample containing aminoglutethimide, an additional spectral response was obtained. This was small with the heavy mitochondria and large with the microsomal fractions. This observation clearly demonstrates that the cytochrome P450 of the heavy mitochondrial and microsomal fractions of the testes are two distinct species. No optical change was observed with the supernatant (105,000 x g), indicating the absence of cytochrome P450 in this fraction. The small spectral change in the light mitochondrial fraction produced with aminoglutethimide and the contrasting large absorbance obtained with metyrapone are consistent with this particulate fraction, being a mixture mainly of microsomes with a small amount of mitochondria.

TABLE 5.2

SPECIFICITY AND SUBCELLULAR DISTRIBUTION OF AMINOGLUTETHIMIDE AND METYRAPONE DETECTABLE CYTOCHROME P450

Expt. No.	AMINES ADDED TO SAMPLE First	Second	SPECTRAL CHANGES $\Delta A(406-425)$ nm x 10^4 Heavy Mitochondria (HMF_5) 3.0 mg/ml	Light Mitochondria 3.0 mg/ml	Microsomal Fraction 3.0 mg/ml	Supernatant Fraction 8.0 mg/ml
1	Aminoglutethimide		11.44	0.88	NM	NM*
	+	Metyrapone	2.20	23.32	22.0	NM
2	Metyrapone		11.44	24.64	22.0	NM
	+	Aminoglutethimide	2.64	NM	NM	NM

*NM = Not measurable (i.e. absorbance change x 10^4 less than 0.44)

Subcellular fractions were prepared, as previously described in Chapter 2 section 2.2, by differential centrifugation, and the heavy mitochondria was further fractionated on a 3-10% ficoll gradient. Spectral determinations were made in an Aminco-Chance spectrophotometer in the dual wavelength mode and at 1% T. The subcellular fractions were suspended in medium B (pH 7.5) to give the above protein concentrations. After establishing the base-line, in Experiment 1 aminoglutethimide (30 μM) was added first and spectral change recorded. With this as the new base-line metyrapone (30 μM) was then added to the same sample. In Experiment 2 the order of amines added was reversed.

The data in Experiment 2 demonstrates the non-specific nature of metyrapone binding. When added first, metyrapone interacted with the heavy mitochondrial (HMF_5) cytochrome P450 producing a spectral response of similar magnitude to that obtained when aminoglutethimide was added first (Experiment 1). In Experiment 2 the subsequent addition of aminoglutethimide to the heavy mitochondrial fraction already containing metyrapone did not produce any further optical change thus indicating that all the cytochrome P450 was bound up with metyrapone. Similarly with the light mitochondria and microsomal fractions only the metyrapone-cytochrome P450 complex was formed. In each of these cases the magnitude of the spectral change observed (in Experiment 2) was the same as in Experiment 1.

These experiments show that both amines produce quantitatively similar spectral changes in the same species of cytochrome P450 and further demonstrate that aminoglutethimide was bound to all the mitochondrial cytochrome P450. These observations indicate that by virtue of its specificity, aminoglutethimide could be used for detecting and quantitating the mitochondrial type cytochrome P450 even in the presence of the microsomal cytochrome species.

5.4.3.2 Distribution of aminoglutethimide detectable cytochrome P450 during ficoll gradient fractionation of heavy mitochondria

When heavy mitochondria obtained by differential centrifugation was subjected to 3-10% ficoll gradient fractionation four particulate subfractions were obtained as described in Chapter 2. These fractions were examined for the distribution of

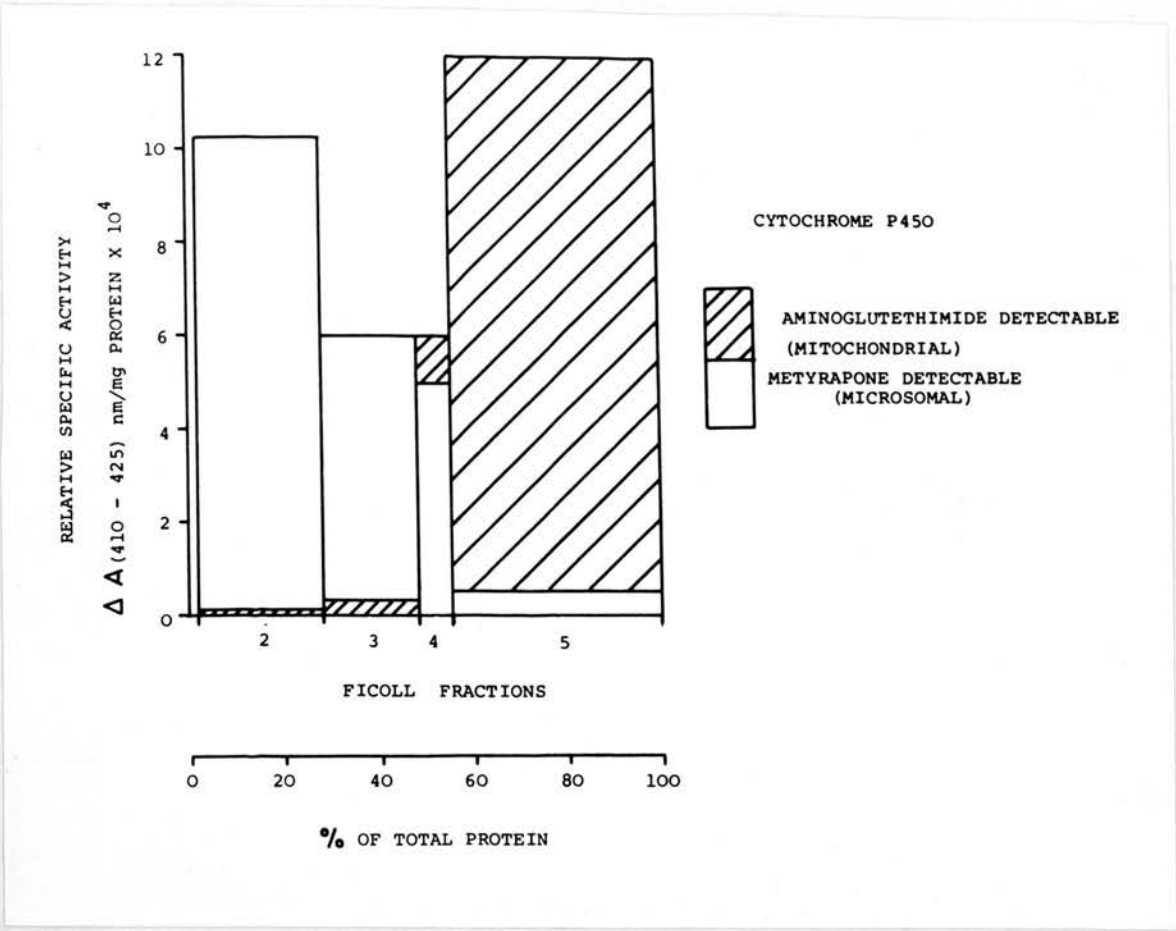


FIG. 5.16 DISTRIBUTION OF CYTOCHROME P450 DURING FICOLL GRADIENT CENTRIFUGATION OF TESTICULAR HEAVY MITOCHONDRIA

Heavy mitochondria prepared by differential centrifugation of testicular homogenate was subjected to gradient fractionation on 3-10% ficoll as described in section 2.3. Mitochondrial type and microsomal cytochrome P450 were detected using aminoglutethimide and metyrapone respectively as described in text (section 5.3.1).

cytochrome P450, with aminoglutethimide and metyrapone added to the same sample as described above. Figure 5.16 shows the results obtained with a typical preparation.

In the homogenate (350 x g supernatant) the amount of aminoglutethimide detectable cytochrome P450 was relatively small compared to that by metyrapone. In the washed heavy mitochondrial fraction (5,000 x g particulate fraction), the mitochondrial species present was contaminated with the microsomal type cytochrome P450. The amount present varied, depending on the number of washings of the particulate fractions. After ficoll fractionation, subfractions 1, 2 and 3 appeared to be mainly microsomal as indicated by the large metyrapone induced absorbance change compared to aminoglutethimide which was insignificant. Subfraction 4 was found to contain aminoglutethimide detectable cytochrome P450 as well as an appreciable amount of microsomal contamination as indicated by the additional metyrapone spectral change. The heavy sedimenting subfraction 5 (HMF_5) however, contained only the aminoglutethimide detectable cytochrome P450. It is interesting to note that the cholesterol side-chain cleavage activity followed a similar distribution pattern to the aminoglutethimide detectable cytochrome P450. Both cholesterol side-chain cleavage activity and mitochondrial cytochrome P450 are therefore localized mainly in ficoll subfraction 5 and to a lesser extent in subfraction 4. The spectral response by subfraction 5 to aminoglutethimide was two to three times greater than the crude heavy mitochondrial fraction (5,000 x g) (Table 5.3). Cholesterol side-chain cleavage activity in ficoll subfraction 5 also exhibited a

TABLE 5.3

PURIFICATION OF RAT TESTIS MITOCHONDRIA BASED ON AMINOGLUTETHIMIDE DETECTABLE CYTOCHROME P450

Subcellular Fraction	ABSORBANCE CHANGE $\Delta A(406\text{-}448)$ nm/mg protein x 10^4		RATIO $\frac{\text{Aminoglutethimide } \Delta A}{\text{Metyrapone } \Delta A}$	Purification over Homogenate
	Aminoglutethimide	Metyrapone		
Homogenate (300xg/20 min.)	0.38	2.12	0.18	-
Heavy Mitochondria (5,000xg/20 min.)	0.96	2.05	0.47	2.5
Heavy Mitochondria Ficoll Fn_5 (HMF_5)	2.96	0.15	1.97	7.8

Rat testes were homogenised and subjected to differential centrifugation as described in Chapter 2. The supernatant from the second low speed centrifugation (300 x g/20 min.) was used as the "homogenate". Heavy mitochondrial fraction (5,000 x g/20 min. pellet) was washed twice. Ficoll gradient fractionation of heavy mitochondria on 3-10% ficoll was performed as previously described (Chapter 2). The resulting pellet fraction 5 was used after washing in homogenising medium.

After recording the aminoglutethimide induced absorbance change metyrapone was added to the same sample.

a similar higher value (see Chapter 3).

5.4.4 Specificity of steroid binding to testicular cytochrome P450

The cytochrome P450 of adrenal cortex mitochondria react specifically with a limited number of steroids producing distinct complexes. The 5-ene-3β-ol steroids such as pregnenolone and 17α-methyl androst-5-ene-3β, 17β-diol (MAD) interact with the cholesterol side-chain cleavage cytochrome P450 in mitochondria to give a difference spectrum with a trough at 390 nm and peak at 420 nm. This inverted type I difference spectrum, which is believed to result from a displacement of substrate cholesterol from cytochrome P450 by the added steroid (Harding et al., 1972) is directly related to the proportion of high spin cytochrome P450 associated with cholesterol side-chain cleavage (Jefcoate & Boyd, 1971; Brownie et al., 1972). On the other hand, potential substrates such as DOC (of 11β-hydroxylase), 25-hydroxycholesterol and 20α,22R-dihydroxy cholesterol (substrates of cholesterol side-chain cleavage system) combine with cytochrome P450 in adrenal cortex mitochondria resulting in an enzyme substrate complex which is identifiable by difference spectroscopy as a type I spectral response (λ max 390 nm and λ min 420 nm). The DOC type I and 5-ene-3β-ol steroid inverted type I binding was suggested to involve two distinct P450 cytochromes (Whysner et al., 1969; Jefcoate & Gaylor, 1970). This was subsequently confirmed by the partial separation of the two P450 cytochromes and their corresponding hydroxylases (Jefcoate et al., 1970).

TABLE 5.4

SPECIFICITY OF DIFFERENT STEROIDS FOR MITOCHONDRIAL AND MICROSOMAL CYTOCHROME P450

STEROIDS	HEAVY MITOCHONDRIA			MICROSOMES	
	Absorbance change $\Delta A_{(390-420)}$ nm/mg protein x 10^4	Type of spectral change	Ratio $\frac{\text{steroid } \Delta A}{\text{MAD } \Delta A}$	Absorbance change $\Delta A_{(390-420)}$ nm/mg protein x 10^4	Type of spectral change
Deoxycorticosterone	NM*	-	-	NM**	-
25-Hydroxycholesterol	1.32	I	0.5	NM**	-
20α,22R-Dihydroxy-cholesterol	6.16	I	2.33	NM**	-
Pregnenolone	0.88	INV I	0.33	4.4	I
MAD	2.64	INV I	1.0	3.9	I

Concentration of subcellular fractions used were: heavy mitochondria (ficoll fraction 5) 3 mg/ml and microsomes 5 mg/ml in medium B (pH 7.5). Approximately 10 μM of steroid was added to the cuvette after establishing the base-line and the spectral change recorded. Further aliquots of the steroid were added until no further spectral change occurred. Controls consisted of samples to which an equal volume of ethanol was added.

NM *, ** = No measurable spectral change at the protein concentrations studied (i.e. absorbance change x 10^4 less than 0.44)

INV = Inverted type I

The ability of certain steroids to complex with testicular mitochondrial or microsomal cytochrome P450 was compared. The results are shown in Table 5.4.

Deoxycorticosterone, which produces a type I spectral change with cytochrome P450 in adrenal mitochondria, was without effect on testis mitochondria reflecting the absence in testis of a cytochrome P450 species associated with 11β- or 18-hydroxylation. The soluble cholesterol analogue, 25-hydroxy-cholesterol produced a small but significant type I absorbance change when added to testicular mitochondria, presumably due to the formation of a typical substrate complex with a cytochrome P450 which is associated with cholesterol side-chain cleavage activity. Such a spectral response to 25-hydroxycholesterol was reported to occur only with intact mitochondria (Jefcoate et al., 1973). 20α,22R-Dihydroxycholesterol, a possible intermediate in the metabolism of cholesterol to pregnenolone (Burstein & Gut, 1971) interacted only with the heavy mitochondria cytochrome P450 of testes producing a type I response. When these type I sterols were added to the testicular microsomal preparations no detectable spectral changes were observed. In bovine and rat adrenal mitochondria such a type I difference spectrum was reported to be specifically due to cytochrome P450 which is associated with cholesterol side-chain cleavage (Jefcoate et al., 1973).

The interaction of methylandrostenediol or pregnenolone with testes mitochondrial cytochrome P450 resulted in the production of an inverted type I spectral change. The magnitude

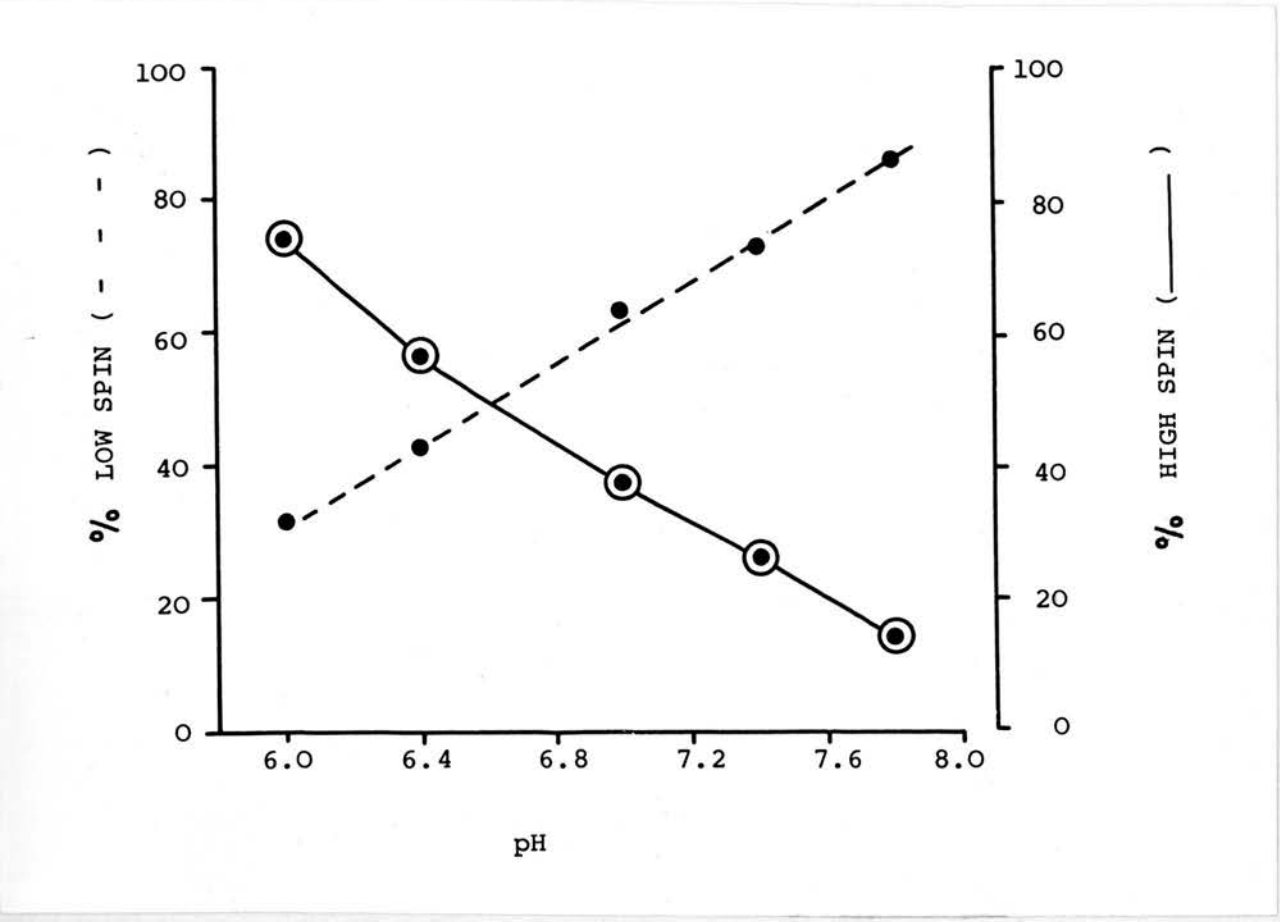


FIG. 5.12 CHANGES IN SPIN STATE OF MITOCHONDRIAL CYTOCHROME P450 INDUCED BY DIFFERENT pH s AND DETECTED BY AMINOGLUTETHIMIDE

Tris buffer was used. HMF_5 protein 2.2 mg/ml. Percentage low spin or high was calculated as described by Jefcoate et al. (1973).

of pregnenolone binding was approximately 30% of that observed with methylandrostenediol, even at pH 6.0. However, when pregnenolone was added to testicular microsomal preparations a type I spectrum characteristic of a substrate induced change was produced. These spectral differences strongly indicate that the mitochondrial and microsomal P450 cytochromes of testes are distinct species. The mitochondrial cytochrome P450 is therefore not a contaminant from the microsomes.

5.4.5 Effect of pH changes on mitochondrial cytochrome P450

Mitani and Horie (1969) first suggested that changes in spin state of the adrenal mitochondrial cytochrome P450 may be affected by alterations to the pH of the environment. Jefcoate et al. (1973, 1974) demonstrated that the pH dependent change in spin state was a property unique to the cholesterol side-chain cleavage cytochrome P450. It was not displayed by cytochrome P450 involved in other reactions. In the amino-glutethimide induced spectrum the absorbance change at 390 nm was reported to be largely derived from the binding to the high spin component and that at 410 nm from the binding to the low spin form. By using the ratio of these absorbances at 390 nm and 410 nm, the proportion of high and low spin cytochrome P450 in adrenal mitochondria was estimated (Jefcoate et al., 1973, 1974). Such a method was used for rat testicular cytochrome P450.

The effect of varying the pH of the medium on the amino-glutethimide induced difference spectra of rat testicular mitochondria was examined. The results of a typical experiment are present in Table 5.5 and Figure 5.12. Table 5.5 shows that with a decrease in pH there was an increase in the ΔA (390-410)nm

TABLE 5.5

EFFECT OF pH ON THE SPIN STATE OF MITOCHONDRIAL CYTOCHROME P450 DETECTED BY AMINOGLUTETHIMIDE

p H	SPECTRAL CHANGE Δ ABSORBANCE x 10^4		SPECTRAL RATIO $\frac{\Delta A(390\text{-}410nm)}{\Delta A(406\text{-}448nm)}$	% CONVERSION of CYTOCHROME P450 to LOW SPIN STATE
	(390-410) nm	(406-448) nm		
6.0	- 10.12	- 9.24	+ 1.10	32
6.4	- 6.6	- 8.6	+ 0.76	43
7.0	- 1.10	- 7.26	+ 0.15	64
7.5	- 1.10	- 7.26	+ 0.15	73
7.8	+ 3.52	- 7.48	- 0.47	84

Heavy mitochondrial protein (ficoll fraction 5) = 2.2 mg/ml. Aminoglutethimide induced spectral changes at the different pHs (Tris buffer) was measured in an Aminco-Chance spectrophotometer in the dual wavelength mode using the wavelength pairs ΔA (390-410)nm and ΔA (406-448nm). The former wavelength pair is dependent on both spin state and concentration of cytochrome P450, while the latter is dependent solely on the concentration of the haemoprotein. From these spectral ratios the percentage low spin was calculated as described by Jefcoate et al. (1973).

The ΔA (406-448) nm remained almost constant. The calculated percentage low spin increased as the pH of the medium was raised from pH 6 to 8 (see Fig. 5.12) suggesting a shift from high to low spin state. At pH 7.5 70-75% of the cytochrome P450 in the heavy mitochondrial ficoll subfraction 5 was found to occur in the low spin state. These pH sensitive changes in spin state of testes mitochondria cytochrome P450 appear to be similar to those observed with a partially solubilized cholesterol side-chain cleavage cytochrome P450 from adrenal mitochondria (Jefcoate & Boyd, 1971).

Since an inverted type I difference spectrum indicates a high to low spin transition of the cytochrome P450 (Jefcoate & Boyd, 1971), the effect of varying pH on the formation of the methylandrostenediol induced inverted type I difference spectrum with testes mitochondrial cytochrome P450 was also examined. A sigmoid curve was obtained. Figure 5.13 demonstrates that as the pH of the medium increased from pH 6 to 8, the magnitude of the inverted type I absorbance change decreased. The spectral change at pH 7.5 was approximately 25% of that at pH 6.0. This pH dependent change in the methylandrostenediol induced spectrum was analogous to that obtained with amino-glutethimide.

In the adrenals a measure of the amount of cholesterol bound to mitochondrial cytochrome P450 was determined from the magnitude of the inverted type I difference spectrum produced by pregnenolone. The magnitude of the spectrum was shown to be dependent on the amount of high spin cytochrome P450 initially present, i.e. the amount of cholesterol bound to cytochrome P450

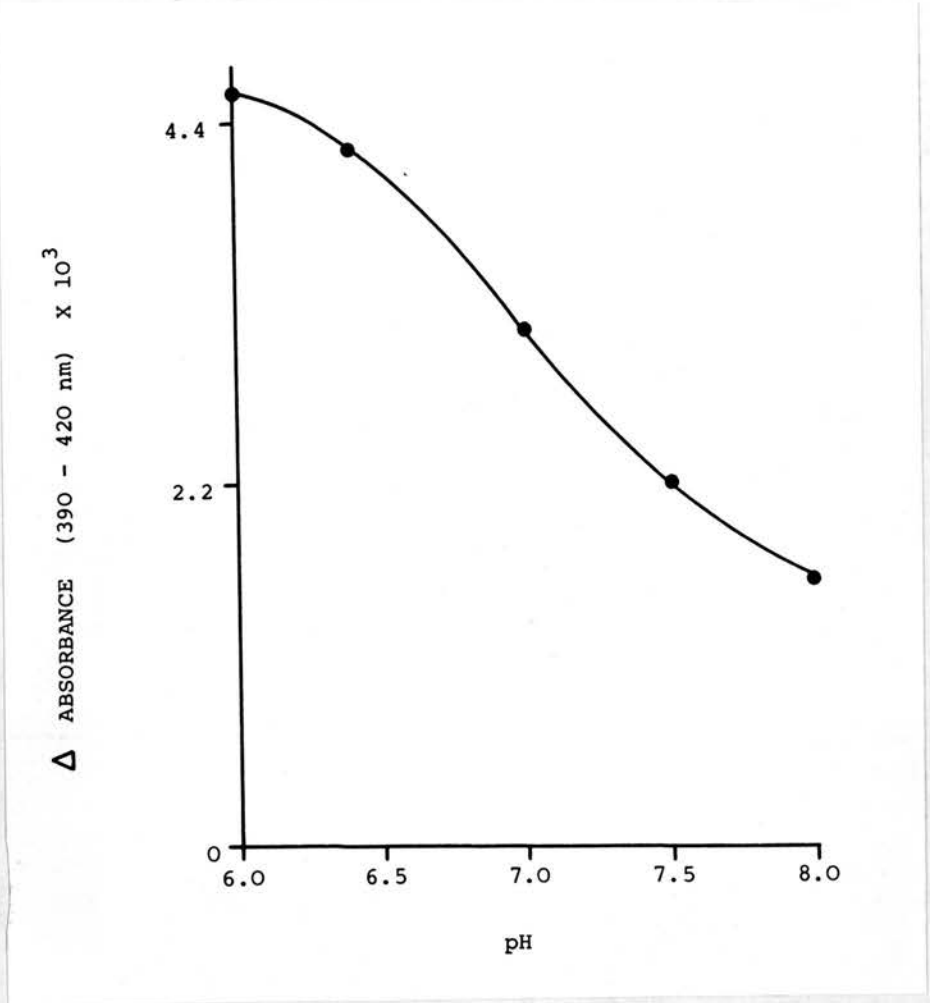


FIGURE 5.13 EFFECT OF pH ON FORMATION OF METHYLANDROSTENEDIOL INDUCED INVERTED TYPE I DIFFERENCE SPECTRA WITH MITOCHONDRIAL CYTOCHROME P450

Ficoll fractionated heavy mitochondria (HMF_5) at protein concentrations of 3.3 mg/ml was used. Buffer was Tris at pH 6.0, 6.4, 7.0, 7.5 and 8.0.

Methylandrostenediol = 10 μM.

TABLE 5.6

CORRELATION BETWEEN AMINOGLUTETHIMIDE AND MAD DETECTABLE CYTOCHROME P450 SPIN CHANGE

EXPT. NO.	HMF_5 protein concentration mg/ml	AMINOGLUTETHIMIDE				METHYLANDROSTENEDIOL	
		Absorbance change $\Delta A \times 10^4$		Ratio a/b	% Low Spin**	Absorbance change $\Delta A \times 10^4$ (390-420) nm	Percent High Spin $\frac{MAD\ \Delta A(390\text{-}420)\,nm}{Amine\ \Delta A(406\text{-}448)\,nm} \times \frac{100}{3.4}$
		(390-410) nm a	(406-448) nm b				
1	2.7	+ 2.20	- 11.0	- 0.2	74.5	11.0	29.4
2	3.0	+ 1.32	- 6.6	- 0.2	74.5	1.72	25.5
3	2.6	+ 2.42	- 6.6	- 0.37	80.0	5.28	23.5
4*	0.9	+ 2.86	- 3.96	- 0.72	91.5	1.32	9.8

*Ficoll fractionated mitochondria was prepared from cycloheximide treated rats.

** Percent low spin was read off from calibration curve correlating the aminoglutethimide spectral ratio to spin state of cytochrome P450 as described by Jefcoate et al. (1973).

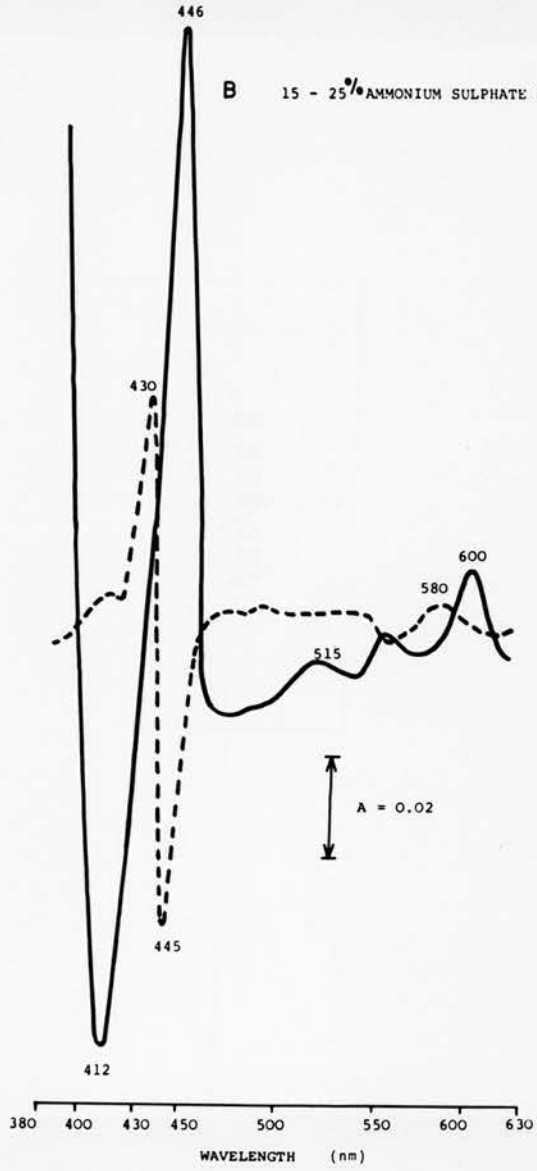
Absorbance change was measured with an Aminco-Chance spectrophotometer in the dual wavelength mode and at 1% T. The heavy mitochondrial preparations (HMF_5) were intact and showed good respiratory control ratios. Spectral measurements were done at pH 7.5 in medium B. The ΔE values used were those determined directly with soluble cytochrome P450 preparations (Jefcoate et al., 1973; Peterson, 1971).

(Jefcoate et al., 1973, 1974). Such a method of estimating the amount of high spin complex of cytochrome P450 seemed to be applicable to testicular mitochondria using the methyl-androstenediol induced inverted type I spectral changes. Table 5.6 shows that there is a very good correlation between the percentage high spin calculated from the inverted type I spectral difference produced with methylandrostenediol and the percentage low spin value obtained by the aminoglutethimide spectral ratio method.

5.4.6 Solubilization of mitochondrial cytochrome P450 from rat testis

A preliminary attempt was made to solubilize cytochrome P450 from rat testes heavy mitochondria using the iso-octane extraction procedure described by Jefcoate et al. (1970) with slight modifications. The procedure was carried out in the presence of 0.1 mM dithiothreitol to protect cytochrome P450.

Ficoll fractionated heavy mitochondria (HMF_5) was obtained as previously described in Chapter 2 (section 2.2 and 2.3) from 12 rats (17 weeks old). The mitochondrial fraction was suspended in 0.1 M phosphate buffer pH 7.4 containing 0.1mM dithiothreitol, sonicated under nitrogen, and then extracted with iso-octane by stirring for 30 minutes. After centrifugation at 105,000 x g for 30 minutes three layers were obtained - a yellow iso-octane supernatant (containing mainly lipid and cholesterol), a brown solid interlayer and a reddish yellow lower phase. The supernatant was aspirated and the remaining layers homogenized and re-extracted with iso-octane, twice, as above. The resulting interlayer was washed with distilled water twice and lyophilized. The brown powder was suspended


446
B 15 - 25% AMMONIUM SULPHATE SATURATION
430
600
580
515
A = 0.02
445
412
380 400 430 450 500 550 600 630
WAVELENGTH (nm)

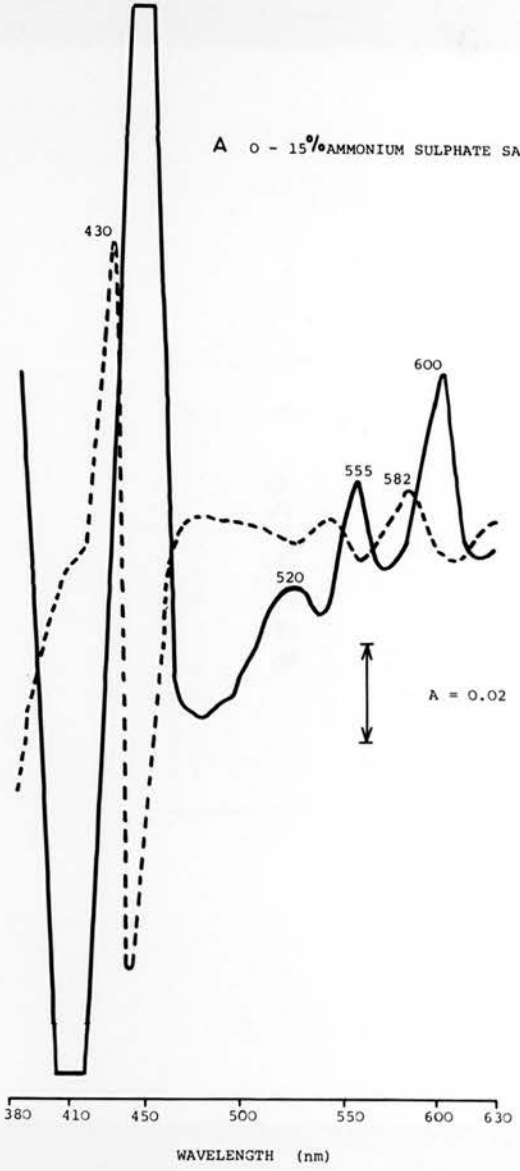

A 0 - 15% AMMONIUM SULPHATE SATURATION
430
600
555
582
520
A = 0.02
380 410 450 500 550 600 630
WAVELENGTH (nm)

FIG. 5.14 SOLUBILIZATION OF MITOCHONDRIAL CYTOCHROME P450

Ammonium sulfate fractionation of iso-octane extracts of HMF_5

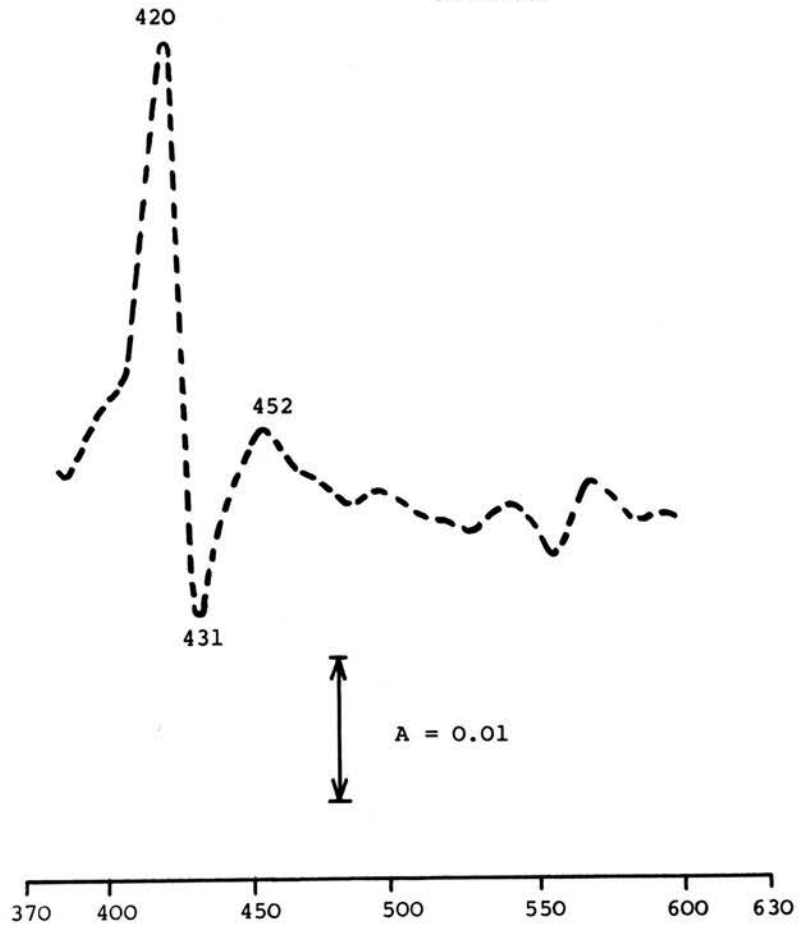
A 0-15% Ammonium sulfate saturation

B 15-25% " " "

——— Dithionite reduced versus oxidized difference spectra

----- Reduced CO minus reduced difference spectra

These recordings were made on a Shimadzu spectrophotometer.


D
35 - 50% AMMONIUM SULPHATE
FRACTION
420
452
431
A = 0.01
370
400
450
500
550
600
630
WAVELENGTH (nm)

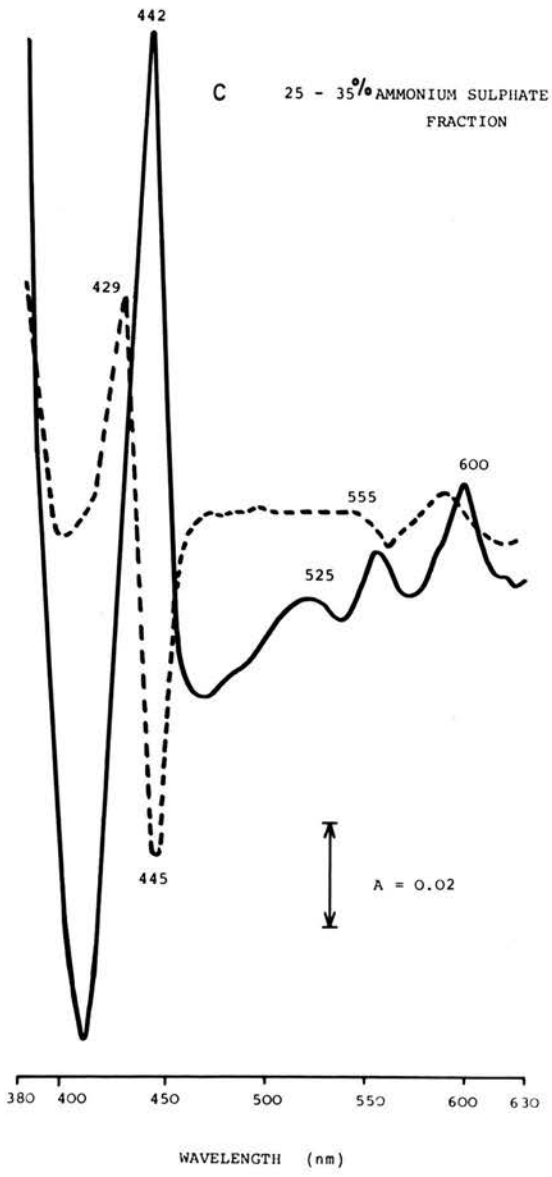

C
25 - 35% AMMONIUM SULPHATE
FRACTION
442
429
600
555
525
445
A = 0.02
380
400
450
500
550
600
630
WAVELENGTH (nm)

FIG. 5.14 SOLUBILIZATION OF MITOCHONDRIAL CYTOCHROME P450

Ammonium sulfate fractionation of iso-octane extracts of HMF_5

C 25-35% Ammonium sulfate saturation

D 35-50% " " "

——— Dithionite reduced versus oxidized difference spectra

----- Reduced CO minus reduced difference spectra

These recordings were made on a Shimadzu spectrophotometer.

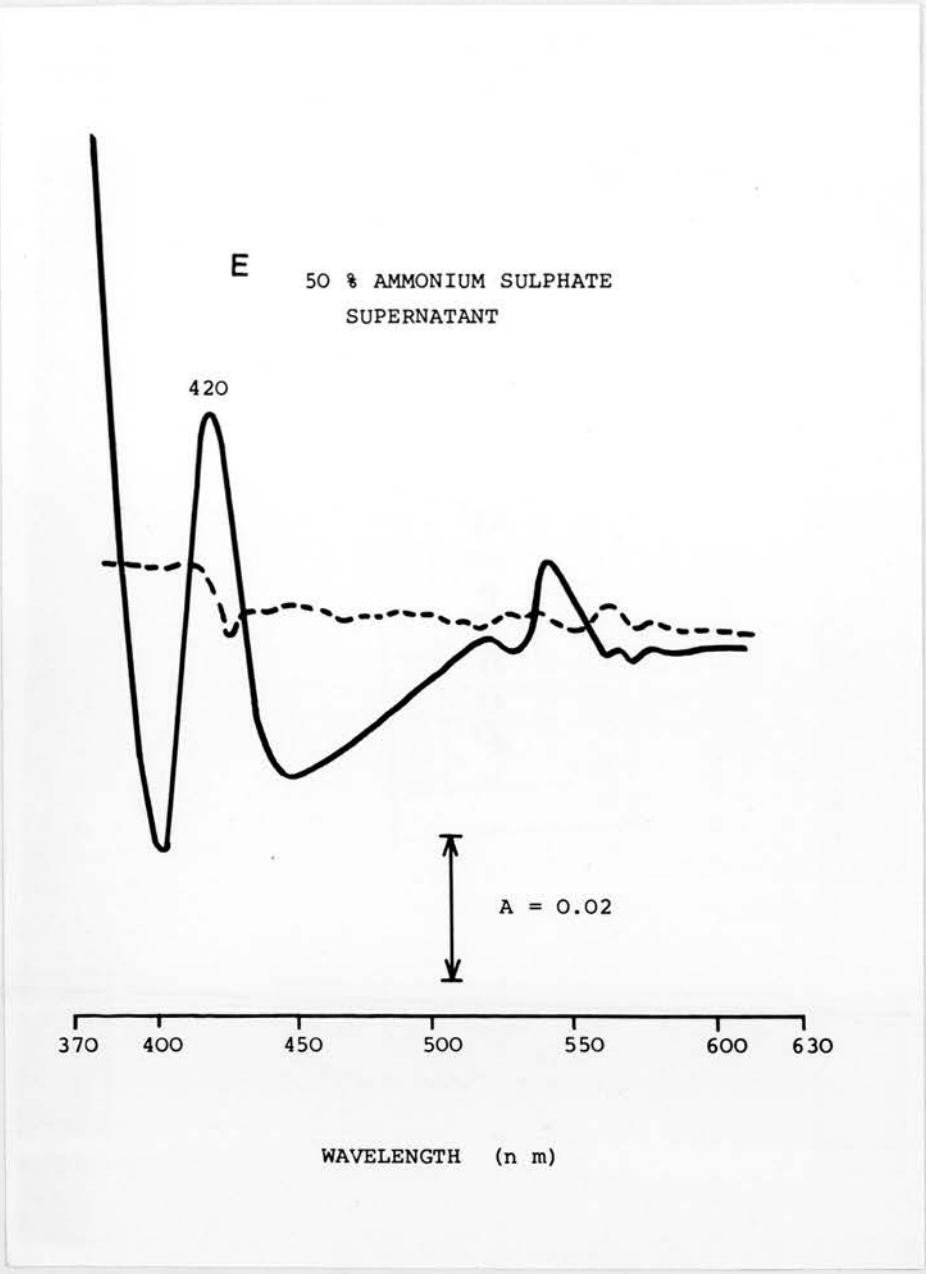


FIG. 5.14 SOLUBILIZATION OF MITOCHONDRIAL CYTOCHROME P450

Ammonium sulfate fractionation of iso-octane extracts of HMF_5

E 50% Ammonium sulfate supernatant fraction

——— Dithionite reduced versus oxidized difference spectra

----- Reduced CO minus reduced difference spectra

These recordings were made on a Shimadzu spectrophotometer.

in 0.1 M Tris buffer 7.4 containing 0.5 mg sodium cholate/mg protein and then subjected to ammonium sulfate fractionation. All fractions were examined for the presence of cytochrome P450 as the reduced CO complex as described earlier.

Figure 5.14 shows that the protein fraction sedimenting between 0-15%, 15-25% and 25-35% ammonium sulfate saturation (Figs. A, B, C) contained mainly cytochromes of the normal respiratory chain and no cytochrome P450. The soluble 35-50% ammonium sulfate fraction (Fig. D) showed the presence of a reduced CO:complex of cytochrome P450 as depicted by the small but definite peak at 452 nm. The absorption peak at 420 nm could be due to cytochrome P420 or hemoglobin. The 50% ammonium sulfate supernatant fraction appeared to be devoid of a CO binding pigment (Fig. E).

In adrenals, it was observed that the fraction precipitating between 15-25% ammonium sulfate saturation yielded mainly cytochrome P450 associated with 11β-hydroxylase activity, whereas the 35-50% ammonium sulfate cut contained the cholesterol side-chain cleavage cytochrome P450 (Jefcoate et al., 1970). This fractionation thus provides conclusive evidence for the presence of cytochrome P450 in normal testes mitochondria and for a similarity of this cytochrome P450 to the adrenal cytochrome P450 which is involved in cholesterol side-chain cleavage.

5.4.7 Cytochrome P450 content in rat testis

The specific cytochrome P450 content in the ficoll fractionated mitochondria of rat testis calculated from the aminoglutethimide difference spectrum ($\triangle$A (406-448)nm and $\triangle$E 28cm^{-1}mM^{-1}) was 0.014 ± 0.003 nmoles/mg protein (mean ± 2 SD of 13 different experiments, each of 8 rats, average age

18 weeks). The corresponding value for microsomal cytochrome P450 using metyrapone was 0.058 ± 0.019 nmoles/mg protein. A similar value of 0.058 ± 0.021 nmoles/mg protein was obtained for the microsomes by the reduced carbonmonoxide method (i.e. ΔA (450-490)nm and ΔE 95$cm^{-1}{}_{m}M^{-1}$). Thus the two methods are comparable. This demonstrates that metyrapone measures all cytochrome P450 in the testis microsomes.

The microsomes contain approximately four times as much cytochrome P450/mg protein as the mitochondria. The specific content of cytochrome P450 in rat testis is very much lower than that reported in other steroid producing tissues (Cammer & Estabrook, 1967; McIntosh et al., 1971; Jefcoate et al., 1973).

The total endogenous cholesterol content (free and esterified cholesterol) in the heavy mitochondrial fraction was found to be 76.5 nmoles/mg protein (70 ± 0.5 nmoles free and 6.5 ± 0.2 nmoles esterified cholesterol). On this basis it appears that there are approximately 7,000 nmoles of cholesterol/nmole of mitochondrial cytochrome P450. However, unlike cytochrome P450, which is localized entirely in the interstitium (see Chapter 7 section 7.4) a sizeable proportion of the cholesterol measured could be of tubular origin.

An approximate estimate of the total mitochondrial and microsomal cytochrome P450 per testis was 0.1 nmoles and 0.9 nmoles respectively.

5.4.8 Involvement of testis mitochondrial cytochrome P450 in cholesterol side-chain cleavage activity.

The spectral properties of cytochrome P450 in rat testis mitochondria show similarities with those of adrenal mitochondrial

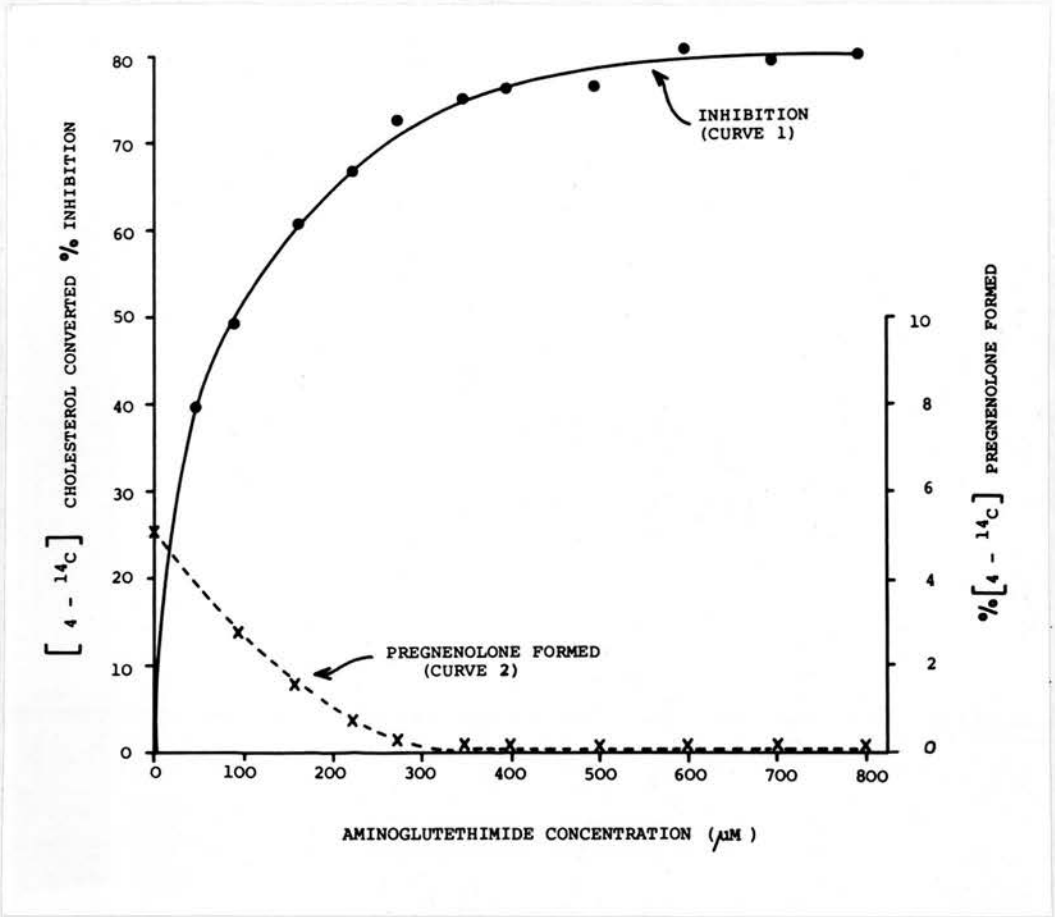


FIG. 5.15 EFFECT OF VARYING CONCENTRATIONS OF AMINOGLUTETHIMIDE ON CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY

Incubations consisted of medium B, [4-^{14}C] cholesterol (Ca 200,000 c/m), DL isocitrate (5 mM), cyanoketone (6 μM), heavy mitochondria (HMF_5) 0.4 mg and varying concentrations (0-800 μM) of aminoglutethimide. Incubations were carried out at 37°C for 60 minutes. In addition to zero minute controls, incubated controls containing boiled enzyme were also included.

cytochrome associated with the cholesterol side-chain cleavage enzyme. Furthermore, the close association of the cholesterol oxygenase activity and the aminoglutethimide detectable cytochrome P450 during subcellular fractionation and their localization entirely in the Leydig cells of the testis (demonstrated in Chapter 7) provide circumstantial evidence for the involvement of cytochrome P450 in steroidogenesis in testis. Direct evidence supporting this view was obtained by the following experiment demonstrating the inhibitory effect of aminoglutethimide on the conversion of cholesterol to pregnenolone in the ficoll fractionated heavy mitochondria from rat testis.

Figure 5.15 shows the effect of different concentrations of aminoglutethimide (0-800 μM) on the cholesterol side-chain cleavage activity in the ficoll fractionated heavy mitochondria of rat testes. For this study cholesterol side-chain cleavage activity was assayed as previously described. Expressed as the percentage of [4-^{14}C] cholesterol converted to labelled steroids the results of Figure 5.15 curve 1 shows that at approximately 80 μM aminoglutethimide inhibited cholesterol metabolism by 50% and at 400 μM by 80%. With higher concentrations up to 800 μM, complete inhibition did not occur. When longer incubation periods of 90 to 150 minutes were used the inhibition observed was much lower (55-66% at 400 μM aminoglutethimide). However, from the nature of products obtained it was observed (curve 2 Fig. 5.15) that the conversion of cholesterol to pregnenolone was completely inhibited, but a

new product was obtained resembling DHA on TLC. The nature and formation of this new product is discussed in Chapter 8. It was therefore concluded that aminoglutethimide appears to bind the mitochondrial cytochrome P450 and thus inhibit the conversion of cholesterol to pregnenolone in rat testis. In other steroidogenic organs, the cholesterol side-chain cleavage enzyme system has been shown to involve cytochrome P450, an iron-sulfur protein (adrenodoxin) and a FAD-containing flavoprotein (NADPH-adrenodoxin reductase) (Simpson & Boyd, 1967b).

Although *in vivo* and *in vitro* studies have demonstrated the inhibitory effect of aminoglutethimide on the conversion of cholesterol to pregnenolone in adrenals of humans (Cash *et al.*, 1967), monolayer of adrenal tumour cells (Kowal, 1969) and ovaries (Behrman *et al.*, 1970), similar effects on testes have not been previously observed.

SUMMARY

1. By difference spectroscopy it was demonstrated that rat testis heavy mitochondria contained a complement of cytochromes b, c + c_1 and a(+ a_3) similar in quantity to other steroidogenic tissue. In contrast the presence of the carbon monoxide binding pigment (cytochrome P450) could not be demonstrated even in mitochondria from perfused testes. Attempts to preferentially reduce and form a CO:complex with cytochrome P450 were unsuccessful. The presence of cytochrome oxidase or hemoglobin was observed to have a masking effect on the cytochrome P450:CO binding spectrum. The molar ratio of cytochrome a(+ a_3) to mitochondrial cytochrome P450 appeared to be unusually high. Cytochrome P450 was present in the microsomal fraction. The fraction called "light mitochondria" was found to be a mixture of mitochondria and microsomes - based on the presence of cytochrome oxidase and cytochrome P450 respectively.

2. The presence of cytochrome P450 in rat testis mitochondria was demonstrated by specific amine binding. Aminoglutethimide produced a type II difference spectra only with mitochondrial cytochrome P450 whereas metyrapone interacted with both mitochondrial and microsomal cytochrome P450. This specificity was utilized for the detection, quantification and distribution of the mitochondrial cytochrome P450.

3. The effect of steroid binding to testicular cytochrome P450 was investigated. DOC did not interact with either mitochondrial or microsomal cytochrome P450 reflecting the absence of the adrenal type cytochrome P450 associated with 11β-or 18-hydroxylation. 25-Hydroxycholesterol and 20α,22R-dihydroxycholesterol

produced a type I spectral change only with the mitochondrial hemoprotein. Methylandrostenediol and pregnenolone both produced an inverted type I spectra with mitochondrial cytochrome P450 and a type I with corresponding cytochrome in the microsomal fraction. These spectral differences clearly indicate that the testes contain at least two very distinct cytochrome P450s, one localized in the mitochondria and the other in the microsomes.

4. Using aminoglutethimide to measure low spin cytochrome P450 and MAD the high spin complex, the effect of pH on the spin state was studied. High pH favours the low spin form and low pH the high spin state.

5. The conversion of cholesterol to pregnenolone by rat testis mitochondria was inhibited by aminoglutethimide (50% at 100 μM).

6. A preliminary attempt to solubilize mitochondrial cytochrome P450 involving sonication, iso-octane extraction and ammonium sulfate fractionation resulted in a partially soluble preparation (35-50% ammonium sulfate fraction) which formed the CO:complex of reduced cytochrome P450.

CHAPTER 6

EFFECT OF DIFFERENT *IN VIVO* TREATMENTS ON TESTICULAR STEROIDOGENESIS *IN VITRO*

CONTENTS

EFFECT OF DIFFERENT IN VIVO TREATMENTS ON TESTICULAR STEROIDOGENESIS IN VITRO

CHAPTER 6

EFFECT OF DIFFERENT IN VIVO TREATMENTS ON TESTICULAR STEROIDOGENESIS IN VITRO

In this chapter the results of the effect of different *in vivo* pretreatments on steroidogenesis in rat testis are presented. The methods used in these investigations and the parameters studied were similar to those developed and described in previous chapters. Mitochondria were prepared from testes of rats subjected to one of the following pretreatments:- a) thyroidectomy, b) stress, c) cycloheximide injections.

6.1. EFFECT OF THYROIDECTOMY ON SOME ASPECTS OF STEROIDOGENESIS IN RAT TESTIS

There have been numerous clinical observations indicating that the adrenals and gonads are affected by thyroid activity. However, in respect to the gonads, there is very little direct experimental evidence to support these observations.

The thyroid gland has long been known to affect the metabolism of adrenal cortical hormones. It has been generally acknowledged, from *in vivo* experiments, that thyroxine brings about an increase of adrenal cortical secretions (D'Angelo & Grodin, 1964; Burstein & Fajer, 1965), while hypothyroidism (surgical or chemical thyroidectomy) is associated with a diminished function of the adrenal cortex (Eik-Nes & Brizzee, 1956; Drucker & Christy, 1963; Gaunt *et al.*, 1970). However, *in vitro* studies by Jao and Koritz (1962) indicated that when thyroxine was added in pharmacological doses to adrenal homogenates, the production of corticosteroids was

inhibited. Strashimirov et al. (1968) observed that thyroidectomy significantly diminished the adrenal 3β-hydroxy steroid dehydrogenase activity.

Ovarian structure and function have also been reported to be affected by thyroid activity (Thorsoe, 1961; Gaunt et al., 1970; Armstrong, 1968b).

In rats, hyperthyroidism appears to alter spermatogenesis, while in hypothyroidism there was disorganization within the tubules with a high incidence of ruptured basement membranes (Johnson, 1969). It has also been reported that the total testis lipid was decreased in hyperthyroid animals whereas hypothyroidism resulted in a marked increase which was associated with the impaired spermatogenesis. Brown et al. (1966) examined certain lipogenic related enzymes in testis of hypophysectomized rats and suggested from their results that thyroxine may not have a direct control over the metabolism of the testis. There has, however, been no report on the effect of thyroid function on steroidogenesis.

The present preliminary study deals with the effect of thyroidectomy on cholesterol side-chain cleavage and 3β-hydroxysteroid dehydrogenase activities, cytochrome P450 and free cholesterol content of the rat testicular mitochondria.

In this experiment adult rats of the Sprague Dawley strain were used. The animals were divided into three groups: one group of 13 rats consisted of thyroidectomized animals; the second "control" group of 11 rats were sham operated, i.e. animals were subjected to the same treatment as the thyroidectomized rats but the thyroids were not removed; the third

TABLE 6.1

EFFECT OF THYROIDECTOMY ON STEROIDOGENESIS IN RAT TESTIS MITOCHONDRIA

Source of Heavy Mitochondria (Ficoll Fn5)	RAT Body Wt gm/rat Mean±SD	Testis Wet Wt gm/testis Mean ± SD	STEROIDOGENIC ACTIVITY % [4-^{14}C] Pregnenolone		MITOCHONDRIAL CYTOCHROME P450 Absorbance Change ($\Delta Ax10^4$/3 mg protein)						Free Cholesterol content nmoles/mg protein Mean ± SD	Respiratory Control Ratios Mean ± SD
					AMINOGLUTETHIMIDE				MAD			
			Formed (CSCC Activity) Mean ± SD	Metabolized (3β-ol dehydrogenase activity) Mean ± SD	390-410 nm (a)	406-448 nm (b)	Ratio a/b	% LS	390-420 nm	% HS		
Thyroidectomy	129±28	1.06±0.11	5.45±0.37	40.2 ± 1.0	+0.89	-6.6	-0.14	73	4.84	21	65 ± 5	1.62±0.03
Sham operated	169±34	1.24±0.15	5.77±0.19	41.2 ± 1.1	+1.32	-7.04	-0.19	75	5.72	24	72 ± 2	1.58±0.05
Normal	256±36	1.61±0.18	7.70±0.23*	62.1 ± 0.66*	-	-	-	-	-	-	66 ± 5	1.56±0.06

*Significantly different from thyroidectomized and sham operated animals ($P<0.001$)

Steroidogenic activity: Results are mean ± standard deviation of 4 determinations. The mitochondrial protein content per incubation tube were:- thyroidectomy 0.46 mg, sham operated 0.5 mg and normal 0.41 mg. Cholesterol side-chain cleavage (CSCC) activity was determined as previously described in Chapter 2. Heavy mitochondria (ficoll fraction 5) were incubated in Medium B with [4-^{14}C] cholesterol, isocitrate and cyanoketone, at 37°C for 90 minutes. Pregnenolone was the only major product formed. 3β-Hydroxysteroid dehydrogenase activity was measured by incubating [4-^{14}C] pregnenolone with heavy mitochondria in Medium B for 60 minutes. Progesterone constituted 75% of the products formed, the remainder were testosterone and androstenedione.
Spectral studies: Optical changes were measured as previously described in Chapter 5. Values are in absorbance and are averages of duplicate determinations. LS = Low spin, HS = High spin.
Free cholesterol content of the mitochondrial fraction was determined by GLC (see Chapter 2).
Respiratory Control Ratios were measured polarographically using succinate as substrate (see Chapter 3).

group consisted of 8 normal, untreated animals. The surgical operations were performed under ether anaesthesia by Dr. G. S. Boyd. All animals were fed on the normal pellet diet and allowed water ad libitum. The rats were sacrificed 6 weeks after the operation. Success of the thyroidectomy was confirmed by the typical bradycardia trace.

Heavy mitochondria were prepared from each group of testes by differential centrifugation and then further fractionated by ficoll gradient centrifugation as described in section 2.2.and 2.3. The resulting subfraction 5 containing intact heavy mitochondria was used. The results are summarized in Table 6.1. No statistically significant difference was observed in the cholesterol side-chain cleavage and 3β-hydroxysteroid dehydrogenase activities between the testis mitochondria from thyroidectomized animals and the sham operated controls; but compared with the normal untreated rats, these activites were significantly reduced ($P<0.001$). The aminoglutethimide detectable cytochrome P450 content (ΔA 406-448 nm) of testes from "thyroidectomized" rats were found to be similar to those from sham operated animals (0.0072 n moles cytochrome P450/mg mitochondrial protein). Furthermore, no significant difference was observed in the spin state of these two groups of mitochondrial cytochrome P450, whether measured by the aminoglutethimide method (determining % low spin) or by MAD (determining % high spin; see Chapter 5 section 4). The values obtained by these two methods showed good correlation. The free cholesterol content of the heavy mitochondria from all three groups of

animals were not significantly different from each other.

The data obtained are preliminary. It appears that thyroidectomy has no influence on testes mitochondrial steroidogenesis when compared with the sham operated controls. Such a conclusion would be in accord with the observations of Menon et al. (1967) that pretreatment of hypophysectomized immature rats with TSH did not affect the cholesterol side-chain cleavage activity of the testis (also see Forchielli et al., 1969). However, when compared with the results of normal untreated rats, thyroidectomy appears to significantly diminish the cholesterol side-chain cleavage and 3β-hydroxysteroid dehydrogenase activities in the rat testis mitochondria. On the basis of such a comparison it has been reported that thyroidectomy inhibits the adrenal 3β-hydroxysteroid dehydrogenase (Strashimirov et al., 1968). Such reports should be treated with some caution in view of the above observations.

6.2 EFFECT OF STRESS

Adult Wistar rats (16-18 weeks old) were used in this study. In each experiment animals of the same age were divided into two groups, each of 8 rats. One group of rats was subjected to a 15-20 minute ether stress prior to death. Such a treatment has been reported to cause an acute increase in the blood level of ACTH (Matsuyama et al., 1971). Rats of the "control" group were killed quickly with a minimum of stress.

Ficoll fractionated heavy mitochondria (fraction 5) were prepared from testis of both stress and normal animals as previously described (Chapter 2, sections 2 and 3) and the different parameters examined and compared. No significant difference was observed in cholesterol side-chain cleavage and 3β-hydroxysteroid dehydrogenase activities, mitochondrial cytochrome P450 and its spin state and free cholesterol content of the two groups. The results obtained were similar to those presented in Table 6.1. The mode of death, whether by ether, decapitation or cervical dislocation, also did not result in any significant difference in the testicular mitochondrial activities studied.

These results suggest that ACTH and/or adrenal corticoids do not have a direct influence on testicular steroidogenesis, at least in the mitochondria. Forchielli et al. (1969) observed that ACTH treatment of hypophysectomized immature rats failed to stimulate the cholesterol side-chain cleavage reaction in the testes.

It has been reported that ether anaesthesis results in a reduction of testosterone secretion in rats (Bardin & Peterson, 1967). This was associated with the reduction of testicular testosterone content, which was demonstrated to occur within 2 minutes and persist for at least 24 hours after ether anaesthesia (Fariss et al., 1969). Eik-Nes (1962) also reported a fall of testosterone in testicular venous blood in pentobarbital anaesthetized dogs. These effects of anaesthesia have been attributed to the inhibition of pituitary release of gonadotrophins rather than a block in in the metabolic pathways for the formation of testosterone (Fariss et al., 1969).

TABLE 6.2

INFLUENCE OF IN VIVO CYCLOHEXIMIDE TREATMENT ON CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY IN RAT TESTIS MITOCHONDRIA IN VITRO

EXPERIMENT No.	SOURCE OF FICOLL FRACTIONATED HEAVY MITOCHONDRIA (HMF_5)	CSCC ACTIVITY % [^{14}C] Pregnenolone formed Mean ± SD (4)	% INHIBITION	RATIO CYCLOHEXIMIDE CONTROL	P
1	CYCLOHEXIMIDE	6.17 ± 0.19	18	0.82	P<0.001
	CONTROL	7.61 ± 0.13			
2	CYCLOHEXIMIDE	5.52 ± 0.11	15	0.85	P<0.001
	CONTROL	6.46 ± 0.18			
3	CYCLOHEXIMIDE	3.47 ± 0.05	16	0.84	P<0.001
	CONTROL	4.12 ± 0.05			
	*CONTROL CYCLOHEXIMIDE (2 mM) IN VITRO	4.21 ± 0.15	-	-	NS

NS = Not significantly different from controls

Cholesterol side-chain cleavage activity was determined as previously described. Ficoll fractionated heavy mitochondria (HMF_5 0.4-0.47 mg/tube) were incubated in medium B with [4-^{14}C] cholesterol (200,000 c/m), isocitrate (5 mM) and cyanoketone (6 µM) at 37°C. For experiments 1 and 2 incubation time was 90 minutes and for experiment 3, 50 minutes. Results are mean ± standard deviation of four determinations. Values were corrected for zero-time incorporation. [^{14}C] Pregnenolone was the only major product formed.

* In this experiment cycloheximide (2 mM) was added (in vitro) to the incubations containing testes HMF_5 from normal control rats. All other conditions were exactly the same as described above for experiment 3.

6.3 EFFECT OF IN VIVO CYCLOHEXIMIDE TREATMENT ON RAT TESTIS CHOLESTEROL SIDE-CHAIN CLEAVAGE ENZYME ACTIVITY

The isolated mitochondria from rat testes, as indicated earlier, are rich in free cholesterol. Most of the cholesterol found might be present in the outer membrane of the mitochondria as observed in liver mitochondria (Graham & Green, 1970). In mitochondria from Leydig tumour cells very little of the cholesterol in mitochondria was found in the inner mitochondrial membrane (Moyle et al., 1973). Cytochrome P450, a component of cholesterol side-chain cleavage enzyme system has been reported to be located in the inner mitochondrial membrane of adrenal cortex and corpus luteum mitochondria (Satre et al., 1969; Yago & Ichii, 1969; Sottocasa & Sandri 1970). Thus it has been suggested that the rate limiting step in steroidogenesis in bovine adrenal cortex might be the penetration of cholesterol into the mitochondria (Kahnt et al., 1974) or transport of cholesterol from other mitochondrial sites to the cholesterol side-chain cleavage cytochrome P450 (Simpson et al., 1971). A "rapid turning over" protein was postulated to be involved in cholesterol transport (Davis & Garren, 1966). Whether a similar situation may occur in the rat testes mitochondria was investigated using cycloheximide - an inhibitor of protein synthesis. Cycloheximide has been shown to prevent the stimulation of steroidogenesis induced by LH or cyclic AMP in ovarian and luteal tissue (Gorski & Padnos, 1966; Hermier et al., 1971, 1972; Flint et al., 1973).

TABLE 6.3

EFFECT OF IN VIVO CYCLOHEXIMIDE TREATMENT ON RAT TESTIS MITOCHONDRIAL CYTOCHROME P450

EXPERIMENT No.	SOURCE OF FICOLL FRACTIONATED HEAVY MITOCHONDRIA (HMF_5)	SPECTRAL CHANGE ΔA/mg PROTEIN x 10^4		MAD RATIO
		AMINOGLUTETHIMIDE (406-448) nm	MAD INVERTED TYPE I (390-420) nm	CYCLOHEXIMIDE TREATED/ CONTROLS
1	CYCLOHEXIMIDE	3.92	4.84	0.5
	CONTROL	3.74	9.68	
2	CYCLOHEXIMIDE	3.43	2.64	0.4
	CONTROL	3.30	6.60	
3	CYCLOHEXIMIDE	3.78	3.52	0.4
	CONTROL	3.83	7.92	

Optical measurements were made in an Aminco-Chance spectrophotometer in the dual wavelength mode and at 1% T as described in Chapter 5. The changes in percentage transmittance were converted to absorbance. Values are averages of duplicate determinations.

Normal adult rats (17-19 weeks old) of the Wistar strain were used in this study. Each experiment consisted of two groups of rats (of at least 8 animals per group) all of the same age. One group of rats was treated with cycloheximide (10 mg/rat; Koch-Light Labs Ltd.) by intraperitoneal injection, while the other control group was either untreated or injected with saline. Animals were killed 40 minutes after the injection. Intact ficoll fractionated testis heavy mitochondria (HMF_5) were prepared from both groups and a comparison made of the ability of each preparation to cleave the side-chain of cholesterol. The total cytochrome P450 content and the spin change due to the cycloheximide treatment were also measured. These results are presented in Tables 6.2 and 6.3 respectively. The data in Table 6.2 show that the cholesterol side-chain cleavage activity in cycloheximide treated animals was significantly reduced compared to controls ($P<0.001$). The spectral data presented in Table 6.3 also shows a corresponding marked decrease (50-60%) in the methylandrost-5-ene-3β,17-diol (MAD) inverted type I absorbance change in the cycloheximide treated group indicating a fall in the amount of cholesterol bound to P450. However, there was no significant difference in the content of free cholesterol and cytochrome P450 of the two groups of mitochondria. (Average cytochrome P450 content = 0.0118 ± 0.00074 n moles/mg protein.) As indicated earlier (in Chapter 5), cytochrome P450 in the high spin state is thought to be a complex with cholesterol (Harding et al., 1972; Jefcoate & Boyd, 1971). In testis, only about 25%

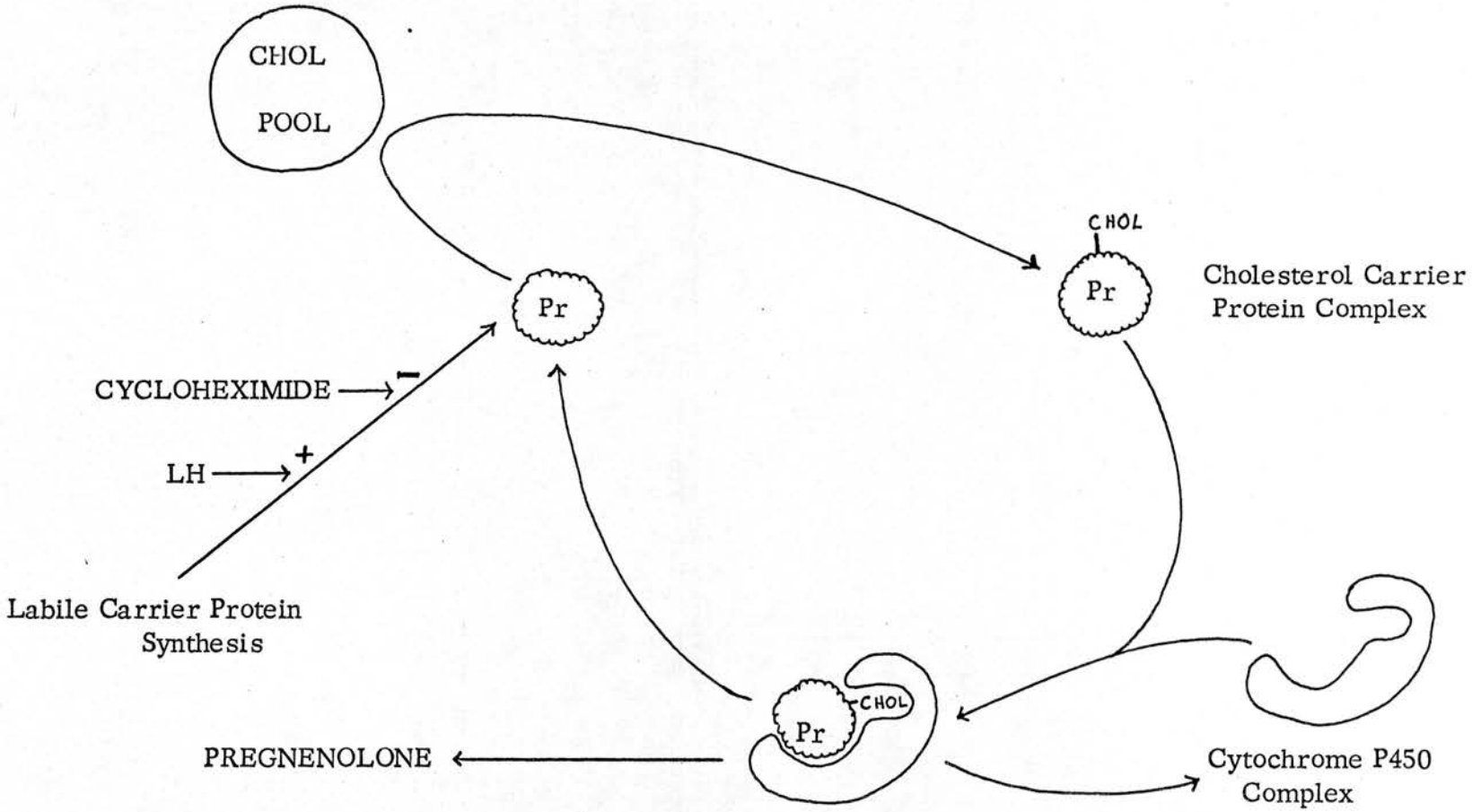


FIGURE 6.1 Role Of Labile Protein Factor In Steroidogenesis In Mitochondria Of Rat Testis.

CHOL = Cholesterol
Pr = Labile Carrier Protein

− = inhibition
+ = stimulation

of the mitochondrial cytochrome P450 was found to occur in the high spin state. Since rat testis mitochondria contain a large amount of free cholesterol (72 ± 6 n moles/mg protein), it is obvious that only a fraction of the available cholesterol is associated with the cytochrome P450. The inverted type I spectral change produced by the interaction of methylandrostenediol with cholesterol side-chain cleavage cytochrome P450 is thought to provide a measure of the proportion of cholesterol bound to the enzyme (Jefcoate & Boyd, 1971). Methylandrostenediol was postulated to alter the interaction of cholesterol with the cytochrome P450 possibly causing the displacement of cholesterol from its binding site (Harding et al., 1972). From the results obtained it can be inferred that cycloheximide, which is a specific protein synthesis inhibitor, reduces pregnenolone biosynthesis by probably affecting the redistribution of cholesterol within the mitochondrion and thereby decreases the amount of precursor cholesterol bound to cytochrome P450. This means that less cholesterol is available for side-chain cleavage (see Fig. 6.1). Since the cytochrome P450 and free cholesterol content of the mitochondria were not significantly altered by in vivo cycloheximide treatment, this inhibitor may exert its rapid influence on steroid biosynthesis by limiting the synthesis of a labile protein factor which possibly transports cholesterol (Ritter & Dempsey, 1973). Evidence for such a protein has been reported in the corpus luteum (Hermier, 1971) and adrenals (Davis & Garren, 1966; Simpson et al., 1972). It is synthesized in response to trophic hormones (Davis & Garren, 1966).

TABLE 6.4

EFFECT OF IN VIVO CYCLOHEXIMIDE TREATMENT ON MITOCHONDRIAL CYTOCHROME P450 FROM RAT ADRENALS

EXPERIMENT No.	SOURCE OF ADRENAL MITOCHONDRIA	SPECTRAL CHANGE $\Delta A(390-420)$ nm/mg protein x 10^3		
		DOC (TYPE I)	25-HOC (TYPE I)	MAD (TYPE I inv)
1	CYCLOHEXIMIDE (a)	3.0	5.3	4.7
	CONTROL (b)	3.0	3.8	8.4
	Ratio a/b	1.0	1.4	0.6
2	CYCLOHEXIMIDE (c)	3.8	5.3	5.0
	CONTROL (d)	3.6	3.1	13.5
	Ratio c/d	1.1	1.7	0.4

Mitochondria from rat adrenals were prepared as described by Simpson et al., (1972). Spectral measurements were made in an Aminco-Chance spectrophotometer in the dual wavelength mode. The final concentrations of substrate added were DOC 20 µM, 25-HOC 25 µM, and MAD 20 µM. Values presented are averages of duplicate determinations. Inv = inverted

6.3.1 Effect of in vivo cycloheximide treatment on rat adrenal mitochondrial cytochrome P450

In order to verify the response of the animal to in vivo cycloheximide treatment, the adrenals were removed from the same group of rats used in the above experiments, and the mitochondria prepared as described by Simpson et al., (1972). With these intact adrenal mitochondria some spectral properties of cytochrome P450 were examined. The results obtained were similar to those previously reported (Brownie et al., 1972; Simpson et al., 1972; Jefcoate et al., 1973) and are summarized in Table 6.4. No difference was observed between adrenal mitochondria from cycloheximide treated and control rats in the magnitude of the type I binding by deoxycorticosterone (DOC) - the substrate of 11β-hydroxylase. The 25-hydroxy cholesterol (25-HOC) type I spectral change, was increased by cycloheximide treatment. This type I spectral change has been attributed to an interaction of 25-HOC with a low-spin cholesterol side-chain cleavage cytochrome P450, which is depleted of cholesterol (Jefcoate et al., 1973). 25-HOC readily undergoes side-chain cleavage in adrenal mitochondria (Burstein & Gut, 1971). Compared to controls, cycloheximide caused a decrease (40-60%) in the MAD inverted type I difference spectrum similar to that observed in the testis. It thus appears that the basic mechanism for control of cholesterol side-chain cleavage activity in testis may be similar to that of the adrenal cortex.

In adrenals the inhibitory effect of cycloheximide on protein and steroid synthesis were reported to occur

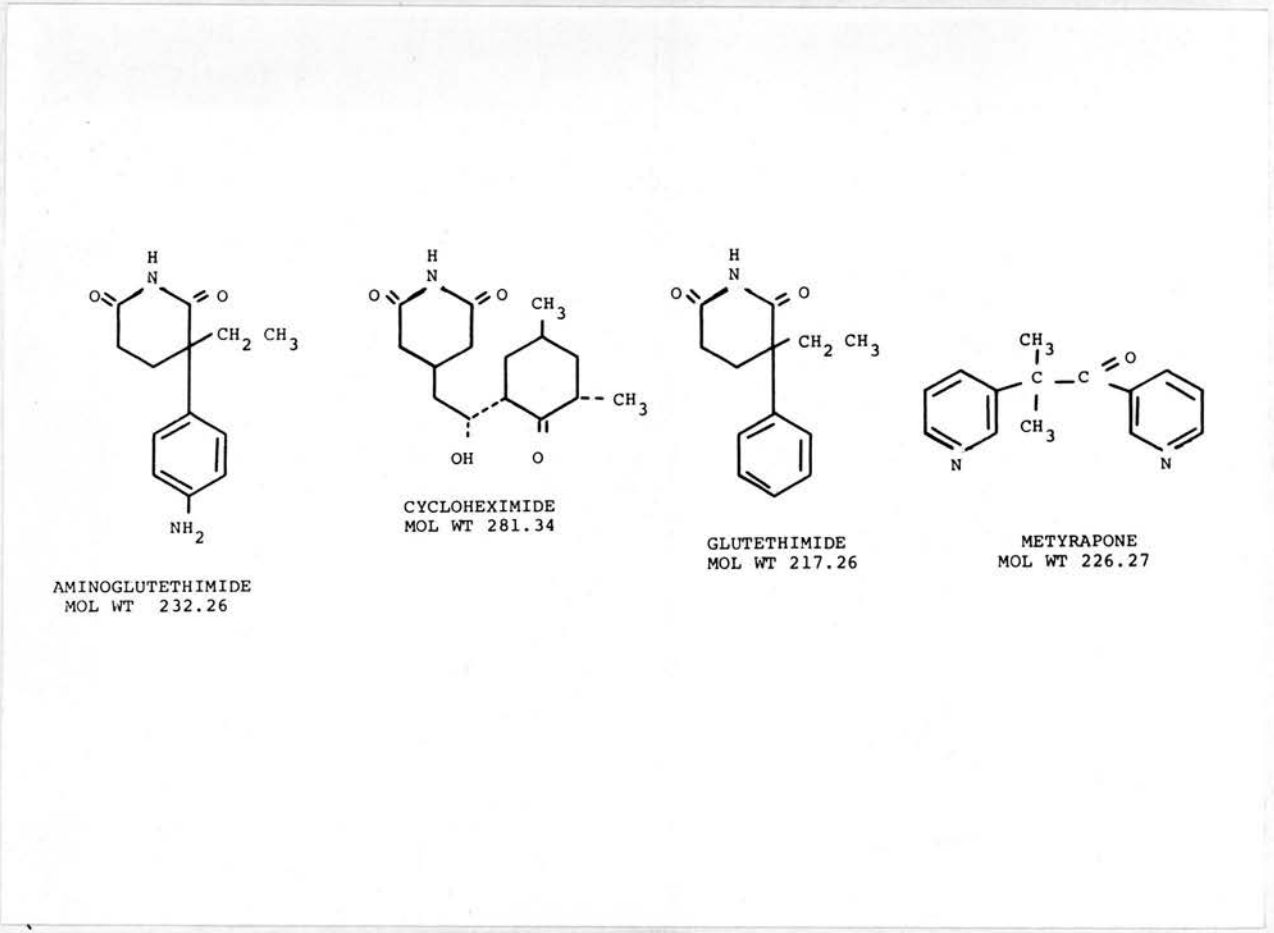


FIGURE 6.2 Chemical Structures Of Inhibitors

immediately ($t \frac{1}{2}$ = 5 mins.; Garren et al., 1965; Kowal, 1970). Simpson et al. (1972) observed changes in adrenal activity within 10 minutes of treatment. However, in rat testis there was no significant effect of cycloheximide within this time period. A longer period of in vivo treatment (40 minutes) was required to produce in vitro measurable changes in the rat testicular steroidogenic activities and spectral properties. It thus appears that cycloheximide acts more slowly on the testis.

6.3.2 Effect of cycloheximide added in vitro to testis mitochondria from control rats

The possibility that cycloheximide could interact directly with cytochrome P450 was considered. When cycloheximide was added to 2 mM concentrations to normal incubations containing ficoll fractionated heavy mitochondria from testes of control rats, no effect on cholesterol side-chain cleavage was observed (Table 6.2, expt. 3). Similarly, at 0.3 mM concentrations, cycloheximide did not induce a spectral change in the mitochondria from controls suggesting an absence of a cycloheximide:cytochrome P450 complex comparable to that induced by aminoglutethimide and metyrapone. As illustrated in Figure 6.2, these three inhibitors are all nitrogenous bases. Cycloheximide resembles aminoglutethimide in that both possess a glutarimide structure but differs from the latter in the absence of a nitrogen on the second ring structure. Glutethimide, which also lacks the amine group, has been reported to be without effect on adrenal function when administered in clinical doses (Gaunt et al., 1968).

SUMMARY

In this chapter the effect of different types of *in vivo* treatments on testicular steroidogenesis was investigated.

1. The results of thyroidectomy were inconclusive. When compared with sham operated animals no significant difference was found in the ability of the mitochondria to convert cholesterol to pregnenolone and to further metabolize pregnenolone. Also no differences were observed in spectral properties of cytochrome P450. However, when compared with normal untreated rats, thyroidectomy appeared to significantly inhibit cholesterol side-chain cleavage and the 3β-hydroxysteroid dehydrogenase activities. No significant differences were found in the free cholesterol content of all three groups.
2. No differences were observed in the various parameters studied with respect to ether stress or mode of sacrifice of the animals.
3. Treatment of intact rats with cycloheximide resulted in a decrease in the conversion of cholesterol to pregnenolone as a result of the reduction in the amount of cholesterol bound to mitochondrial cytochrome P450. The cytochrome P450 content and free cholesterol content were not significantly different from controls. Cycloheximide added *in vitro* to normal mitochondria did not produce any significant effect in enzyme activity or spectral properties. From these observations it was concluded that cycloheximide did not have a direct effect on the cholesterol side-chain cleavage enzyme or cytochrome P450 but exerted its effect via a labile protein which is probably involved in cholesterol binding and transport.

CHAPTER 7

CELLULAR LOCALIZATION OF CHOLESTEROL SIDE-CHAIN CLEAVAGE ENZYME ACTIVITY AND CYTOCHROME P450 OF RAT TESTES

CONTENTS

CELLULAR LOCALIZATION OF CHOLESTEROL SIDE-CHAIN CLEAVAGE ENZYME ACTIVITY AND CYTOCHROME P450 OF RAT TESTES

CHAPTER 7

CELLULAR LOCALIZATION OF CHOLESTEROL SIDE-CHAIN CLEAVAGE ENZYME ACTIVITY AND CYTOCHROME P450 OF RAT TESTES

Although it is generally accepted that interstitial or Leydig cells of the testis are the principal source of testicular steroids (see Chapter 1), there are suggestions based on some histochemical (Baillie & Mack,1966) and biochemical studies (Lacy, 1967; Ellis, 1969; Lacy & Pettit, 1970; Dufau et al., 1971) that the Sertoli cells and the germinal epithelial cells of the seminiferous tubules may also be capable of de novo steroid production.

Employing the technique previously used by Christensen and Mason (1965) for separating seminiferous tubules from interstitial elements of rat testis, Hall et al. (1969), Bell et al. (1968a,b; 1971), as well as Christensen and Mason, demonstrated that the seminiferous tubules converted added labelled pregnenolone and progesterone to testosterone. On the basis of such studies Ellis (1969), and Lacy and Pettit (1970) suggested that the Sertoli cells might convert cholesterol to steroids which are required for maintenance of spermatogenesis. However, Hall et al. (1969) could not demonstrate any significant conversion of radioactive cholesterol to steroids by the isolated intact seminiferous tubules.

All the above mentioned biochemical studies were performed with intact tissues. The failure of seminiferous tubules to convert exogenous cholesterol to androgens may reflect the restricted penetration of cholesterol into the tubules

(Parvinen et al., 1970) as well as dilution effects rather than the absence of enzyme activity. Furthermore, in the experiments with separated, histologically intact components of the testis, less than 50% of the added radioactivity was recovered (Hall et al., 1969; Christensen & Mason, 1965). In order to circumvent such difficulties, studies were performed employing subcellular fractions from the separated testicular components instead of the isolated whole intact tissue. Since steroids are normally synthesized from cholesterol (Menon et al., 1965) and since the heavy mitochondrial fraction from testis has been shown (see Chapters 3 and 4) to convert cholesterol to androgens in vitro and to contain cytochrome P450 (Chapter 5), it was of interest too, to discover the cellular origin of the heavy mitochondrial fraction responsible for the cholesterol side-chain cleavage reactions in the testes. Such experiments could prove whether the seminiferous tubules (Sertoli cells) are capable of de novo steroid synthesis from cholesterol.

7.1.1 Preparation of tissue

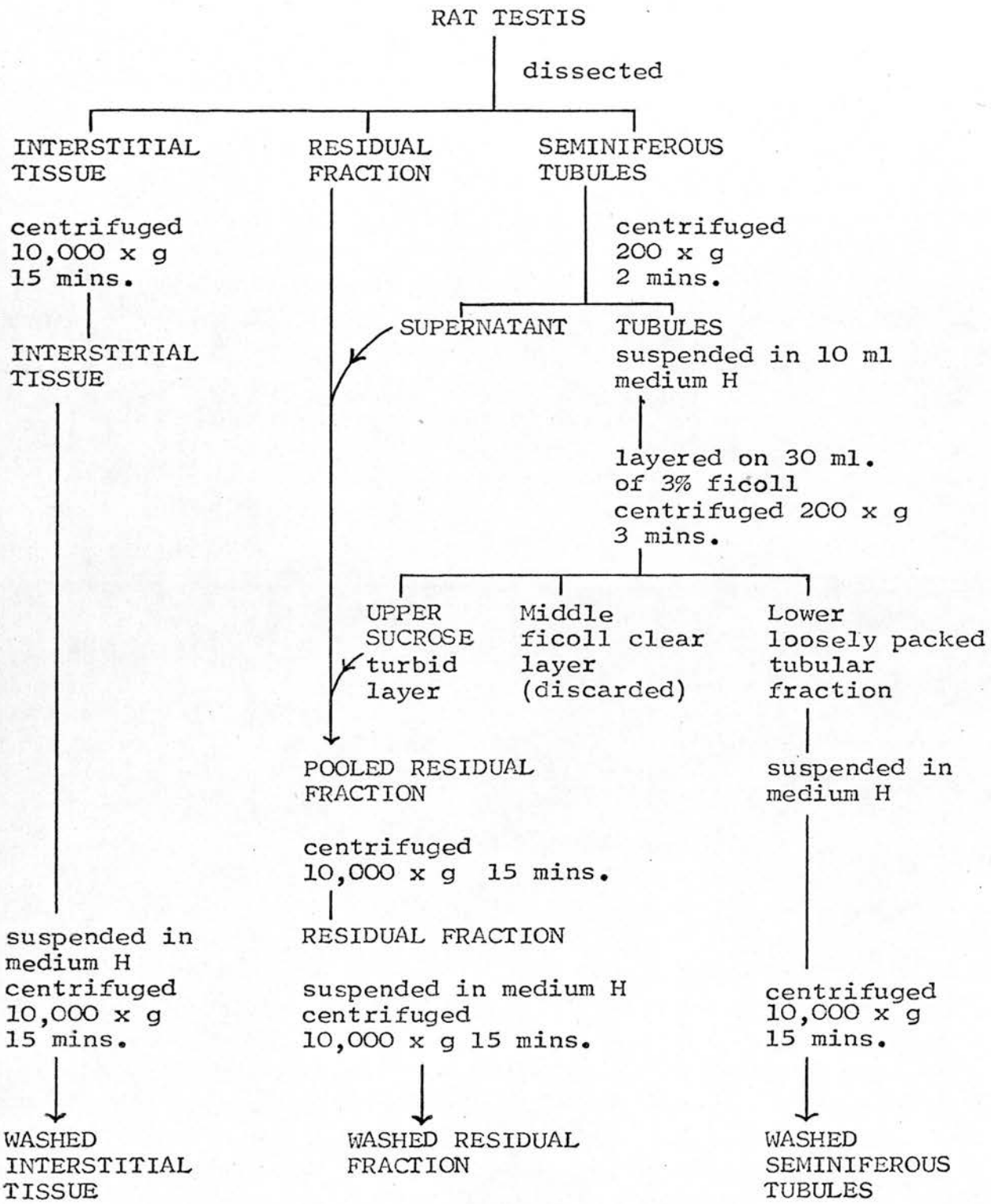
Testis from two adult rats 12-15 weeks old were used for each experiment. Rats were killed and the testis removed as described in Chapter 2. After removal of the tunica albuginea and spermatic artery, the testis tissue was placed in a Petri dish with ice cold medium H (see Chapter 2) containing glucose (2 mg/ml) and kept on ice (or at $0^{o}C$).

7.1.2 Dissection of the testis

Rat testis interstitial tissue and seminiferous tubules were separated by the wet dissection technique similar to that previously described by Christensen and Mason (1965)

FIGURE 7.1

WASHING OF SEPARATED HISTOLOGICAL TESTICULAR TISSUE COMPONENTS



ALL PROCEDURES WERE CARRIED OUT AT 0-4^{o}C

and modified by Hall et al. (1969). All dissection procedures were performed in ice (or at $0^{o}C$).

Small pieces of testis were very gently teased apart and the reddish web of interstitial tissue removed with the aid of jeweller's forceps, retained and then examined under a dissecting microscope in order to remove the remaining small fragment of tubules. The tubules were removed from the rest of the tissue mass, transferred to another container with ice cold medium H plus glucose and also examined under the microscope for adhering interstitial tissue. When all the tubules were removed, the small tissue fragments remaining on the dissection petri dishes were collected by rinsing with the above medium. This was referred to as the "Residual Fraction". Complete dissection of one testis by one person required approximately 2 hours. However, with the technical assistance of colleagues, the dissection of four testes was usually completed in 4-5 hours.

7.1.3 Washing of separated testicular tissues

The tubules were centrifuged at 200 x g for 2 minutes and the supernatant aspirated and pooled with the residual fraction. The tubular mass was then suspended in 10 ml of medium H and layered onto 30 ml of 5% ficoll containing medium H and centrifuged at 200 x g for 3 minutes. Three layers were obtained (a) the upper turbid (whitish) sucrose layer was aspirated and pooled with the residual fraction, (b) the middle clear ficoll layer was discarded and (c) the loosely packed tubular layer at the bottom of the tube was suspended in medium H (see Fig. 7.1). All three testicular fractions

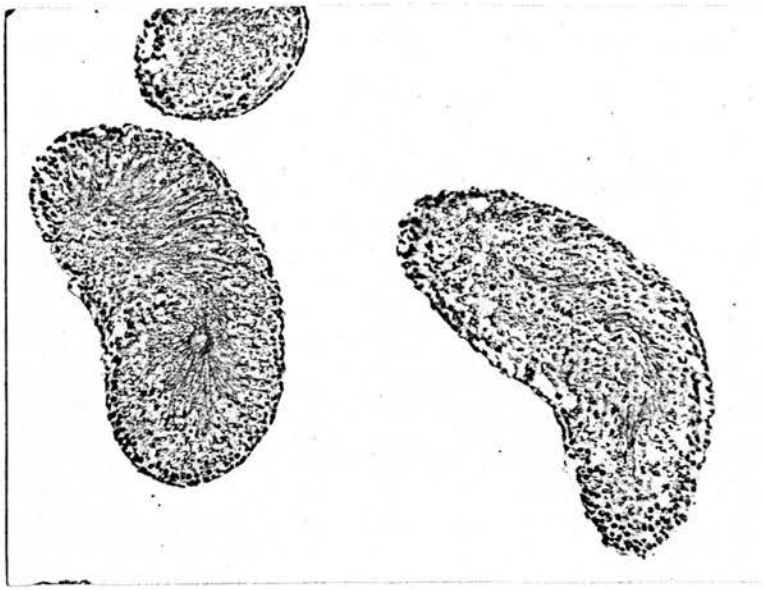


Fig 1a

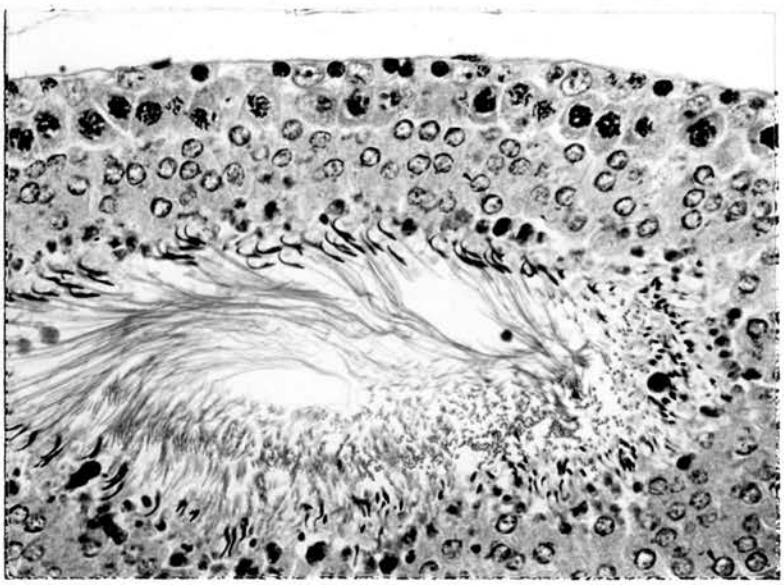


Fig 1b

FIG. 7.2 Microscopic appearance of dissected fractions from testes of adult rats (stained with hematoxylin and eosin).

1. Photomicrograph of the seminiferous tubules after separation from intertubular tissue. Figures 1.a (x 100) showing tubules free of interstitial tissue. In Figure 1.b (x 400) shows that the lamina propria forming the boundary of the tubules are free of adhering interstitial tissue. The germinal cells within the tubules appear normal.

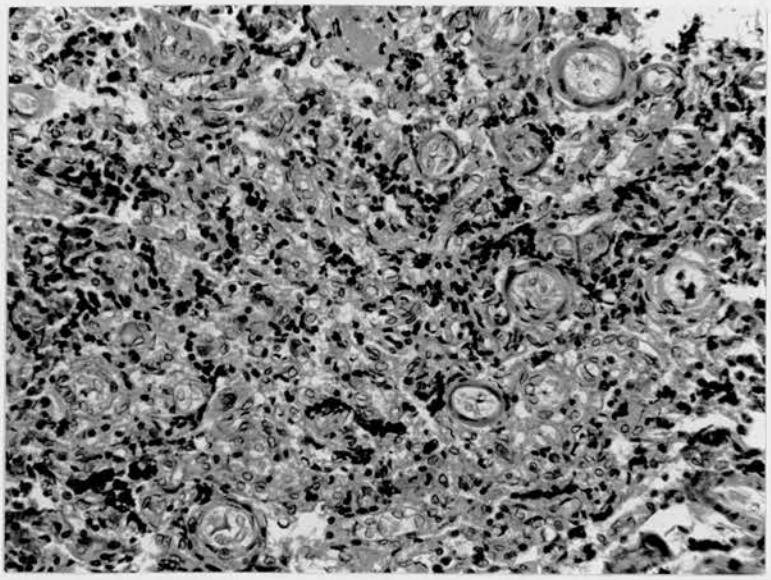


Fig 2a

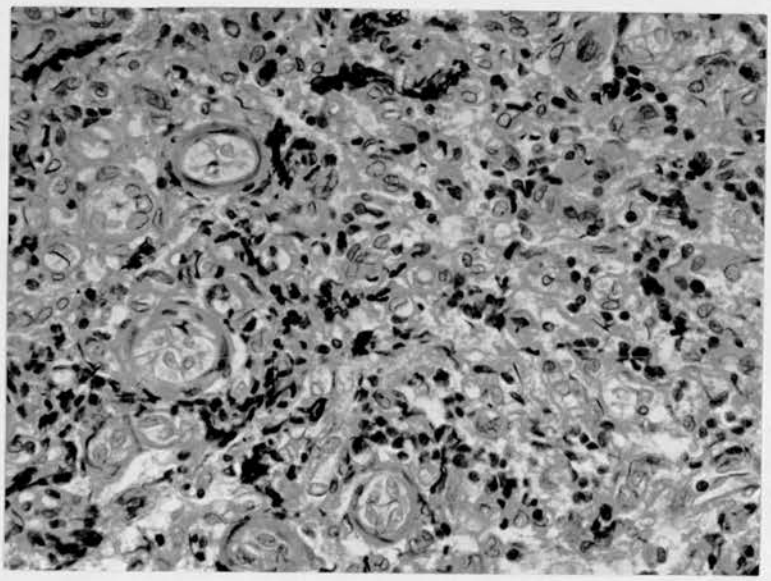


Fig 2b

FIG. 7.2 cont'd

2. Photomicrograph of the interstitial tissue fraction from the same testes containing Leydig cells, fibroblastic cells, blood vessels and occasional contaminating spermatozoa, spermatids and germ cells from broken tubules. No tubules present. Figures 2.a (x 250) and 2.b (x 400).

(interstitial, pooled residual and tubular suspensions) were centrifuged at 10,000 x g for 15 minutes. Each fraction was then washed again by resuspending in medium H and re-centrifuging as above.

7.1.4 Subcellular fractionation of washed testicular tissues

Each washed testicular fraction was separately homogenized in medium H. Using the differential centrifugation procedure developed, each isolated testicular component was fractionated into heavy mitochondria, light mitochondria, microsome and supernatant fractions, as described in Chapter 2.

7.2 HISTOLOGY OF DISSECTED TESTICULAR TISSUE COMPONENTS

Aliquots from the three washed testis fractions obtained after dissection were fixed in Bouin's solution for 18 hours and subsequently dehydrated in graded concentrations of alcohol. The tissues were then embedded in paraffin, sectioned, stained with hematoxylin and eosin or Giemsa's stain and examined under the light microscope.

Figure 7.2.1 shows a typical photomicrograph view of the tubules and indicates that the tubules are free of contamination by interstitial tissue, i.e. the walls (lamina propria) of the tubules show no adhering cells or connective tissue. The cells within the tubules appear normal. Figure 7.2.2 shows a photomicrograph of the intertubular fraction (interstitial tissue) containing Leydig cells and fibroblastic cells. Occasional spermatozoa, spermatids and germ cells from broken seminiferous tubules are present but no tubules.

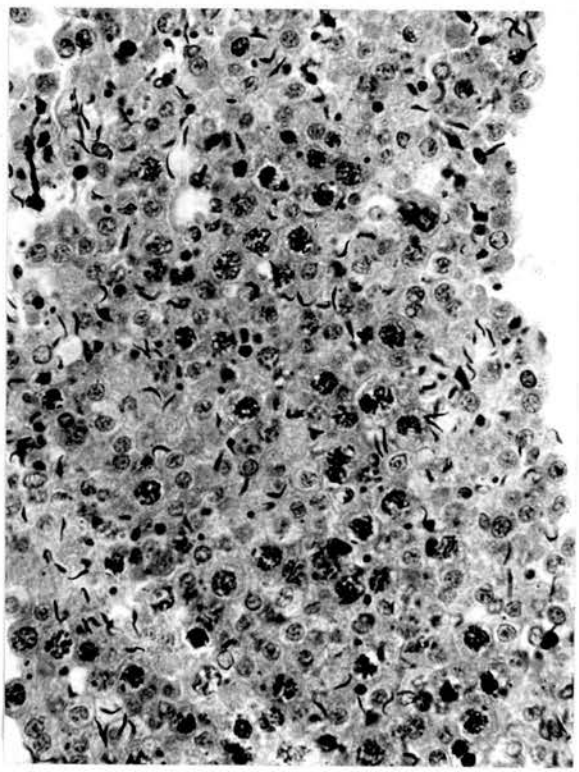


FIG. 7.2 cont'd

3. Photomicrograph of the residual fraction (x 400) comprising mainly of components of the seminiferous tubules such as spermatozoa, spermatids, other germ cells and some contaminating interstitial tissue constituents.

(The histology processing was kindly performed by Mr. J. Masson of the Department of Neuropathology)

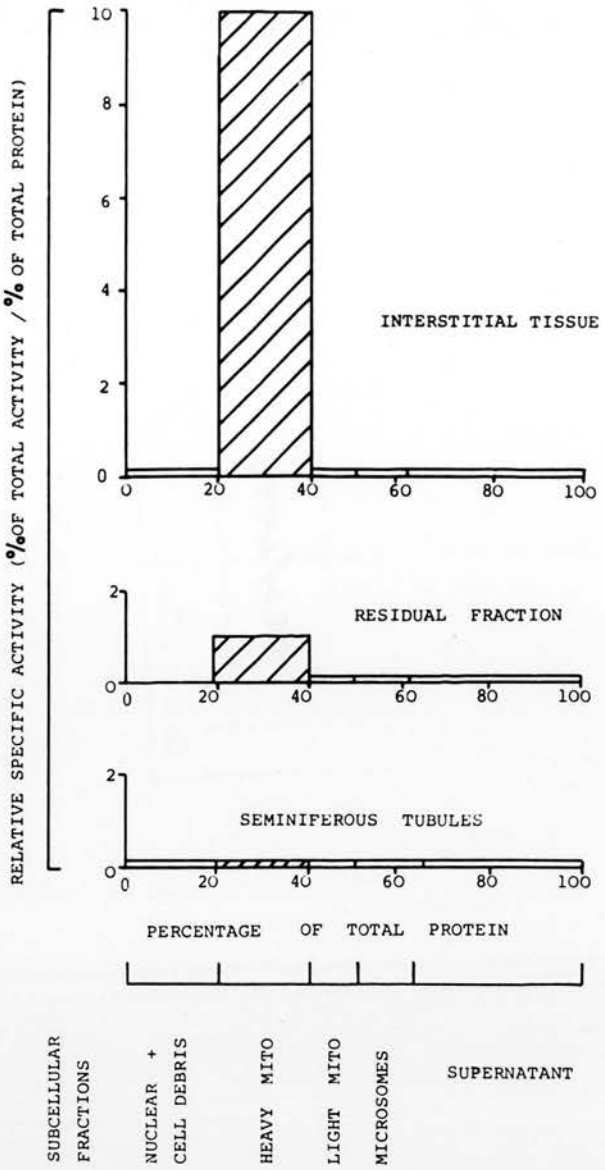


FIG. 7.3 SUBCELLULAR DISTRIBUTION OF CHOLESTEROL SIDE-CHAIN CLEAVAGE ENZYME SYSTEM ISOLATED FROM DISSECTED RAT TESTIS COMPONENTS

Graph shows the relative specific activity of cholesterol side-chain cleavage enzyme in the subellular fractions obtained from the isolated interstitial tissue, residual fraction and seminiferous tubules. Aliquots of each subcellular fraction (0.3-0.5 mg protein) were incubated for 90 mins. at 37°C in medium B with [4-^{14}C]cholesterol (2 x 10^5 cpm), DL isocitrate (5 mM) and cyanoketone (5.5 µM - an inhibitor of 3β hydroxysteroid dehydrogenase). Labelled pregnenolone was the only product formed. Activity is expressed as per cent of [4-^{14}C]cholesterol converted. Determinations were carried out in duplicate. Zero time values from unincubated samples were subtracted from the values of incubated samples. Ordinate: shows the mean relative specific activity of subcellular fractions. Abscissa: subcellular fractions are represented by their relative protein content in the order in which they were isolated. Rats were 15 weeks old. With different groups of rats the results were essentially similar.

Figure 7.2.3 shows a photomicrograph of the residual fraction. This fraction was found to comprise mainly of components of seminiferous tubules such as sperms, spermatids, spermatogonia and other germ cells (compare with the intact tubular section Fig. 7.2.1) and tubular fragments as well as some dislodged interstitial tissue constituents (Leydig cells). Most of the components of the residual fraction are quite distinct from those of the interstitial fraction.

7.3 LOCALIZATION OF CHOLESTEROL SIDE-CHAIN CLEAVAGE ENZYME ACTIVITY

The relative cholesterol side-chain cleavage activity was measured as described in Chapter 2.4. The results obtained are presented in Figure 7.3 and Table 7.1. Illustrated in Figure 7.3 is the subcellular distribution pattern of cholesterol side-chain cleavage activity in each of the three fractionated testicular tissue components. The comparative ability of the mitochondria isolated from the three testicular tissue components to convert $[4\text{-}^{14}C]$ cholesterol to labelled pregnenolone is demonstrated in Table 7.1. These data show that the cholesterol side-chain cleavage enzyme activity is localized only in the heavy mitochondrial fraction obtained from interstitial tissue and residual fraction. The heavy mitochondria from the seminiferous tubules failed to convert the exogenous labelled cholesterol to pregnenolone even when NADPH was used as an alternative electron source. This failure suggests that the enzymesinvolved in the conversion are not present in the cells

TABLE 7.1

TESTICULAR DISTRIBUTION OF CHOLESTEROL SIDE-CHAIN CLEAVAGE ENZYME ACTIVITY

EXPT. NO.	SOURCE OF HEAVY MITOCHONDRIA	HEAVY MITOCHONDRIAL PROTEIN		% PREGNENOLONE FORMED	
		Total Protein(mg)	Protein(mg)/ incubation	TIME OF INCUBATION (minutes) 45	90
1	INTERSTITIAL TISSUE	2.0	0.3	1.4 ± 0.2	3.1 ± 0.3
	RESIDUAL FRACTION	6.4	0.3	0.2 ± 0.1	0.3 ± 0.1
	SEMINIFEROUS TUBULES	10.1	0.5	ND	ND
			0.5+	-	ND
2	INTERSTITIAL TISSUE	3.1	0.4	2.2 ± 0.2	4.6 ± 0.2
	RESIDUAL FRACTION	3.8	0.5	0.3 ± 0.1	0.8 ± 0.1
	SEMINIFEROUS TUBULES	10.9	0.5	ND	ND
			0.5+	-	ND

ND = Not Detectable
- = not done

Rats in Experiment 1 were 15 weeks old and those in Experiment 2, 12 weeks old. In each experiment testis from two rats were used. The interstitial tissue, residual fraction and seminiferous tubules were separated from each testis by the wet dissection technique as described in the text. Washed heavy mitochondrial fractio was prepared from the isolated testicular components as described in Chapter 2.2. Aliquots of washed heavy mitochondrial fraction were incubated in medium B for 45 and 90 mins. at 37°C with [4-^{14}C]cholesterol, DL isocitrate and cyanoketone. The percent of [4-^{14}C]pregnenolone formed from cholesterol was measured as described in Chapter 2. Zerotime values from unincubated samples were measured and subtracted from the values of incubated samples. Values presented represent the mean ± SEM of four determinations.

+In this case, additional incubations were carried out in which isocitrate was replaced with an NADPH-generating system. All other conditions were the same.

of the tubules. The other subcellular fractions, namely light mitochondria, microsome and supernatant, showed no cholesterol side-chain cleavage activity.

7.4 TISSUE DISTRIBUTION OF TESTICULAR CYTOCHROME P450

Using the procedure developed for detecting and measuring cytochrome P450 (described in Chapter 5.3), the subcellular fractions prepared from the three dissected testicular constituents were examined in order to establish the nature and type of cells from which cytochrome P450 was derived. For these studies light mitochondrial and microsomal fractions were pooled ("microsomal fraction"). The content of mitochondrial and microsomal cytochrome P450's were measured from the amine-difference spectra induced by aminoglutethimide and metyrapone respectively, using the wavelength pair (406-448)nm and the reported extinction co-efficient of $\Delta E = 28\ mM^{-1}\ cm^{-1}$ (Jefcoate et al., 1973). The inverted type I spectral change produced by methyl-androstenediol and the type I binding spectrum induced by 20α,22R-dihydroxy-cholesterol were measured using the wavelength pair (390-420)nm. The results are presented in Table 7.2 and 7.3.

The data in Table 7.2 show that only the heavy mitochondrial fraction from the interstitial cells of testis possess cytochrome P450 of the species which specifically binds aminoglutethimide producing a type II difference spectrum (λ min. 390 nm, λmax. 425 nm). No spectral changes were detectable in the mitochondrial fractions of seminiferous

TABLE 7.2

CELLULAR DISTRIBUTION OF RAT TESTIS MITOCHONDRIAL CYTOCHROME P450

EXPERIMENT No.	MITOCHONDRIAL SOURCE	SPECTRAL CHANGES Δ ABSORBANCE/mg protein x 10^4			
		(406-448) nm		(390-420) nm	
		AMINOGLUTETHIMIDE (Type II)	METYRAPONE (Type II)	METHYLANDROSTENEDIOL (Inverted Type I)	20α,22R-DIHYDROXY CHOLESTEROL (Type I).
1	INTERSTITIAL TISSUE	4.7	0.2	9.4	13.9
	RESIDUAL FRACTION	0.7	0.4	0.9	1.0
	SEMINIFEROUS TUBULES	ND	ND	ND	ND
2	INTERSTITIAL TISSUE	4.1	0.9	7.9	14.6
	RESIDUAL FRACTION	1.0	0.9	0.5	1.2
	SEMINIFEROUS TUBULES	ND	ND	ND	ND

ND = Not Detectable (i.e. Δ A = less than 0.4 x 10^4)

Spectral changes were measured as described in Chapter 5. 1-2 mg mitochondrial protein were suspended in medium B in a final volume of 2.5 ml. In the case of seminiferous tubules, protein concentrations of up to 5 mg/ml were used. After establishing the base-line enough amine or steroid was added to produce maximal spectral changes. After recording the aminoglutethimide spectral change metyrapone was then added to the same sample and a spectral change, if any, recorded. Amine-induced difference spectra were determined at pH 7.4 an and those produced by steroid at pH 6.4 in order to obtain maximal optical change.
Results are means of duplicate determinations.

TABLE 7.3

CELLULAR LOCALIZATION OF TESTICULAR MICROSOMAL CYTOCHROME P450

Microsomal Source	△A(406-448)/mg protein x 10^4			
	Aminoglutethimide Expt. No.		Metyrapone Expt. No.	
	1	2	1	2
Interstitial Tissue	ND	ND	15.6	17.8
Residual Fraction	ND	ND	3.1	6.2
Seminiferous Tubules	ND	ND	ND	ND

ND = Not Detectable

The amine induced spectral changes produced with the microsomes were made on an Aminco-Chance spectrophotometer in the dual wavelength mode and at 1% transmittance using the wavelength pair (406-448)nm. 0.2-0.5 ml aliquots of microsomal fraction (2-5 mg protein) was suspended in medium B (pH 7.4) in a final volume of 2.5 ml. After establishing a base-line, 25 µg of aminoglutethimide or metyrapone in aqueous solution (5 µl) was added to give a maximal spectral change. All determinations were carried out in duplicates. In this study light mitochondrial and microsomal fractions were pooled. These subcellular fractions were isolated from each separated testicular tissue compartment as described in Chapter 2.2.

tubules, with either aminoglutethimide and/or metyrapone. The spectral changes shown in Table 7.3 were obtained only with the microsomal fraction from interstitial tissue. Unlike the mitochondrial cytochrome P450 the type II difference spectrum produced in this case was induced only by metyrapone. Aminoglutethimide did not react with the microsomal cytochrome P450. These results suggest that the cytochrome P450 rich organelles appear to be derived exclusively from the interstitial tissue of the testis. The observations also clearly demonstrate the relative differential selectivity and specificity of the amines (aminoglutethimide and metyrapone) and provide confirmation for the earlier observations (see Chapter 5) with subcellular fractions from whole testis of rat that there are two distinct species of cytochrome P450 - one mitochondrial and the other microsomal.

Further supporting evidence for the interstitium being the sole source of the cholesterol side-chain cleavage enzyme in testis was provided by the additional spectral results shown in Table 7.2. Methylandrostenediol and 20α,22R-dihydroxycholesterol each reacted only with the heavy mitochondrial cytochrome P450 from the interstitium. No spectral changes were induced with the corresponding subcellular fraction from the seminiferous tubules. As discussed in Chapter 5 these spectral changes have been attributed to the cytochrome P450 associated with cholesterol side-chain cleavage activity (Whysner et al., 1969; Jefcoate et al., 1973; Burstein et al., 1972; Harding et al., 1970; Simpson et al., 1971).

The small spectral changes observed with the heavy mitochondrial and microsomal fractions obtained from the residual fraction could, in view of the above histological observations, be ascribed to Leydig cell contamination.

Conclusion

The metabolic data based on cholesterol side-chain cleavage activity and the spectralevidence obtained with the amines aminoglutethimide and metyrapone and further supported by methylandrostenediol and 20α,22R-dihydroxycholesterol clearly indicate that the interstitial cells possess the cholesterol side-chain cleavage enzyme complex and hence are the major source of testicular steroids. The failure to detect cytochrome P450 in both the mitochondrial and microsomal fractions of the seminiferous tubules, coupled with its inability to convert cholesterol to pregnenolone, strongly suggests that the key steroid hydroxylating enzyme systems, namely cholesterol side-chain cleavage enzyme and 17α-hydroxylase, may not be present in the cells of the seminiferous tubules.

The evidence obtained in this chapter also establish and confirm that the observations made in previous chapters with subcellular fractions from whole testes relate solely to the steroidogenic capacity of the Leydig cells.

SUMMARY

1. Seminiferous tubules and interstitial tissue were dissected out from rat testis by a wet dissection method. In addition to these two components a third "Residual Fraction" consisting mainly of tubular components and some Leydig cells was obtained. The tubules were "cleaned" by layering on a ficoll gradient. Each isolated tissue component was separately homogenized and subcellular fractions prepared by differential centrifugation.

2. Cholesterol side-chain cleavage activity was localized only in the heavy mitochondrial fraction from interstitial tissue. Tubular mitochondria failed to convert [4-^{14}C]cholesterol to labelled steroids.

3. The presence of two distinct species of cytochrome P450 in rat testis was confirmed by selective amine binding spectral evidence. The aminoglutethimide specific mitochondrial cytochrome P450 and metyrapone detected microsomal cytochrome P450 were both located exclusively in the interstitial tissue. No cytochrome P450 was detectable in the seminiferous tubular fractions.

These results, supported by spectral changes produced by methylandrostenediol and 20α,22R-dihydroxy-cholesterol, establish that the interstitium is the only testicular tissue capable of de novo steroid synthesis from cholesterol. Seminiferous tubules appear to lack the enzyme systems involved in the side-chain cleavage of cholesterol and 17α-hydroxylation of progesterone — two key rate limiting steps in the steroid biosynthetic pathway and the possible sites of control by trophic hormones.

CHAPTER 8

A POSSIBLE ALTERNATIVE PATHWAY FOR CHOLESTEROL METABOLISM IN RAT TESTIS MITOCHONDRIA

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CHAPTER 8

A POSSIBLE ALTERNATIVE PATHWAY FOR CHOLESTEROL METABOLISM IN RAT TESTIS MITOCHONDRIA

8.1 INTRODUCTION

Stimulated by the suggestions of Dorfman and colleagues (Burstein & Dorfman, 1962; Gual et al., 1962; Dorfman 1960), Jungman (1968a,b, 1969) reported the existence of a new pathway for the biosynthesis of androgens from cholesterol. Unlike the traditional route, by which cholesterol is transformed into pregnenolone by cleavage of a six carbon side-chain fragment, this alternative route involved the cleavage of all eight carbon atoms of the side-chain of cholesterol, splitting off 6-methyl-heptan-2-one with the direct formation of a C_{19} steroid product - dehydro-epiandrostenedione (DHA ; see Fig. 1.3, Chapter 1). Evidence for the existence of this pathway was adduced from the accumulation of radioactivity in the eight carbon fragment 6-methyl-heptan-2-one, which was supposedly formed by incubating /26-^{14}C/ cholesterol with homogenates of testes and other steroid producing glands from several species. However, Hochberg et al. (1971) and Burstein et al. (1971) were unable to prove unequivocally that 2-methyl-heptan-6-one was formed from cholesterol and thus failed to support the existence of the alternative pathway in which cholesterol is converted to androgens by way of a side-chain cleavage between C-17 and C-20.

Another pathway of steroid hormone biosynthesis which bypasses pregnenolone (or pregnenolone sulfate) has been reported to proceed as follows:
cholesterol→20α-hydroxycholesterol→17,20α-dihydroxy-cholesterol →17α-hydroxypregnenolone. Evidence for this was based on the

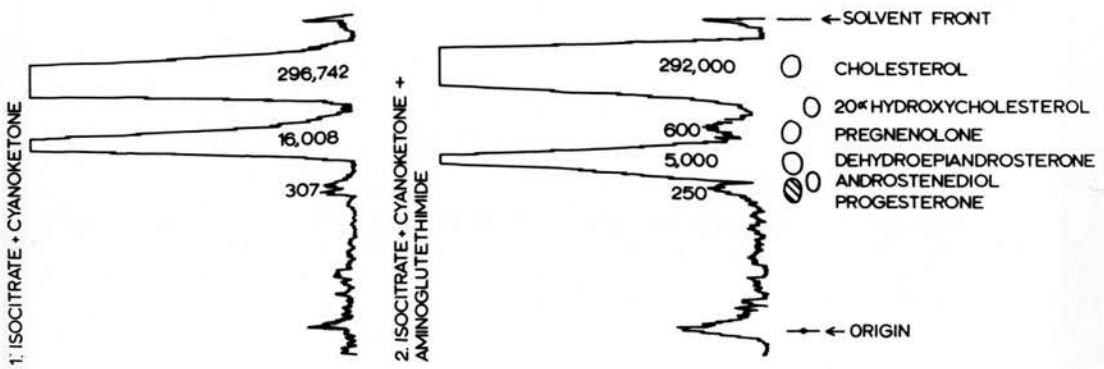


FIG. 8.1 Typical radiochromatographic scan traces obtained after TLC of extracts from incubations of testicular heavy mitochondria (0.4 mg ficoll fraction 5) with [4-^{14}C] cholesterol (approximately 3 x 10^5 cpm) in the presence of:

1. Isocitrate (5 mM) plus cyanoketone (5.5 μM).
2. Isocitrate (5 mM) plus cyanoketone (5.5 μM) plus aminoglutethimide (400 μM).

The thin layer plates were developed four times in solvent 1V as described in Chapters 2 and 3.

metabolism of 20α-hydroxycholesterol and 17,20α-dihydroxycholesterol to steroids by human fetal adrenal tissue (Shimizu et al., 1965). However, the proposed pathway of formation of C_{19} steroids directly from 17,20α-dihydroxycholesterol has yet to be demonstrated.

8.2 PRELIMINARY EVIDENCE FOR AN ALTERNATIVE PATHWAY IN RAT TESTIS

In the course of studies presented earlier (section 5.3) it was observed that when testicular mitochondria were incubated with [4-^{14}C] cholesterol and isocitrate in the presence of 5.5 μM cyanoketone to inhibit 3β-hydroxysteroid dehydrogenase and 400 μM aminoglutethimide to inhibit the conversion of cholesterol to pregnenolone, there was no accumulation of pregnenolone, but another radioactive labelled product (compound X) having an identical mobility to authentic DHA on TLC was formed as shown in Figure 8.1.2. (Also see Fig. 5.14.) Figure 8.1.1 shows a typical radiochromatographic scan trace obtained after TLC of the extract from testicular heavy mitochondria (HMF_5) incubated in the presence of isocitrate and cyanoketone (5.5 μM). It can be seen that only pregnenolone was formed. No DHA or any other steroids were obtained. From these observations aminoglutethimide (400-800 μM) appeared to fully inhibit the orthodox cholesterol side-chain cleavage reaction but not the conversion of [4-^{14}C] cholesterol to radioactive product 'X' thus suggesting the existence of another pathway for cholesterol metabolism. Preliminary studies were therefore conducted to verify the enzymic production of compound X, and

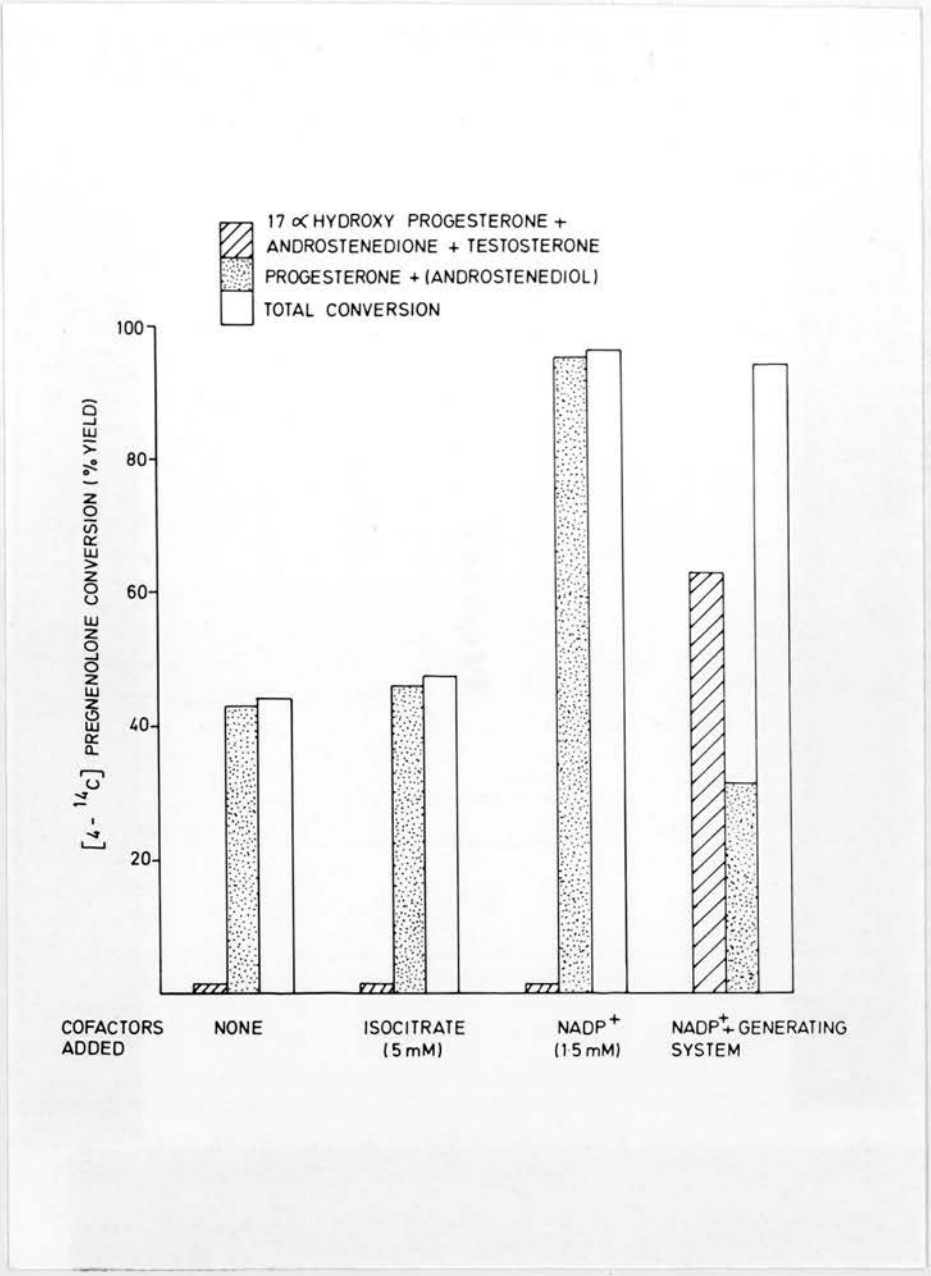


FIG. 8.2 The effect of cofactors on the rate of pregnenolone metabolism and nature of products formed by rat testis mitochondria.

Incubations were carried out for 60 minutes of 37°C in medium B with [4-^{14}C] pregnenolone and 0.4 mg protein of heavy mitochondria ficoll fraction 5. The following cofactors were added where indicated: DL isocitrate (5 mM); $NADP^+$ (1.5 mM) or NADPH-generating system (see Table 2.2 Chapter 2).

the existence of an alternative pathway(s) for steroid biosynthesis from cholesterol. On the basis of its mobility on TLC in different solvent systems (III, IV, V, VI), compound X was tentatively regarded as DHA.

8.2.1 Effect of different cofactors on the rate of pregnenolone metabolism and steroid products formed

In the course of previous studies (see chapters 3 & 4) circumstantial evidence was obtained for the presence in rat testes mitochondria of significant amounts of enzymes involved in the metabolism of pregnenolone to androgens. With a view to determining whether pregnenolone could be converted to DHA by the rat testes mitochondrial preparations, the 5-ene-3β-hydroxysteroid dehydrogenase activity was measured using /4-^{14}C/ pregnenolone as substrate as described in Chapter 2.6. Activity is expressed as the percentage conversion of pregnenolone to steroid products. The steroids formed being presented in 3 groups based on TLC separation:

1. progesterone and androstenediol
2. DHA
3. testosterone, androstenedione and 17α-hydroxyprogesterone

The results in Figure 8.2 and Table 8.1 illustrate the effect of different cofactors (DL isocitrate 5 mM; NADP+ 1.5 mM; extra-mitochondrial NADPH generating system) on the rate and products of pregnenolone metabolism in intact rat testicular heavy mitochondrial fraction. In the absence of added cofactor the rate of pregnenolone conversion was linear up to 2 hours. The presence of isocitrate had no effect on the rate of conversion. In both cases 44-47% of /4-^{14}C/

pregnenolone was transformed to progesterone and about 1% to labelled testosterone in 1 hour. These results confirm the presence of an active 3β-hydroxysteroid dehydrogenase in rat testicular heavy mitochondria. In the presence of $NADP^+$ the rate of 3β-hydroxysteroid dehydrogenase activity was greatly enhanced. In 1 hour 97% of the added pregnenolone was consumed. Of this 95% was transformed to progesterone and a further 2% metabolized to testosterone. $NADP^+$ thus appears to be the cofactor in the conversion of pregnenolone to progesterone. The 3β-hydroxysteroid dehydrogenase from bovine adrenal microsomes (Beyer & Samuels, 1956), rat ovarian mitochondria and microsomes (Sulimovici & Boyd, 1969) were reported to use NAD^+ as the preferred cofactor.

When an extra mitochondrial NADPH-generating system was used as cofactor, a similar stimulation to that of $NADP^+$ was observed, but in this case the major product was testosterone (63%). The amount of progesterone accumulated was 31%. This observation indicates that NADPH is a cofactor in the metabolism of progesterone to testosterone in rat testis.

In these studies no DHA was detected. However, these studies no not prove the absence of the pregnenolone $\longrightarrow$ 17α-hydroxypregnenolone $\longrightarrow$ DHA pathway, since DHA if formed, could be rapidly converted to androgens. In an attempt to verify whether pregnenolone could be metabolized via DHA, an inhibitor of 3β-hydroxysteroid dehydrogenases — cyanoketone was employed. Cyanoketone would therefore inhibit progesterone formation and hence the progesterone to testosterone pathway.

TABLE 8.1

EFFECT OF CYANOKETONE AND AMINOGLUTETHIMIDE ON PREGNENOLONE METABOLISM

EXPERIMENT No.	ADDITIONS	% PREGNENOLONE CONVERSION TO		TOTAL % CONVERSION
		TESTOSTERONE ANDROSTENEDIONE 17αHYDROXYPROGESTERONE	PROGESTERONE ANDROSTENEDIOL	
1 a	None	1.0	43.0	44.0
b	cyanoketone (CTA)	<0.1	<0.1	<0.1
c	aminoglutethimide (AMG)	0.9	42.8	43.7
d	CTA + AMG	<0.1	<0.1	<0.1
2 a	ISOCITRATE	1.3	46.0	47.3
b	" + CTA	<0.1	<0.1	<0.1
c	" + AMG	1.3	42.5	43.8
d	" + CTA + AMG	<0.1	<0.1	<0.1
3 a	$NADP^+$	1.0	95.0	96.0
b	" + isocitrate	3.1	92.6	95.7
c	" + " + CTA	0.1	1.8	1.9
d	" + " + AMG	3.5	92.5	96.0
e	" + " + CTA + AMG	<0.1	2.0	2.0
4 a	$NADPH^+$-Gen. Sys.	63.0	31.0	94.0
b	" " " + CTA	1.0	42.5	43.5
c	" " " + AMG	56.6	35.0	91.6
d	" " " + CTA + AMG	1.5	42.2	43.7

Incubations were carried out for 60 minutes at 37°C in medium B with [4-^{14}C] pregnenolone, and ficoll fractionated mitochondria (HMF_5) (0.4 mg protein). The following cofactors and inhibitors were added as indicated: DL isocitrate 5 mM ; $NADP^+$ 1.5 mM ; extramitochondrial NADPH generating system (NADPH-G.Sys.; see Table 2.2), cyanoketone (CTA) 5.5 µM and aminoglutethimide (AMG) 400 µM . Results are typical of those obtained repeatedly.

The only products expected from pregnenolone in the presence of cyanoketone would be 17α-hydroxypregnenolone, DHA and androstenediol (see Figure 8.4).

8.2.2 Effect of inhibitors on pregnenolone metabolism

Incubations were carried out for 60 minutes, as described above except that the following inhibitor(s) were included in the incubation: cyanoketone (5.5 μM) and/or aminoglutethimide (400 μM). The results in Table 8.1 show that in the absence (experiment 1) or presence (experiment 2 and 3) of cofactors (isocitrate or $NADP^+$), cyanoketone completely inhibited 3β-hydroxysteroid dehydrogenase activity and no radioactive pregnenolone was consumed. No labelled products were detected. Aminoglutethimide did not have any significant inhibitory effect on the metabolism of pregnenolone indicating that the site of inhibition of aminoglutethimide is at a point prior to the synthesis of pregnenolone. When cyanoketone and aminoglutethimide were both added to the incubations, the effect was the same as that observed in the presence of cyanoketone alone. No labelled peak corresponding to DHA or any other steroid was produced. These results establish that pregnenolone conversion to DHA via 17α-hydroxypregnenolone does not occur in testicular mitochondria from mature animals, at least, under the conditions of these studies.

Except for the labelled substrate used the incubation conditions employed in the studies presented above (Table 8.1, experiment 2.d), were identical to those under which compound X ('DHA') was formed. These results in conjunction with observations in sections 8.1 and 8.3 strongly imply that

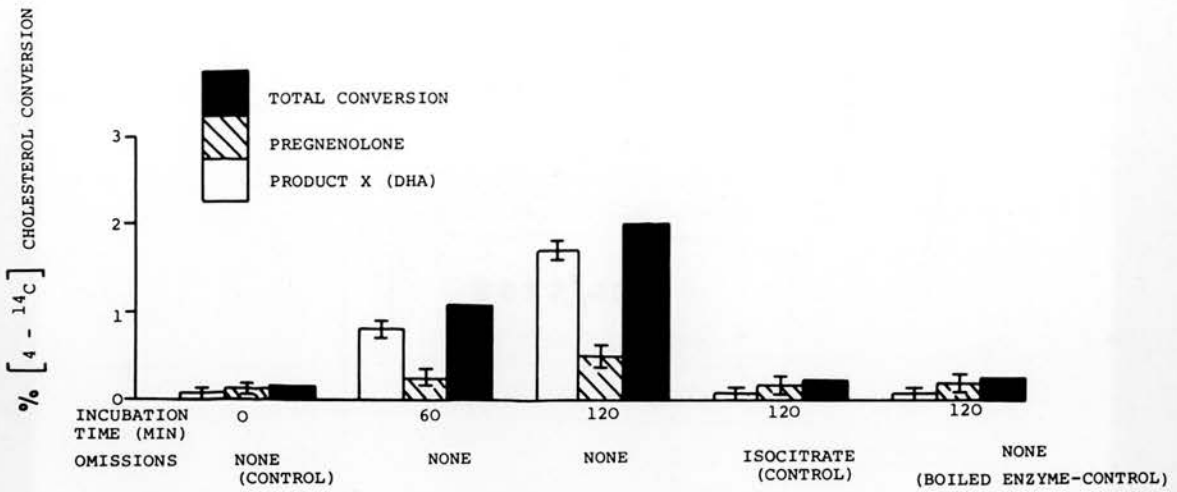


FIG. 8.3 EFFECT OF TIME ON THE RATE OF FORMATION OF COMPOUND X

Ficoll fractionated heavy mitochondria (HMF_5 0.4 mg) were incubated at 37°C for 0, 60 and 120 minutes in medium B with [4-^{14}C] cholesterol, isocitrate (5 mM), cyanoketone (5.5 μM) and aminoglutethimide (400 μM). In addition to the zero minute control incubations, two other controls were included, each incubated for 120 minutes. One consisted of the complete system but with a boiled mitochondrial fraction, while in the other, isocitrate was omitted from the complete system. Each data point in the figure represents the mean and range of duplicate determinations from two different groups of rats of the same age (8-10 animals/group). Results shown are typical of those observed repeatedly.

compound X ('DHA') could only be derived from cholesterol and not from pregnenolone.

8.3 EFFECT OF TIME ON THE RATE OF FORMATION OF COMPOUND X ('DHA')

Ficoll fractionated mitochondria (0.4 mg) were incubated at 37°C for 0, 60 and 120 minutes in medium B with [4-^{14}C] cholesterol, isocitrate (5 mM), cyanoketone (5.5 μM) and in the presence of aminoglutethimide (400 μM). The results are shown in Figure 8.3. Compound X was the main product formed and its rate of formation was linear with time. Compared with controls the small increase in pregnenolone formation at 120 minutes incubations may be due to partial reversal of the inhibition (due to metabolism of the inhibitor - Kowal, 1970). The amount of compound X formed in 60 minutes (0.81%) was approximately one fifth of that of pregnenolone (4.3%) obtained under similar conditions but in the absence of aminoglutethimide. These observations indicate that the relative rates of the two pathways viz pregnenolone: DHA is of the order of 5:1.

8.4 SUBCELLULAR DISTRIBUTION OF THE ENZYME SYSTEM INVOLVED IN THE BIOSYNTHESIS OF COMPOUND X

The subcellular distribution of the enzyme involved in the synthesis of compound X is shown in Table 8.2. The subcellular fractions were obtained by differential centrifugation and ficoll gradient fractionation as described previously (section 2.2). Experiment 1 shows that the enzyme system

TABLE 8.2

SUBCELLULAR DISTRIBUTION OF THE ENZYME INVOLVED IN THE BIOSYNTHESIS OF COMPOUND X

EXPT. NO.	SUBCELLULAR FRACTION mg/tube	% [4-^{14}C] CHOLESTEROL CONVERTED IN PRESENCE OF		RATIO b/a
		CTA	AMG + CTA	
		TO PRODUCT		
		PREG (a)	X (b)	
1	Crude HM (0.5 mg)	2.4	0.58	.24
	Light mito (0.5 mg)	0	0	-
	Microsomes (0.7 mg)	0	0	-
	Supernatant (0.5 mg)	0	0	-
	Ficoll gradient mitochondria HMF_5 (0.47 mg)	4.4	1.1	.25
2	Second ficoll gradient fractionation of HMF_5			
	Sub Fraction 1 (Upper layer 0.24 mg)	1.9	0.4	.20
	Sub Fraction 2 (Middle layer 0.29 mg)	6.9	1.6	.23
	Sub Fraction 3 (pellet 0.24 mg)	5.6	1.0	.17

The different subcellular fractions were incubated for 90 minutes at 37°C in medium B with [4-^{14}C] cholesterol, 5 mM isocitrate, 5.5 µM cyanoketone and/or the presence of 400 µM aminoglutethimide.
In Expt. 1 the testis subcellular fractions were obtained by differential centrifugation as described in Chapter 2. Heavy mitochondrial fraction 5 was obtained after discontinuous ficoll gradient (3-6-10% ficoll) fractionation of the crude heavy mitochondria.
In Expt. 2 subfractions 1, 2 and 3 represent the different layers obtained after subjecting HMF_5 to a second (10-15-20%) ficoll gradient centrifugation as described in Chapter 3.
These results are typical of those obtained repeatedly.

concerned with the production of compound X is localized exclusively in the heavy mitochondrial fraction and is closely associated with the cholesterol side-chain cleavage activity. The other fractions viz light mitochondria, microsomal and supernatant fractions were completely devoid of activity.

In an attempt to separate the cholesterol side-chain cleavage activity from the enzyme system producing compound X, the mitochondria (fraction 5) obtained from the 3-10% ficoll gradient was further subjected to a second fractionation on a discontinuous 10-20% ficoll gradient as described in Chapter 2. The three subfractions obtained were washed and assayed as above for the two activities. The results are shown in Table 8.2 experiment 2. The ratios indicate that the two activities are not readily dissociable by the ficoll gradient procedure.

8.5 ENZYMATIC NATURE OF COMPOUND X FORMATION

The results in Figure 8.3 also show that the reaction involved in the production of compound X is enzymatic since no product was synthesized in the complete incubation system terminated at zero time or after incubation for 120 minutes with the boiled heavy mitochondrial fraction. Furthermore, in the absence of isocitrate, compound X was not produced indicating a requirement for reducing equivalents. The subcellular distribution and ficoll gradient fractionation studies (section 8.4) lend further support for the enzymatic formation of compound X. This new product was detected only

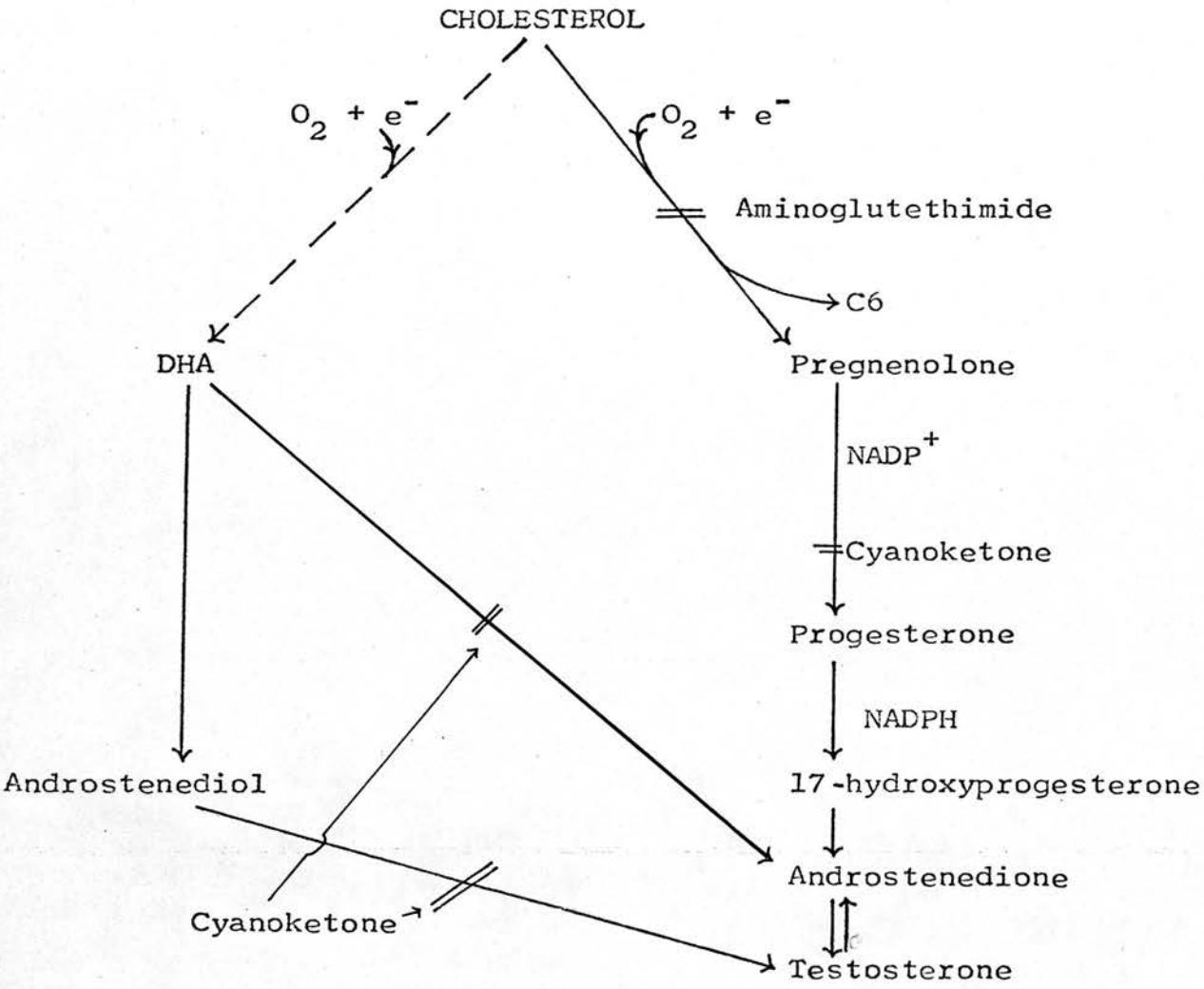


FIG. 8.4 Sites of inhibition of cyanoketone and aminoglutethimide in the biosynthesis of testosterone from cholesterol.

in the presence of inhibitors cyanoketone and aminoglutethimide. Since cyanoketone inhibited pregnenolone conversion to androgen via progesterone and 17α-hydroxyprogesterone pathway and aminoglutethimide prevented the formation of pregnenolone from cholesterol, 'DHA' (X) could only have been derived directly from cholesterol (see Figure 8.4).

The data presented in this chapter establish that a compound which behaves like DHA on TLC appears to be enzymatically synthesized from cholesterol in the presence of reducing equivalents by an enzyme localized in the heavy mitochondrial fraction from rat testis.

The studies presented in this chapter are far from complete and hence do not allow any firm conclusions to be drawn. Further investigations need to be carried out to establish the identity and nature of X. Since the mechanism of removal of the side-chain of cholesterol is not clear and since the present studies have not proved the identity of compound X as DHA, it is possible that compound X may be an intermediate in the formation of pregnenolone or DHA from cholesterol such as 20α 22R-dihydroxy-cholesterol or a hydroperoxide intermediate. If X was DHA, its formation would have to occur by a different mechanism - probably involving hydroperoxide formation, e.g. cholesterol 20α-hydroperoxide, since the postulated enzymic removal of the entire C_8 side-chain of cholesterol could not be confirmed (Hochberg et al., 1971; Burstein et al., 1971). Thermal decomposition of cholesterol 20α-hydroperoxide has been reported (Van Lier & Smith, 1970c) to result in the formation of androst-5-ene-

3β,17β-diol and other similar products. Furthermore, the isolation of DHA from air aged cholesterol (Van Lier et al., 1972) suggests a prior formation of a cholesterol 17α-hydroperoxide from which DHA could be derived by β scission of the C_{17}-C_{20} bond. It is possible therefore that similar direct enzymatic pathways could exist and is elaborated (or induced) under certain conditions.

The investigations on the nature and identity of X could present a considerable problem.

An approach to the problem of the identity of X would be to accumulate X and (1) make derivatives by subjecting X to chemical treatments such as acetylation, oxidation, reduction etc. and (a) determine molecular weight (b) identify X and its derivatives by TLC in different solvent systems, radio-GLC and mass spectrometry. (2) Enzymatic oxidation using α-or β-hydroxysteroid dehydrogenase from Pseudomonas testosteroni. If X were DHA only the free steroid would be oxidized whereas the acetate would not react.(Stempfel & Sidbury, 1964).

As regards the source of X: by using cholesterol [26-^{14}C] instead of [4-^{14}C] or double labelled cholesterol, it should be possible to demonstrate whether X is still a derivative of cholesterol with the side-chain lost - i.e. would X accumulate when [^{14}C] cholesterol labelled at C_{26} is used as substrate.

Since aminoglutethimide and metyrapone both bind mitochondrial cytochrome P450 it would be of interest to determine whether the accumulation of 'X' could be induced by metyrapone as well.

SUMMARY

1. Data presented in this chapter established that in the presence of aminoglutethimide and cyanoketone, no pregnenolone was formed from cholesterol indicating that aminoglutethimide fully inhibits the orthodox side-chain cleavage reaction. However, the formation of a new radicactive product - compound X which behaves like DHA on TLC - suggested the existence of another pathway for cholesterol metabolism not involving pregnenolone as an intermediate. The relative rates of the two pathways (Pregnenolone/DHA) is 5:1. Since compound X was detected only in the presence of aminoglutethimide the alternative pathway may not occur under normal conditio

2. Formation of compound X was enzymatic; was linear with time and required an electron source.

3. The enzymatic system involved was localized in the heavy mitochondrial fraction from rat testis and was closely associated with the cholesterol side-chain cleavage enzyme complex.

4. Studies on pregnenolone metabolism confirmed that the rat testis heavy mitochondrial fraction contains an active 5-ene-3β-hydroxy steroid dehydrogenase as well as other enzymes involved in the further conversion of progesterone to androgens. These latter enzymes were activated by NADPH while the former was stimulated by $NADP^+$.

5. Pregnenolone metabolism was completely inhibited by cyanoketone (a known potent inhibitor of 3β-hydroxysteroid dehydrogenase) but not affected by aminoglutethimide.

6. Under the <u>in vitro</u> conditions used, the inhibitor studies indicated that in these testes mitochondria the transformation o pregnenolone to DHA via 17α-hydroxypregnenolone or pregnenolone to 'X' did not occur.

CHAPTER 9

GENERAL DISCUSSION

C O N T E N T S

GENERAL DISCUSSION

CHAPTER 9

GENERAL DISCUSSION

9.1 INTRODUCTION

The investigation of steroidogenesis in testes proved to be difficult in comparison to similar studies in the adrenals and ovaries. The major handicap was that in the testes the androgen synthesizing cells constituted only a small fraction of the organ. In adult rats the seminiferous tubules make up 90% of the whole testis and the interstitium only about 10% by volume (or 6% by weight). Of this the Leydig cells were estimated to constitute about 3% (Roosen-Runge, 1955, 1956; Christensen & Mason, 1965). Therefore, for a proper evaluation of the results of steroidogenesis in testes it was desirable to work with a homogeneous population of cells. Reported attempts at preferentially isolating or increasing the concentration of Leydig cells employing techniques such as radiation (Oakberg, 1959; Rugh & Grupp, 1960), induction of spontaneous interstitial cell tumours (Inano et al., 1968) and cell culturing (Kodani & Kodani, 1966; Steinberger et al., 1966, 1970) were unsatisfactor as they resulted either in the loss of some specific enzyme activities or in the production of abnormal steroids. Micro-dissection (Christensen & Mason, 1965), although apparently the best available method, was found to be time consuming and laborious, requiring a dissecting time of about 4 h per testis. This procedure was not practical for routine use. In this present study, these difficulties were circumvented by preparing heavy mitochondria from whole testes and then attempting to isolate the mitochondria of steroidogenic cells before any

investigations were carried out on the mechanism of control of steroidogenesis or on how steroid biosynthetic activity was affected. Some of the findings were then confirmed by using the subcellular preparations obtained from dissected out interstitial tissue and seminiferous tubules.

The rat was chosen as the source of testicular tissue because in comparison to other animal species it offered several advantages - some of these being (a) the age of the rats of each experimental group could be strictly controlled. This was important in view of the variation of the steroid biosynthetic capacity of the testes with the age of the animal. (b) Steroidogenesis in rat testes did not appear to be influenced by seasonal changes. (c) Compared to other species such as cats, dogs, rabbits etc., the separation of interstitial tissue from seminiferous tubules by microdissection was most easily accomplished with rat testes (Christensen & Mason, 1965).

9.2 THE ASSAY SYSTEM

In earlier studies on the cholesterol side-chain cleavage in testes and other steroid producing organs, the assay methods used were based on either the estimation of the C_6 side-chain fragment, isocaproic acid (Ichii *et al.*, 1963) or measurement of pregnenolone, the first C_{21} steroid product of the cleavage reaction by means of the Pettenkofer colour reaction (Hall & Koritz, 1964; Koritz, 1962). The first method had the disadvantage that it yielded no information on the nature of the steroids produced in the reaction and thus could not provide any indication of the presence of the alternative pathway of androgen synthesis as proposed by Jungman (1968a,b). The second

method assumed that pregnenolone was the sole product of the reaction accumulating. This however was not always the case. These problems were largely overcome by the use of a radiometric assay method based on the conversion of the substrate [4-^{14}C] cholesterol to labelled steroid products which were separated by thin-layer chromatography as described in Chapters 2 and 3.

In view of the low cholesterol side-chain cleavage activity in testes in vitro and the formation of a number of steroid products compared to other tissues, it was necessary to reduce procedural losses of compounds during routine analysis and yet achieve good separation of the steroid products formed. For this purpose a quantitative extraction procedure (described in Chapter 2) and a reproducible one plate thin layer separation of steroids (described in Chapter 3.5) was developed. Radiochromatogram scanning and liquid scintillation spectrometry were used to detect and quantitate the products. The assay procedure was quantitative, reproducible and sensitive allowing the detection of metabolites which might occur in small amounts. However, since it is not known to what extent the exogenous cholesterol added as substrate mixes with the endogenous cholesterol nor how large the endogenous cholesterol pool is, this assay method did not permit the determination of the absolute rates of cholesterol side-chain cleavage.

9.3 SUBCELLULAR LOCALIZATION OF CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY

It has generally been observed that cholesterol side-chain cleavage activity occurs in the mitochondrial fraction of most steroid synthesizing tissues (Sulimovici & Boyd, 1969a). Howev

Flint and Armstrong (1971a,b) reported that the microsomal fraction obtained from rat lutealized ovaries was also capable of catalyzing the cleavage of the cholesterol side-chain. In the majority of published investigations the particulate fraction which sedimented between 800-10,000 x g on differential centrifugation was employed as the mitochondrial fraction. However, such a subcellular preparation from rat testes was found to be contaminated with sperms, spermatids and other cell fragments. In view of the fact that sperms and spermatids are rich in mitochondria and constitute the bulk of the cells in the mature testis (Roosen-Runge, 1956), their removal was important. This was achieved by the differential centrifugation technique described in Chapter 2. This procedure also achieved an initial fractionation of the mitochondria into "heavy" and "light" fractions. The cholesterol side-chain cleavage activity was found to be localized only in the "heavy" mitochondrial fraction (470-6,000 x g) whereas the "light" mitochondrial (6,000-11,000 x g and microsomal fractions were completely devoid of the enzyme activity.

With the view of further separating out the mitochondria not involved in cholesterol side-chain cleavage from those containing the enzyme, the heavy mitochondrial fraction was subjected to a ficoll gradient centrifugation as proposed in Chapters 2 and 3. This fractionation procedure not only achieved the removal of subcellular organelles equivalent to about 40% of the total protein present in the original heavy mitochondrial fraction but also resulted in a mitochondrial pellet (HMF_5) which contained most of the cholesterol oxygenase activity and which was free of microsomal contamination as indicated by

spectral studies (see Chapter 5). Furthermore, this ficoll gradient purification of the heavy mitochondria made possible the detection of cytochrome P450 involved in cholesterol hydroxylation as demonstrated in Chapter 5.

Evidence that the mitochondria catalyzing cholesterol side-chain cleavage reaction were intact was provided by measurement of respiratory control and ADP/O ratios, inability of NADH and NADPH to stimulate oxygen utilization, demonstration of the presence of normal respiratory chain cytochromes and of a specific mitochondrial type cytochrome P450, and by electron microscopy.

9.4 RAT TESTES MITOCHONDRIAL CYTOCHROME P450

The cholesterol side-chain cleavage enzyme system of most steroid producing organs have been resolved by partial purificati into three separable components, namely a flavoprotein, an iron sulfur protein and cytochrome P450. However, with testes, such attempts were not successful. The failure to demonstrate the presence of cytochrome P450 in the rat testicular mitochondria (Machino et al., 1969; Inano et al., 1970; Mason et al., 1973) cast some doubt on the nature and mechanism of the cholesterol side-chain cleavage reaction. The studies presented in Chapter 5 demonstrated that normal rat testes mitochondria contained a relatively small amount of cytochrome P450 and that it was not possible to detect this cytochrome P450 by the conventional reduced-carbonmonoxy difference spectra. There appeared to be some spectral interference from the relatively high cytochrome oxidase content. This problem was overcome by using the ficoll fractionated mitochondria and employing the

difference spectra changes selectively induced by amines which interact with the cytochrome P450 and not with the respiratory chain cytochromes. Aminoglutethimide was observed to form a typical cytochrome P450-amine complex with all the cytochrome P450 in the testes mitochondria. It did not bind to the microsomal cytochrome P450. Metyrapone, on the other hand, was a general ligandand bound to both the mitochondrial and microsomal cytochrome P450 species. This specificity formed the basis of the method developed for the detection and estimation of the two species of testicular cytochrome P450.

The specific cytochrome P450 content in the ficoll fractionated mitochondria of rat testes calculated from the aminoglutethimide difference spectrum (Δ A 406-448 nm and ΔE 28 cm^{-1} nM^{-1}) was 0.0137 ± 0.003 nmoles/mg protein (mean ± SD of 13 different experiments, each of 8 rats, average age 18 weeks). The corresponding value for microsomal cytochrome P450 using metyrapone was found to be 0.058 ± 0.019 nmoles/mg protein. The microsomes thus contained approximately four times as much cytochrome P450/mg protein as the mitochondria. A similar value of 0.058 ± 0.021 nmoles/mg protein was obtained for the microsomal P450 content by the reduced carbonmonoxide method (i.e. ΔA 450-490 nm and ΔE 95$cm^{-1}nM^{-1}$). The amine methods of measuring cytochrome P450 was thus comparable to the reduced-carbon monoxide method. With mitochondria prepared from isolated interstial tissue, a higher and much truer value (0.026 ± .003 nmoles cytochrome P450 per mg of mitochondrial protein) was obtained (data from 5 experiments). This value

however was approximately only two-fold higher than that found with ficoll fractionated mitochondria from whole testes. These observations imply that the ficoll gradient fractionation does achieve a good degree of purification of the crude mitochondria from whole testes - separating out the bulk of the tubular mitochondria from the interstitial ones.

The content of cytochrome P450 in rat testis mitochondria is considerably lower ($^1/_{10}$-$^1/_{100}$th) than that reported in other steroid producing tissues (Cammer & Estabrook, 1967; McIntosh et al., 1971; Jefcoate et al., 1973). This relatively low cytochrome P450 content of the testis could therefore account for the very low cholesterol side chain cleavage activity observed in vitro.

That virtually all the aminoglutethimide-dectectable cytochrome P450 in testes mitochondria is involved in the mixed function oxidation of cholesterol was indicated by several lines of evidence:-

The aminoglutethimide induced spectral change exhibited pH dependence. As pH was lowered there was an increase in the high spin cytochrome P450. So also the magnitude of the methyl-androstenediol induced inverse Type I difference spectral change, which is attributed to the conversion of high spin cytochrome P450 to the low spin state (Whysner et al., 1969; Jefcoate et al., 1973), increased with a decrease in pH. In contrast the Type I difference spectrum induced by 20,22R-dihydroxycholesterol was observed to be independent of pH. Since the rate of cholesterol side-chain cleavage appears to depend on the amount of high spin cytochrome P450 in the mitochondria (Simpson et al., 1972) and also since the high spin form of cytochrome P450 is thought to be due to the cytochrome complexing with substrate cholesterol,

changes in the amount of cholesterol bound to cytochrome P450 as a high spin complex would change the rate of cholesterol side-chain cleavage. The pH changes induced around the mitochondria would cause an increase or decrease in the amount of high spin cytochrome P450 and hence activate or inhibit pregnenolone synthesis. These pH dependent changes in spin state appear to be a property unique to the cholesterol side-chain cleavage cytochrome P450. It is not displayed by the cytochrome P450s involved in other reactions.

The sum of the cholesterol-bound and cholesterol-free cytochrome P450 at pH 6.0 as determined by the spectral responses to methylandrostenediol and 20,22R-dihydroxycholesterol respectivel agreed closely with the total testes mitochondrial cytochrome P450 which was detected with aminoglutethimide. The magnitude of the methylandrostenediol binding spectrum is thought to depend on the amount of cholesterol bound to cytochrome P450. 20,22R-dihydroxycholesterol has been suggested to occupy a specific site on cytochrome P450 and thus reflect the proportion of mitochondrial cytochrome P450 which is not bound by cholesterol (Jefcoate et al., 1973).

Deoxycorticosterone, the substrate in 11β-or 18-hydroxylations in the adrenal cortex failed to produce a Type I spectral response with testicular mitochondria implying the absence of the cytochrome P450 species associated with such hydroxylation reactions.

During subcellular fractionation procedures the cholesterol side-chain cleavage activity and the aminoglutethimide detectable cytochrome P450 showed a similar distribution pattern. There

were also similarities in the fractionation behaviour of cytochrome P450 of testes mitochondria during the partial solubilization procedure, with those of adrenal cholesterol side-chain cleavage cytochrome P450. Therefore, there appeared to be a very close correlation between the mitochondrial cytochrome P450 based on spectral studies and cholesterol side-chain cleavage activity. Further spectral studies (presented in Chapter 6) demonstrated that the pretreatment of rats with cycloheximide, reduced by half the proportion of cholesterol, which is bound by the mitochondrial cytochrome P450. Correspondingly, the rate of cholesterol side-chain cleavage activity was also reduced in these testes mitochondria from animals previously treated with cycloheximide. These observations in conjunction with the *in vitro* studies which demonstrated that aminoglutethimide fully inhibited the conversion of cholesterol to pregnenolone *in vitro* (see Chapter 4) provided the evidence for the involvement of mitochondrial cytochrome P450 in cholesterol side-chain cleavage reaction.

9.5 USE OF INHIBITORS

In the present study on steroidogenesis in testes, the inhibitors - cyanoketone, cycloheximide, metyrapone and aminoglutethimide proved to be very useful chemical tools. Cyanoketone an inhibitor of the 3β-hydroxysteroid dehydrogenase (Goldman *et al.*, 1967) was used to overcome the difficulties encountered in the measurement of the rate and pathway of cholesterol side-chain cleavage when three or more steroid products were formed. Pregnenolone was the only product of side-chain cleavage under normal conditions (see Chapters 4 and 8). Cycloheximide, a known

inhibitor of protein synthesis, aided in elucidating the possible mechanism of control of steroidogenesis in testes as discussed in Chapter 6. The two amine inhibitors, aminoglutethimide and metyrapone in particular, helped to highlight differences between the individual cytochrome P450s, thereby revealing two distinct cytochrome P450 species in the testes - one mitochondrial and the other of microsomal origin.

The precise action of aminoglutethimide in the inhibition of cholesterol side-chain cleavage activity is poorly understood. It is probable that the inhibitory effect could be the result of the intervention of the compound at several sites:-

(a) Alteration of the mitochondrial structure, particularly the internal membrane of the cristae as observed in the adrenal cortex (Kadioglu & Harrison, 1971; Racela et al., 1969). The 20α-, 11β-and 18-hydroxylases, which are all of mitochondrial origin, are affected by aminoglutethimide.

(b) Competitive inhibitory effect with the substrate as has been shown to occur with 11β-hydroxylase (Kowal, 1969a).

(c) Inhibition of hydroxylation by the action on cytochrome P450; the reduction of the oxidized form of cytochrome P450-substrate complex is considered to be the rate limiting step of the reaction. Aminoglutethimide, which forms a complex with the oxidized form of cytochrome P450, considerably slows the speed of the reduction and blocks the hydroxylation (Whysner & Harding, 1968; McIntosh & Salhanick, 1969).

Metyrapone and aminoglutethimide both induce a similar Type II spectral change but unlike aminoglutethimide metyrapone interacts with oxidized and reduced cytochrome P450 (Cooper

et al., 1968; Sweat et al., 1969; Peterson et al., 1971) and interferes with the interaction of CO and O_2 with the cytochrome.

9.6 CELLULAR LOCALIZATION OF THE CHOLESTEROL SIDE-CHAIN CLEAVAGE ENZYME COMPLEX

Having established that the cholesterol side-chain cleavage enzyme and its associated cytochrome P450 are localized exclusively in the mitochondria of testes, it was of interest to determine the type of cells from which these mitochondria were derived. Since cholesterol is regarded as an obligatory precursor of steroids, the presence or absence of cholesterol side-chain cleavage activity in a particular tissue could be regarded as reflecting the steroid biosynthetic ability of that tissue. Although the Leydig cells have generally been considered as the source of testicular steroids, it was reported that the Sertoli cells in the seminiferous tubules were also capable of synthesizing steroids de novo.(Lacy & Pettit, 1970). Most of the evidence for this was based on the metabolism pregnenolone and progesterone (Bell et al., 1968; Christensen & Mason, 1965). Other studies based on the conversion of labelled cholesterol to androgens were inconclusive because of the very low rates of conversion (<0.6%) (Hall et al., 1969). This apparent steroid biosynthetic activity attributed to the seminiferous tubules could easily have resulted from contamination by a small amount of highly active interstitial cell enzymes. To overcome such a problem in the present studies the testes were dissected out into the two main components - the interstitium and seminiferous tubules, and a third minor residual fraction of mixed cells from tubules and interstitium. The seminiferous tubules were thorough

washed using ficoll. Unlike previous investigations which were performed with whole tissues in this present study, subcellular fractions were prepared from these washed isolated tissues. This approach also had the advantage of avoiding the problems of differential penetration of exogenously added substrates. Studies were carried out with these subcellular fractions from isolated testicular tissue, applying the techniques developed with whole testes.

The data presented in Chapter 7 demonstrates that the mitochondria containing the enzyme system, which catalyzes the first step in steroid biosynthesis from cholesterol, was derived exclusively from the interstitial tissue. The evidence for this was based on measurement of the cholesterol side-chain cleavage activity as well as spectral studies on cytochrome P450. The mitochondria obtained from the seminiferous tubules did not exhibit any such detectable enzyme activity, and did not possess cytochrome P450. The spectral studies also revealed that the microsomal fractions isolated from the interstitial tissue were rich in cytochrome P450, whereas those from seminiferous tubules lacked the cytochrome. In view of the reported involvement of microsomal cytochrome P450 in the hydroxylation of progesterone (Menard & Purvis, 1972) this finding implies that the seminiferous tubules may also lack the steroid 17α-hydroxylase enzyme system. In view of the relatively larger proportion of tubular tissue to interstitial tissue, the number of Sertoli cells in the tubules may, at the very least, be similar to the number of Leydig cells present (Heller et al., 1971). Thus, if Sertoli cells were capable of de novo steroid synthesis from cholesterol

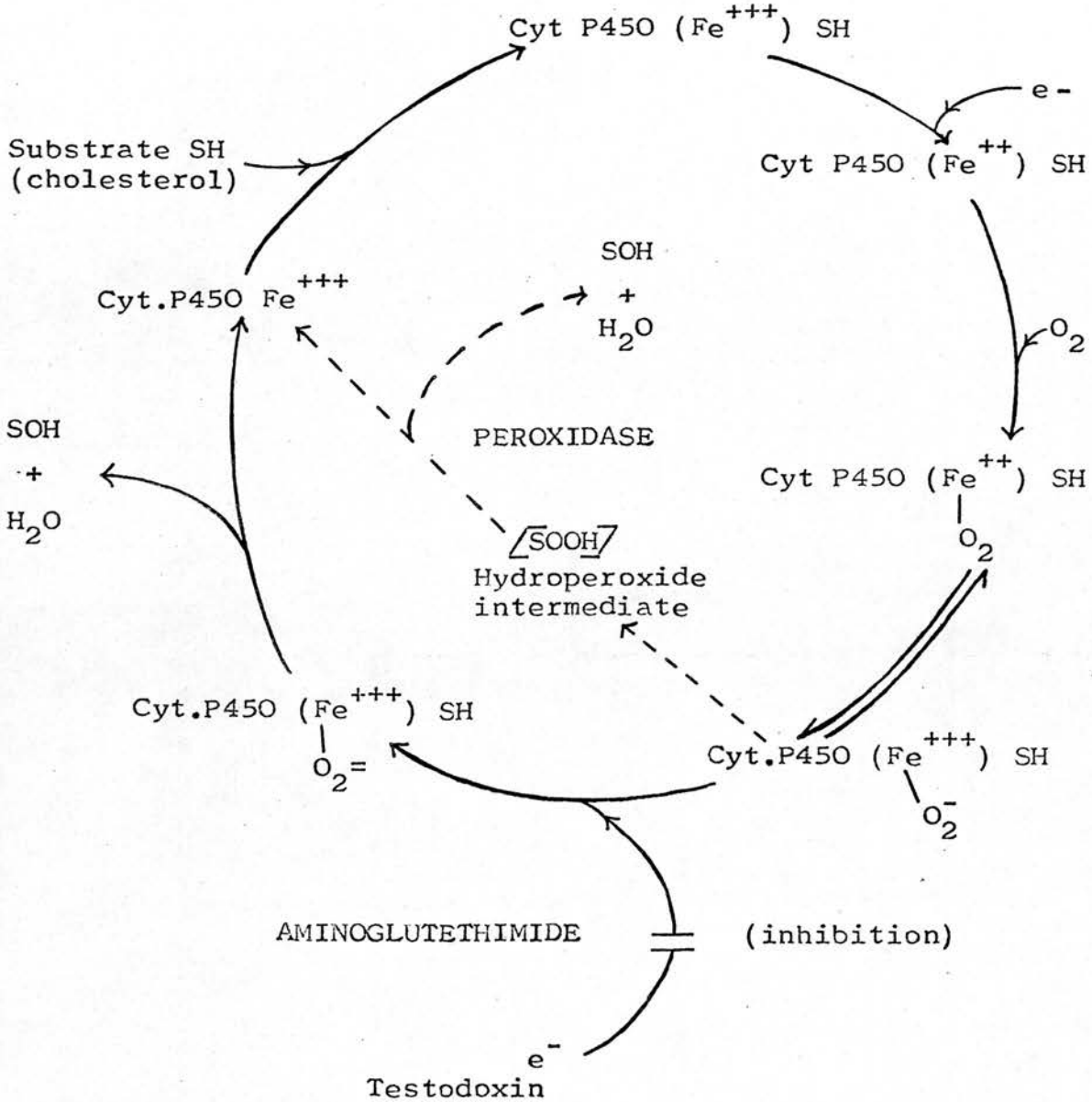


FIGURE 9.1 Postulated mechanism for the inhibitory effect of aminoglutethimide and the formation of dehydroepiandrosterone. (---- postulated pathway)

it should have been possible to detect both cholesterol side-chain cleavage activity and cytochrome P450 if present in the seminiferous tubular fractions. Menard and Purvis (1972) also observed that rat testis microsomal cytochrome P450 measured as the CO complex and the associated steroid 17α-hydroxylase were located in the interstitial cells. Dufau et al. (1971) demonstrated that cell cultures derived from the seminiferous tubules were deficient in 17α-hydroxylase. The reported observations that aminoglutethimide injected into rats produce hyperplasia of the interstitial cells without appreciable changes in the spermatogenic elements of the testes (Limanowski & Miskowiak, 1972) are, in view of the present studies, of particular significance. The cells of the seminiferous tubules, although lacking the biosynthetic capability may, however, utilize the steroids synthesized by the Leydig cells and thereby maintain spermatogenesis.

9.7 POSSIBLE MECHANISM FOR THE FORMATION OF DEHYDROEPIANDROSTENEDIONE

A mechanism is proposed involving cytochrome P450 as depicted in Figure 9.1 assuming that product X is dehydroepiandrostenedione (see Chapter 8), and taking into account (a) the formation of DHA only in the presence of aminoglutethimide, and (b) the necessity of an electron source. Although aminoglutethimide is postulated to inhibit the reduction of oxy-cytochrome P450 by the iron sulfur protein testodoxin (reduced), steroid formation could however occur via a hydroperoxide intermediate. Van Lier and Smith (1970b) have demonstrated that in adrenal cortex mitochondria, cytochrome P450 is involved in certain hydroperoxide reactions of cholesterol

20α-hydroperoxide. Hrycay and O'Brien (1971) have suggested that cytochrome P450 acts as a peroxidase in adrenal cortex microsomal metabolism of sterol hydroperoxides.

Recently Dufau et al. (1971) found that cell cultures derived from seminiferous tubules could form testosterone from certain steroid precursors. The cell cultures showed minor conversions of pregnenolone to progesterone and 17α-hydroxyprogesterone to testosterone but in contrast substantial conversions of DHA to testosterone and androstenedione, and of androstenediol to testosterone were observed. This generation of high local concentrations of testosterone from precursor DHA may be an important function of the tubules and significant in the maintenance of spermatogenesis. These authors also observed the absence of 17α-hydroxylation of pregnenolone and progesterone Further, it was reported that no significant conversion of labelled pregnenolone to 17α-hydroxypregnenolone occurred in rat testis microsomes (Shikita et al., 1964) or in rat testis interstitial tissue freed from tubules (Bell et al., 1968). In view of these findings, the existence in the testis of an alternative pathway for androgen biosynthesis from cholesterol via DHA which bypasses pregnenolone, could be physiologically highly significant.

9.8 SUPPLY OF SUBSTRATES FOR CHOLESTEROL SIDE-CHAIN CLEAVAGE

During the course of the present study it was decided to investigate some of the potential sources of the reactants - cholesterol + O_2 + NADPH $\longrightarrow$ pregnenolone + isocaproic acid.

It was conceivable that control of the rate of cholesterol side-chain cleavage (and the overall steroidogenic activity)

could be exerted (presumably via LH *in vivo*) by some initial effect on the supply of the reactants involved.

(A) CHOLESTEROL

It has long been held that the profuse lipid droplets present in adrenal cortex, corpus luteum and immature testes are stored precursors for steroidogenesis (Deane *et al.*, 1948; see Christensen & Gillim, 1969) and their presence and abundance thought to reflect the secretory activity of the cells. These droplets are known to be rich in cholesterol, present mainly in an esterified form with long chain (and often poly-unsaturated) fatty acids (Claesson, 1954; Garren *et al.*, 1971; Johnson, 1969 Boyd & Trzeciak, 1973). Cholesteryl ester depletion and steroid production in adrenal (Garren *et al.*, 1971) and luteal tissues (Armstrong, 1968a) induced by ACTH and LH respectively suggest that synthesis of cholesteryl esters may be an important regulatory site in steroid biosynthesis - providing a source of cholesterol for cholesterol side-chain cleavage. The possibility that a comparable effect of gonadotrophin on testicular cholester or cholesteryl ester might be of importance in the action of gonadotrophins on steroid production in testis was investigated by Van der Molen *et al.* (1973). Their results did not indicate a specific effect of HCG on any cholesterol pool in testis although testosterone levels in peripheral blood of the rats reflected the effect of hypophysectomy and HCG administration. Moyle and Armstrong (1970) also reported that LH had no effect on the content of free and esterified cholesterol in Leydig-cell tumours of mouse although stimulation of steroidogenesis was observed.

The adult rat testicular heavy mitochondria used in this present study contained very little cholesteryl esters (6.5 ± 0.2 nmoles/mg protein) compared to free cholesterol (70 ± 0.5 nmoles/mg protein). In addition these washed mitochondria were found to possess an active sterol ester hydrolase but less than 0.8% of the cholesterol so released was oxidized to steroids. These findings, in conjunction with the reported electron microscopic observations that the interstitial cells from mature rats (in comparison to mouse), are virtually without lipid droplets (Christensen & Gillim, 1969), imply that cholesteryl esters may not play as significant a role in testes as the source of cholesterol for steroid synthesis as in the adrenals (Dailey et al., 1963) and corpus luteum (Robinson & Stevenson, 1971). In this regard it is of interest to note that the rat adrenal cortex (Werbin & Chaikoff, 1961), like many other tissues of the body (Morris & Chaikoff, 1959), make virtually none of their own free cholesterol, but take it up from the plasma. In contrast the rat testicular interstitial cells rely far more heavily on endogenous synthesis - from acetate in the endoplasmic reticulum (Christensen, 1965; Christensen & Gillim, 1969).

The nature and size of the cholesterol pool associated with steroidogenesis in testicular mitochondria is not known. However, on the basis of the total cholesterol content (76.5 nmoles/mg protein) and cytochrome P450 content (see section 9.4) in the mitochondria of adult rat testis it was estimated that there are potentially about 7,000 nmoles of cholesterol/nmole of cytochrome P450. This extremely high cholesterol to cytochrome P450 ratio could therefore account for the very low

rates of cholesterol side-chain cleavage observed. It must be assumed that the cholesterol which is available for steroidogenesis is only a small portion of the total cholesterol pool of the mitochondria. As demonstrated in Chapter 6.3, the translocation of cholesterol to the cytochrome P450 in the mitochondria appears to be a cycloheximide sensitive process. The pretreatment of rats with cycloheximide decreased the rate of the cholesterol side-chain cleavage activity and also reduced the proportion of the cholesterol which was bound to the mitochondrial cytochrome P450 (by half). Thus there was less "reactive" cholesterol in these mitochondria. Davis and Garren (1966) postulated that for the transport of cholesterol to the cytochrome P450 site, a new protein is required, the synthesis of which is inhibited by cycloheximide and stimulated by LH. A similar cholesterol transport step is therefore one of the mechanisms and probable sites for the control of steroid synthesis in testes.

(B) OXYGEN

No attempt was made to investigate the supply of oxygen for practical reasons. It is interesting to note however that the Leydig cells are generally found in the testis in clusters around blood vessels, thus ensuring a constant supply of oxygen

(C) NADPH

It was observed (see Chapter 4) that the rat testis cholesterol side-chain cleavage enzyme system had an absolute requirement for NADPH - a characteristic shared by most other mammalian mixed function oxidases. NADPH generated extramitochondrially involves glucose-6-phosphate dehydrogenase. Contrary to earlier reports (Menon et al., 1967), in the present study glucose-6-phosphate dehydrogenase was not found to be associated

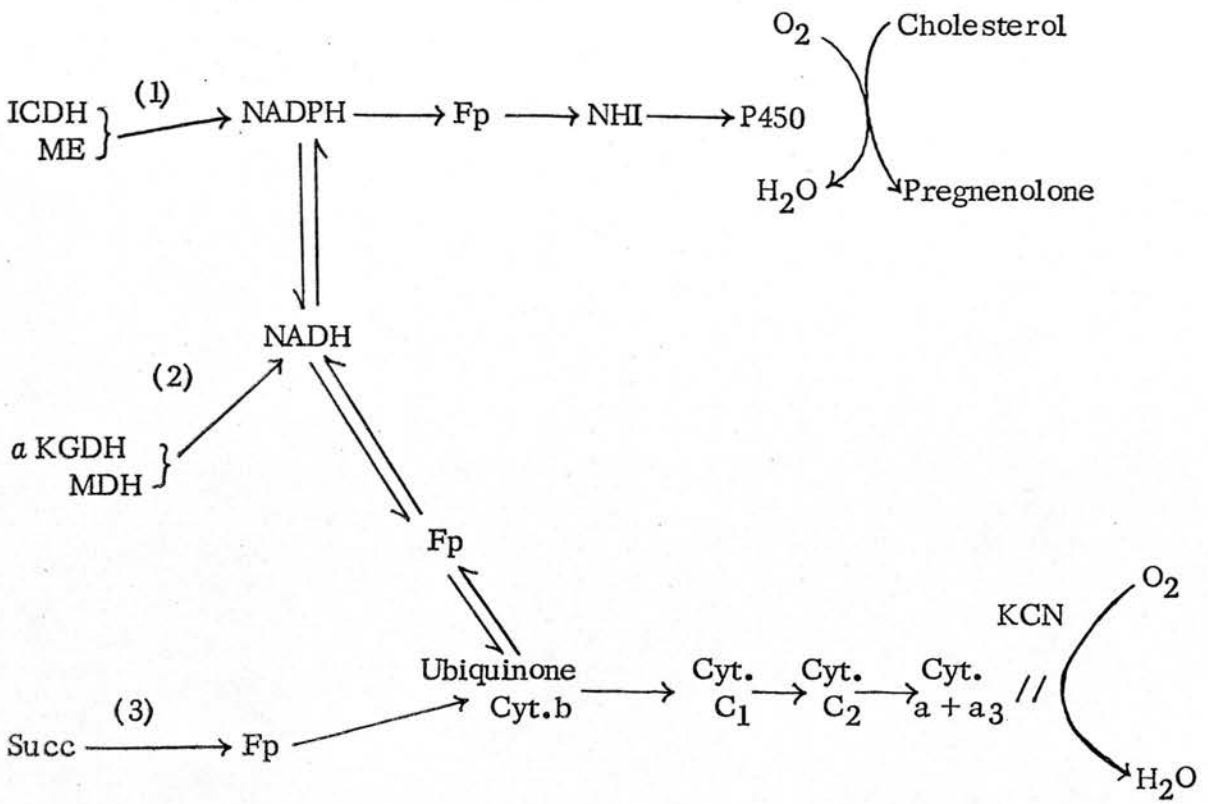


FIGURE 9.2 A SCHEMATIC REPRESENTATION OF POSSIBLE PATHWAYS FOR THE PRODUCTION OF NADPH IN RELATION TO CHOLESTEROL SIDE-CHAIN CLEAVAGE

(1) Via $NADP^+$-linked substrates (isocitrate or malate.

(2) Via NAD^+ dependent dehydrogenase coupled with NADPH:NADH transhydrogenase.

(3) Via flavoprotein dehydrogenases - involving reverse electron transport from succinate and transhydrogenation.

Arrows ($\rightarrow$, $\leftrightharpoons$) indicate direction of electron flow and // site of inhibition.

NHI = nonheme iron protein; Fp various flavoproteins; isocitrate dehydrogenase, malate dehydrogenase, α Ketoglutarate dehydrogenase and malic enzyme are designated ICDH, MDH, αKGDH and ME respectively.

with the mitochondrial fraction. Since this dehydrogenase is not a mitochondrial enzyme, and as mitochondria are relatively impermeable to pyridine nucleotides, the physiological supply of intramitochondrial reducing power for cholesterol side-chain cleavage could arise from some other sources. Based on studies in other steroidogenic organs the production of intramitochondrial NADPH could occur via three possible pathways as depicted in Figure 9.2.

On the basis of studies on adrenal cortex, Hall (1969) and Scoon and Major (1972) implied that malate and isocitrate were not likely to supply reducing equivalents for cholesterol side-chain cleavage in testes. However, the studies with rat testis mitochondria demonstrated that the Krebs cycle intermediate may be the main source of supply of reducing equivalents for cholesterol oxidation. Isocitrate, malate, and to a lesser extent succinate, were able to replace the requirement for extramitochondrially generated NADPH. Isocitrate was found to be the most effective electron donor. The marked stimulation of cleavage activity observed when isocitrate and $NADP^+$ were both present in the incubation indicated the presence of an active $NADP^+$ dependent isocitrate dehydrogenase. Spectrophotometric assay confirmed the presence of such an enzyme in these testis mitochondria (Stevenson, unpublished results). In this regard the reported histological observations that the Leydig cells are the principal sites of isocitrate dehydrogenase (Hitzeman, 1962; Ito, 1966) and malate dehydrogenase (Ambadkar & George, 1964) are of interest.

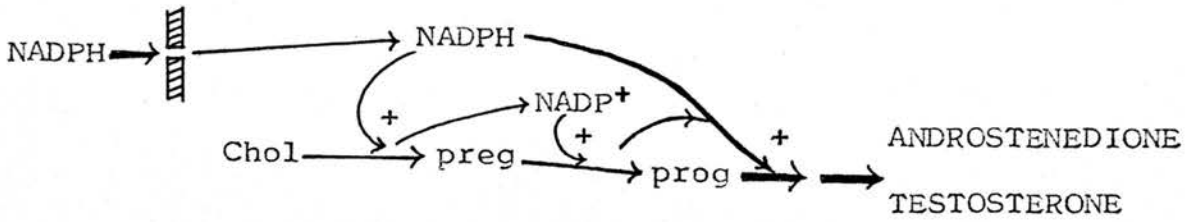
9.9 NATURE OF STEROID PRODUCTS FORMED FROM CHOLESTEROL

When NADPH-generating system was used as the electron source for cholesterol side-chain cleavage, the products formed were testosterone, androstenedione and 17α-hydroxyprogesterone, progesterone and pregnenolone. The latter two products generally constituted a smaller proportion of the total products. When NADPH was replaced by isocitrate as the electron donor the products now were mainly pregnenolone and progesterone. The yield and ratio of steroids accumulating were found to be further influenced by the presence of certain factors in the incubations such as $NADP^+$, Ca^{++}, BSA, ADP and cyanide (see Chapter 4, Figure 4.8). These substances could therefore, by their respective influences, provide a possible mechanism of control of steroid synthesis by regulating the accessibility of NADPH and other substrates to the cholesterol side-chain cleavage enzyme complex. The observations that magnesium and other reagents which uncouple the mitochondria stimulate cholesterol side-chain cleavage activity, suggest that the integrity of the mitochondria could also contribute to the regulation of steroid biosynthesis. It is of interest that coupling could only be demonstrated when potassium phosphate, ethylenediamine tetraacetic acid and bovine serum albumin were present in the medium together. Since the cholesterol side-chain cleavage enzyme has been reported to be located in the inner mitochondrial membrane (Yago & Ichii, 1969), the influence of the above factors on cholesterol oxygenase activity could be highly significant.

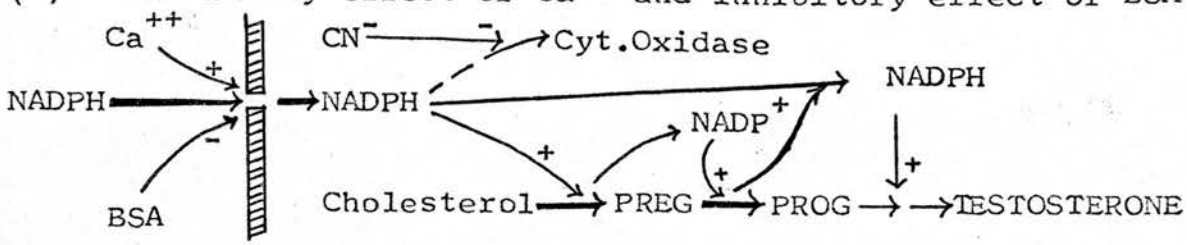
On the basis of studies discussed in Chapter 4, the differen effects of cations, anions, pyridine nucleotides and BSA can be

A. With NADPH-generating system as the electron source

(1) No additional factors

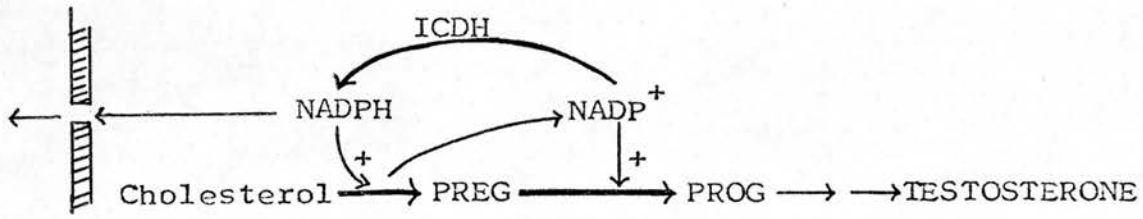


(2) Stimulatory effect of Ca^{++} and inhibitory effect of BSA



B. With Isocitrate as electron donor

(3) No additions



(4) Stimulatory effect of $NADP^{+}$, BSA and ADP plus Ca^{++} plus KCN and inhibitory effect of Ca^{++}

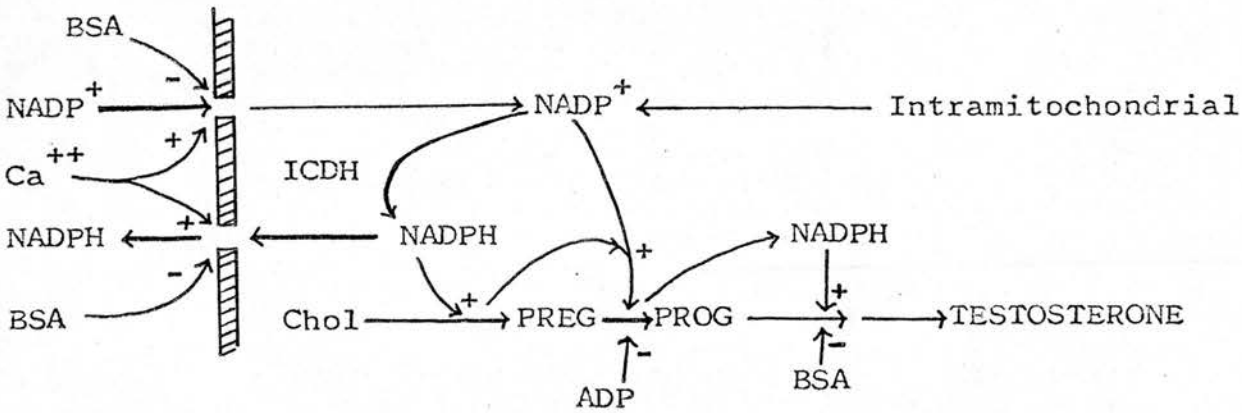


FIGURE 9.3 Schematic representation of the influence of various factors on the formation of steroid products from cholesterol.

mitochondrial membrane

\+ = stimulation

\- = inhibition

schematically represented as set out in Figure 9.3. From the multiple nature of steroid products formed it can be inferred that rat testis heavy mitochondrial fraction may, in addition to the cholesterol oxygenase enzyme, also contain the enzymes involved in the conversion of pregnenolone to testosterone. These latter enzymes are usually assumed to be microsomal enzymes. However, since repeated washings of the ficoll fractionated mitochondrial pellet did not influence the nature of the reaction products formed, there may well be two sets of dehydrogenase-isomerase, 17α-hydroxylaseand C17, 20 lyase enzyme systems - one associated with the mitochondrial and the other with microsomal components of rat testes. Such a dual existence of distinct dehydrogenase-isomerase enzymes have been reported in immature rat ovarian mitochondria and microsomes (Sulimovici & Boyd, 1969b). However, the possibility that the testicular mitochondrial fractions could be contaminated by a small amount of active enzymes of microsomal origin, which may be closely adhering to the mitochondria, cannot be ruled out. Furthermore, the possibility that seminiferous tubules may possess different enzyme systems to that of interstitium must also be considered. Investigations involving kinetic studies and enzyme purification will need to be carried out with a homogeneous population of cells to confirm or rule out these possibilities.

9.10 CONCLUSIONS

The results presented in this thesis establish that the first step in steroid biosynthesis in testes, namely the side-chain cleavage of cholesterol, occurs exclusively in the "heavy" mitochondria derived solely from the interstitial tissue. The

presence of cytochrome P450 was demonstrated in these mitochondria for the first time and shown to be involved in cholesterol hydroxylation. Evidence also indicated that the regulation of the synthesis of a labile carrier protein, which participates in the translocation of cholesterol to the cytochrome P450 sites, appears to be one of the possible modes of action of gonadotrophins in the control of steroidogenesis. A regulatory influence on the synthesis of steroids could also be exerted by altering the nature of the electron donor, cofactors, ions such as calcium and magnesium, pH around the mitochondrion and in the immediate vicinity of cholesterol side-chain cleavage cytochrome P450, and/or integrity of the mitochondrial membrane. These factors markedly affected the activity of the cholesterol side-chain cleavage complex in vitro.

It is hoped that the work presented in this thesis will aid and pave the way to further investigations on steroidogenesis in testes. Although some answers have been obtained in this present study there are many points that still need to be clarified and investigated further:-

1. Alternative pathway
   (a) Identity and nature of compound X
   (b) Can metyrapone also induce the formation of X?
   (c) Is cholesterol sulfate a precursor?
   (d) If there are two pathways of androgen biosynthesis from cholesterol, does one pathway predominate over the other with respect to the development and maturation of the testis?

2. Components of the cholesterol side-chain cleavage enzyme complex
   (a) The presence of flavoprotein and non heme iron have yet to be demonstrated
   (b) Can metyrapone also inhibit cholesterol side-chain cleavage activity?
3. Can foetal and postnatal testes convert cholesterol to pregnenolone?
4. Is cytochrome P450 present in foetal and postnatal testis, and if so when does it appear?
5. During development of the testes is the cholesterol oxygenase enzyme or its components, such as cytochrome P450, in any way affected or altered?
6. What is the part played by gonadotrophic hormones during the development and maturation of the testis? Do they:
   (a) Stimulate net synthesis of particular enzymes, cytochrome P450 or cholesterol carrier protein?
   (b) Stimulate cofactor generation systems (NADPH)?
   (c) Remove inhibitor(s) of cholesterol metabolism?
   (d) Alter cyclic AMP activity?

Since the changes in the histology of Leydig cells observed during early development of the testis reflect gonadotrophin activity, age studies could provide a useful and natural model for the investigation of the mechanisms of control of steroidogenesis. Such information will be of interest not only from the viewpoint of basic endocrinology, but also for sciences related to clinical endocrinology and reproduction.

APPENDIX

The following chemicals were purchased from the Sigma Chemical Company Ltd., London:- $NADP^+$ monosodium salt, glucose 6-phosphate disodium salt; glucose-6-phosphate dehydrogenase (type XII), NADH, NAD^+, ATP, ADP, bovine serum albumin (free of fatty acids - Type F), bovine plasma albumin (fraction V), steroids, DL isocitrate, L-malate, succinate, EDTA, EGTA, Trizma HCl, Trizma base and Pseudomonas testosteroni (Type I). Sucrose, phosphate salts, magnesium chloride, potassium cyanide and potassium chloride were purchased from BDH Chemicals Ltd., Poole, England.

Silica gel G and thin layer chromatography silica gel precoated plates were obtained from E. Merck A.G., Darmstadt, Germany.

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

PPO and POPOP (Scintillation grade) and Soluene 100 were from Packard Instrument Co. Inc., U.S.A.

Aminoglutethimide (Elipten) and metyrapone (SU 4885) were obtained from CIBA.

Ficoll (M wt 400,000) was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden.

Cycloheximide-1-P was from Koch-Light Laboratories Ltd., Bucks., England.

Cyanoketone was a gift from Dr. J. L. McCarthy, Southern Methodist University, Dallas, Texas.

APPENDIX - Cont'd

Gas chromatography column packing was obtained from Applied Science Laboratories Inc., U.S.A.

Ethanol, methanol and acetone were redistilled before use. Ethanol and methanol were refluxed for 2 hours with 0.1% KOH and acetone with 0.1% potassium permanganate before redistillation.

All other reagents and solvents were of analar grade. Hemoglobin solution was prepared from 1.0 ml heparinized rat blood by washing the red blood cells with isotonic saline, lysing in 3.0 ml distilled water and sedimenting the erythrocyte ghosts at 10,000 x g.

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