SOME BIOCHEMICAL STUDIES RELATED TO STEROIDOGENESIS IN THE PORCINE CORPUS LUTEUM

by

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ABBREVIATIONS USED IN THE TEXT.

The following abbreviations will be used throughout the text:-

ACTH	-	adrenocorticotrophic hormone
ADP	-	adenosine 5'-diphosphate
AMP	-	adenosine 5'-monophosphate
3'5'AMP	-	adenosine 3':5'-cyclic monophosphate
ATP	-	adenosine 5'-triphosphate
BSA	-	bovine serum albumin
CoA	-	coenzyme A
DNP	-	2,4 - dinitrophenol
EDTA	-	ethylenediamine tetraacetate
ER	-	endoplasmic reticulum
FFA	-	free fatty acid
GC	-	gas chromatography
G-I-P	-	D-glucose-1-phosphate
G-6-P	-	D-glucose-6-phosphate
LH	-	luteinizing hormone
NAD^+	-	nicotinamide adenine dinucleotide, oxidized
NADH	-	" " " reduced
$NADP^+$	-	" " " phosphate, oxidized
NADPH	-	" " " phosphate reduced
PC	-	palmitylcarnitine
PMS	-	N-methyl phenazonium methosulphate
RQ	-	respiratory quotient
TCA	-	tricarboxylic acid cycle
TMPD	-	tetramethylphenylene diamine
TMS	-	trimethylsilyl ether -

pregnenolone - 3β-hydroxypregn-5-ene-20-one

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

SUMMARY.

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

1. The cholesterol side-chain cleavage reaction was investigated in preparations of porcine luteal mitochondria. The enzyme system was found to be similar to that studied in other steroidogenic tissues in that it was located in the mitochondrial subcellular fraction, was associated with cytochrome P450 and required NADPH as an electron donor.

2. A method for determining the oxygen content of incubation media was described; it was based on the stoichiometric oxidation of added NADH, catalysed by phenazine methosulphate. Using values for oxygen concentration obtained by this method, some respiratory characteristics of luteal mitochondrial preparations were investigated. Respiratory control and ADP:O ratios of these preparations were found to be significantly lower than those measured for porcine liver mitochondrial preparations.

3. The efficiency of several tricarboxylic acid cycle intermediates as electron donors for cholesterol side-chain cleavage activity was investigated: citrate, isocitrate, succinate, fumarate and malate supported greater activity than NADPH.

4. Studies with respiratory inhibitors and uncoupling agents indicated that NAD^+- linked substrates could

donate electrons to the NADPH - cytochrome P450 reductase by an energy-independent pyridine nucleotide transhydrogenase. In contrast, succinate supported the reaction via an energy-dependent electron transfer pathway. Experimental evidence was presented which indicated that this latter route might not involve reduction of NADH.

5. In view of the natural abundance of cholesterol-fatty acid esters in luteal tissues, it was thought relevant to investigate the utilization of such compounds by porcine luteal mitochondrial preparations. [4-^{14}C] cholesteryl oleate was shown to be hydrolysed, and the [4-^{14}C] cholesterol thus liberated underwent side-chain cleavage. The capability of the fatty acid moiety to act as an electron donor for this reaction was also demonstrated. Palmitylcarnitine was shown to support high levels of cholesterol side-chain cleavage activity, via an energy dependent electron transfer process.

6. A schematic hypothesis of electron transfer pathways in porcine luteal mitochondria was presented. Its main features embodied electron transfer connections between the mitochondrial "respiratory" and "steroidogenic" chains at two different levels: (a) a reversible, non-energy dependent pyridine nucleotide transhydrogenase, and (b) an energy-dependent electron transfer route from reduced flavoprotein to the NADPH-cytochrome P450 reductase.

7. As a speculation it was suggested that LH might stimulate cholesterol side-chain cleavage activity, and hence steroidogenesis, by promoting electron transfer from respiratory substrates to the NADPH-cytochrome P450 reductase via these connections, at the expense of mitochondrial respiratory electron flow.

CHAPTER I

CHAPTER 1.

INTRODUCTION

1.1 Some notes on porcine ovarian endocrinology.

In the adult female mammal characteristic cyclic changes are observed in the morphology of the reproductive organs and in the behaviour of the animal: these recurring phenomena associated with reproduction are termed oestrous cycles. In domestic animals the oestrous cycle is divided into various phases which are referred to as "proestrus", "oestrus", "metoestrus" and "dioestrus"; the meaning of these terms has been standardized by Asdell (1946). Among the larger domestic mammals, the porcine species is unique in being polytocous i.e. giving birth to several offspring at one time. The sow is polyoestrous and will breed at anytime of the year.

The porcine oestrous cycle has a mean duration of 20.7 days (Asdell, 1964). The first stage, or oestrus phase, of the cycle lasts for 59.3 hours and during this time the female will accept coitus. Ovulation of follicles, which had matured towards the end of the previous oestrus cycle, is usually begun 36 hours after the start of this phase and may take 6 or 7 hours to complete (Burger, 1952). Several ova are shed in each oestrous cycle, the usual litter size in the sow being 8 - 12 (Catchpole, 1969). Metoestrus, a short transitional

phase of the cycle follows in which the recently ruptured follicles are re-organized into corpora lutea. When a follicle ruptures, its walls collapse and their granulosa and thecal layers are thrown into folds; the corpus luteum is formed from cells of both layers. As the gland develops, small blood vessels from the theca invade the granulosa, and the granulosal cells begin to hypertrophy or luteinize. At the end of metoestrus, which lasts about 3 days, progesterone secretion by the ovary is rising rapidly, showing that the transition to dioestrus has occurred. The length of dioestrus primarily determines the length of the oestrus cycle and it is in this phase that the corpus luteum is functional. In the sow significant amounts of progesterone are secreted from the fourth day (after oestrus) onwards (Masuda et al., 1967) and maximal luteal secretory activity is attained by mid-cycle, between day 8 and 12 (Duncan et al., 1960) (Masuda et al., 1966, 1967; Cook et al., 1967).

Unless pregnancy has occurred the corpus luteum ceases to function fairly abruptly on about day 16 of the cycle, usually before any histological changes are evident in the gland (Bjersing, 1967). The arrival of proestrus usually on day 17 or 18 is heralded by a rise in the secretion of oestrogens, whose origin is in the maturing follicles.

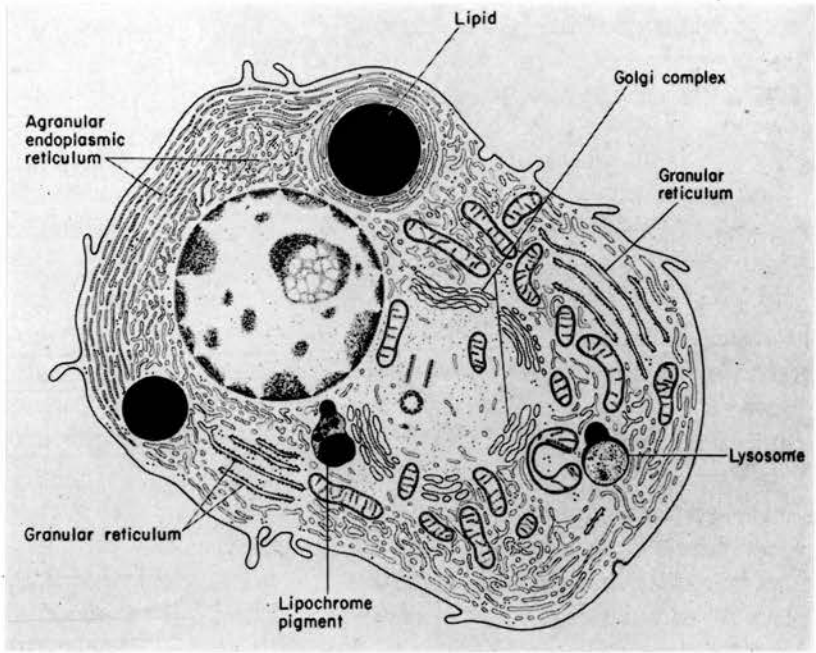


FIG. 1.1 Schematic drawing of the characteristic cytological features of a steroid-secreting cell, a large Golgi complex, pleomorphic mitochondria, a few cisternal profiles of granular endoplasmic reticulum, and a very extensive smooth surfaced reticulum. There are also variable numbers of lipid droplets, lysosomes, and lipochrome pigment. (From Fawcett et al., 1969

The porcine corpus luteum generally reaches a maximum diameter of 8-10 mm (Duncan et al., 1960), and a maximum weight of about 500 mg. (Masuda et al., 1967). Corpora lutea have been classified into those which produce many steroids, including oestrogens as well as progestogens, and those which produce few steroids, progestogens only (Savard et al., 1965). The porcine gland falls into the latter category: in the luteal phase of the porcine oestrous cycle, the synthesis of only two steroids, progesterone and its reduction product 20 α hydroxy - 4 - pregnen - 3 - one, has been demonstrated, the latter steroid comprising less than 5% of the total (Cook et al., 1967).

1.2 Cellular ultrastructure of steroid-secreting cells, with particular reference to the porcine corpus luteum.

Cells that specialize in producing steroid hormones show some features in their ultrastructure which are distinct from those of other endocrine cells; these characteristics may reflect the steroidogenic capabilities of such cells. (see Christensen and Gillim, 1969; Fawcett et al., 1969).

The typical cytological organization of a generalized steroid-secreting cell is shown in Fig. 1.1. The ultrastructure of cells of the porcine corpus luteum has been investigated by electron microscopy, using tissue taken sequentially throughout the oestrous cycle, in

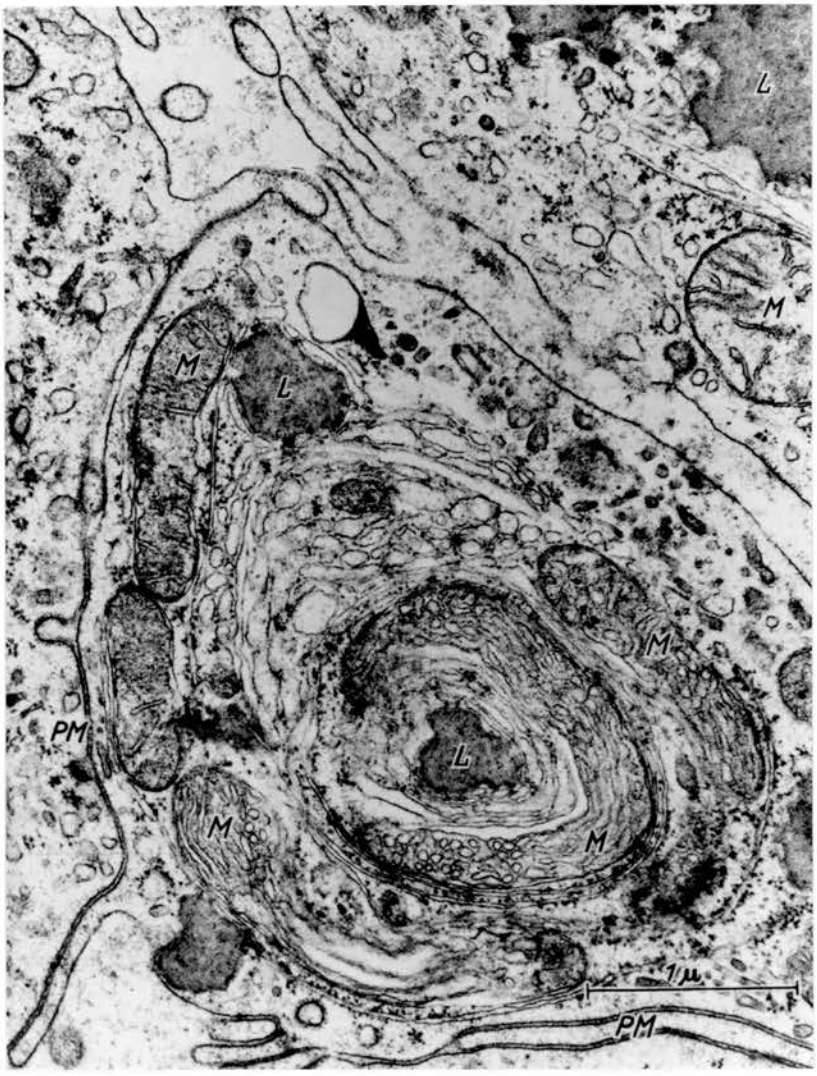


FIG. 1.2: An electron-micrograph of a porcine granulosa lutein cell, 3 to 4 days after ovulation (from Bjersing, 1967). Centrally, part of a cell with elongated and cup-shaped mitochondria (M). A lipid droplet (L) surrounded by agranular membranes of endoplasmic reticulum, and more peripherally a cup-shaped mitochondrion containing tubular inner structures of the same appearance as part of the agranular endoplasmic reticulum. Some free and membrane associated ribosomes are seen. PM is plasma membrane.

an attempt to correlate the changes in fine structure with steroid hormone synthesis (Bjersing, 1967). The sub-cellular morphology of such lutein cells (see Fig. 1.2) shows several examples of typical steroidogenic ultrastructure:-

(i) Endoplasmic reticulum:

In the very early stages of the corpus luteum, granular (rough) endoplasmic reticulum (ER) predominates over the agranular (smooth) variety; it is likely that protein synthesis is a major activity of the growing gland. In the mature corpus luteum however, agranular ER is very extensively developed. It is possible that some of this agranular ER is derived from preformed granular ER: James et al,(1969) have recently shown that the two morphological forms of ER are inter-convertible in liver tissue, and that steroid sex hormones are involved in this process.

A well developed agranular ER is characteristic of most steroid-secreting cells. In lutein cells (and other steroidogenic tissues) it forms a vast meshwork of interconnecting tubules that fills much of the cytoplasm. Occasionally it is organised into whorls of concentric cisternae, which are often arranged around cytoplasmic lipid droplets, mitochondria or some other cytoplasmic structure (Christensen, 1965; Blanchette, 1966; Bjersing, 1967). Throughout the first half of the porcine oestrous cycle, at a time when progesterone

synthesis is known to be increasing (Duncan et al., 1960; Brinkley and Young, 1965; Gomes et al., 1965; Masuda et al., 1966), a parallel increase in smooth ER is seen (Bjersing, 1967). The development of this organelle is apparently related to trophic hormone stimulation of the gland; after hypophysectomy it shows rapid regression, (Christensen and Gillim, 1969), whereas administration of gonadotrophins is followed by a rapid increase in its development (Ashworth et al., 1959; Schwarz et al., 1962; Nishikawa et al., 1963; Enders and Lyons, 1964; Rennels, 1966).

The extent of the development of smooth ER seems far greater than would seem to be required for the relatively small amounts of hormone secreted (Fawcett et al., 1969). It has been suggested (Fawcett, 1965; Christensen and Fawcett, 1966) that it might act as a reservoir for newly synthesized cholesterol; in favour of this hypothesis, a correlation can be seen between the amount of smooth ER present in a steroidogenic cell, and the amount of cholesterol biosynthesis undertaken by that cell (see Christensen, 1965, 1969), i.e. if the smooth ER is well developed the cell seems to have the capability of synthesizing all the cholesterol necessary for its steroid biosyntheses, whereas when smooth ER is not so abundant cholesterol is taken up from the plasma.

(ii) Golgi Complex:

The Golgi complex is usually prominent in steroid

secreting cells. Although this organelle can be seen to be well developed in these cells, there is as yet no definite evidence suggesting a secretory function for it, comparable to its role in other secretory tissues. The extent of development of the Golgi complex is however, related to trophic hormone stimulation and the functional status of steroidogenic cells (Reese and Moon, 1938; McDonald and Goldfien, 1965). In a recent study on the fine structure of porcine corpus luteum, Belt et al., 1971) have described the appearance and accumulation of dense granules in the cytoplasm of granulosa luteal cells in early and midgestation, and their disappearance in the last days of pregnancy. They demonstrated that these granules contained the polypeptide hormone relaxin. The Golgi apparatus of the cell became increasingly prominent as pregnancy progressed, and appeared to contain structures which were interpreted to be developing granules. It is therefore possible that in the corpus luteum the Golgi complex is associated with the secretion of relaxin, in a manner analogous to the role of this organelle in the exocrine secretion of zymogens in pancreatic tissue.

It has also been suggested that in adrenocortical tissue, the Golgi complex may be a site for steroid conjugation (Long and Jones, 1967).

(iii) Mitochondria:

It is in the mitochondria of steroid secreting cells that the most striking qualitative changes appear, especially after hypophysectomy or trophic hormone administration (Christensen and Gillim, 1969); this is consistent with the view that LH and ACTH act at the site of cholesterol side-chain cleavage (Hall and Koritz, 1965; Stone and Hechter, 1954) which occurs in the mitochondria (Sulimovici and Boyd, 1969). The mitochondria increase in number and size during dioestrus, in the porcine ovary, and are often seen close to, or even around cytoplasmic lipid droplets (Bjersing, 1967) (see Fig. 1.2). These mitochondria, and those from other steroidogenic cells (Fawcett et al., 1969; Christensen and Gillim, 1969), have cristae that are characteristically different from those of mitochondria isolated from other tissues; the cristae are usually tubular or even vesicular in appearance, rather than lamellar (Palade, 1953; Belt and Pease, 1956), and closely resemble the tubules of smooth ER of steroidogenic cells. The presence, in these mitochondria, of tubules similar to those of the smooth ER may reflect active engagement in steroid metabolism, e.g. cholesterol side-chain cleavage (Bjersing, 1967); in this context it is interesting to note that the vesicular cristae shape is modified to a "non-steroidogenic" tubular shape by hypophysectomy, and can be regained by trophic hormone administration (Sabatini

et al., 1962; Idelman, 1966; Kahri, 1968).

(iv) Cytoplasmic lipid droplets:

These cellular inclusions are present in steroid secreting cells of a great many species, including those of the porcine corpus luteum (Bjersing, 1967). The abundance of such lipid droplets varies with the secretory activity of the gland; during dioestrus, when progesterone synthesis is maximal, the droplets appear to diminish; similarly, administration of gonadotrophin leads to a rapid disappearance of these inclusions and conversely, hypophysectomy is followed by an increase in their numbers (Christensen and Gillim, 1969). The presence of cholesterol, especially in an esterified form, in these lipids, has been inferred from cytochemical considerations (see Bjersing, 1967), and they have been considered to be stores of steroid hormone precursor materials (see Deane, 1958; Armstrong, 1966). Their close spatial relationship with smooth ER (Schwarz et al., 1962; Brenner, 1966; Blanchette, 1966; Christensen and Fawcett, 1966; Bjersing, 1967) and also with mitochondria (Bjersing, 1967) has suggested to several investigators a functional association concerned with steroidogenesis (Idelman, 1966; Bjersing, 1967; Christensen and Gillim, 1969).

(v) Lysosomes:

These organelles are characteristically associated

with steroid secreting cells at certain stages of their development: in the porcine ovary (Bjersing, 1967) they are least common in the corpora lutea of metoestrus and early dioestrus, but become more evident in late dioestrus and proestrus as the functional status of the gland declines and its involution begins.

1.3 Some hypotheses concerning the mechanism of action of trophic hormones on steroidogenesis.

The problem of how the pituitary trophic hormones concerned with steroidogenesis regulate the metabolism of their target organs has received considerable attention since the early 1950's. Over this period of time several hypotheses concerning the mechanism of action of such hormones have been suggested, but to date none has gained universal acceptance. Although the following account is concerned primarily with LH and luteal steroidogenesis, repeated reference to ACTH will be made, since the mechanism of action of these trophic hormones on their respective target organs may be similar (see Hirshfield and Koritz, 1966; Hall and Young, 1968; McKerns, 1969).

(1.3.1) Involvement of a "second messenger".

Although there are numerous reports of positive effects of LH (or ACTH) in stimulating steroidogenesis *in vivo*, or *in vitro* in perfusion or tissue slice preparations (Armstrong, 1968) there have been very few substantiated claims of similar effects of the hormone on subcellular

fractions: the probable explanation for this discrepancy is that ACTH and, more especially LH, being relatively large polypeptides, do not penetrate their target cells but act at a site on the cell membrane. It therefore becomes necessary to postulate the involvement of an intracellular mediator of trophic hormone action on the steroidogenic process. Currently, two possible candidates have been suggested for this role: there is a considerable body of evidence indicating that the nucleotide cyclic 3'5' adenosine monophosphate (3'5' AMP) may be involved in the steroidogenic actions of ACTH and LH (Karaboyas and Koritz, 1965; Marsh et al., 1966; Grahame-Smith, et al., 1967; Sutherland et al., 1968; and Robison et al., 1968); alternatively the involvement of a "labile protein" whose synthesis is stimulated by ACTH (or LH) and which in turn mediates the intracellular effect of these trophic hormones, has also been suggested (Ferguson, 1962; Farese, 1964; Garren et al., 1965, and Garren 1968). The choice of either hypothesis is to some extent peripheral to this account, since neither gives any direct suggestion as to how the effects of the trophic hormones are actually achieved at the level of the target cells' steroidogenic metabolism. Furthermore, the two hypotheses are not mutually exclusive, and there is some evidence that indicates their sequential involvement in this process; i.e. ACTH (or LH) → 3' 5' AMP → "labile" protein → stimulation of steroidogenesis (see Schulster et al., 1970).

(1.3.2) Effects on the metabolism of the steroidogenic cell.

Attempts to locate the site of action of trophic hormones upon steroidogenesis have been made by several groups of investigators: Stone and Hechter (1954) presented evidence that ACTH stimulated steroidogenesis by increasing the rate of conversion of cholesterol to pregnenolone, and this finding has since been confirmed and extended to other steroid secreting tissues (Hall and Eik-Nes, 1964; Karaboyas and Koritz, 1965; Hall and Koritz, 1965; and Hall, 1966) including the bovine corpus luteum (Hall and Koritz, 1965). However, results obtained by Savard and co-workers (Mason and Savard, 1964; Savard and Casey, 1964; Savard et al., 1965;) have indicated that, in some species at least, LH might also exert a stimulatory effect on ovarian cholesterol biosynthesis.

Several theories have been proposed to explain more precisely how the rate of steroidogensis is increased.

(i) via control of NADPH availability.

Many of the reactions involved in both cholesterol synthesis (see Popjak and Cornforth, 1960) and in the transformation of cholesterol to steroid hormones (Koritz and Péron, 1958) depend on electrons from NADPH for reductive, and hydroxylation reactions. The obvious importance of this cofactor in the steroidogenic process has lead to the proposal of several theories of the mechanism

of action of LH or ACTH based on some control of the availability of NADPH at the site of its utilization for steroidogenesis.

One of the earliest theories concerning the biochemical mode of action of a steroidogenic trophic hormone was that proposed by Haynes and Berthet (1957). Stated briefly, their concept was that ACTH acted initially on adenyl cyclase of the adreno-cortical cell (Haynes, 1958) leading to an increased production intracellularly of 3'5'AMP, which in turn activated a phosphorylase; the increased phosphorylase activity would break down adrenal glycogen to produce G-I-P and G-6-P, and the latter substrate, when metabolized via the pentose phosphate pathway would produce increased amounts of NADPH and so stimulate adrenal corticosteroidogenesis. Since that time several criticisms of the original theory have been made: although LH stimulates intracellular production of 3'5'AMP in its target cells (Marsh et al., 1966), and also activates an ovarian phosphorylase (Marsh and Savard, 1964), the very small amounts of glycogen present in the tissue suggests that stimulation of its utilization is unlikely to have a significant effect on levels of G-6-P (see Armstrong, 1968). The validity of the theory was challenged even more basically by Savard and co-workers (Savard et al., 1963; Savard and Casey, 1964; Mason and Savard, 1964; Savard et al., 1965), whose results showed that even in the presence of saturating

amounts of NADPH, addition of LH in vitro to ovarian preparations could elicit a further stimulation of progesterone biosynthesis, leading them to conclude that the stimulating actions of LH and NADPH on ovarian steroidogenesis were separate and independent. It should be emphasized however, that these conclusions were based on experiments performed on tissue slice preparations. The stimulation by exogenous NADPH of steroidogenesis in such preparations is probably more related to cell damage than to a potential role in the mechanism of action of LH, since the nucleotide does not readily penetrate intact cells; the possibility that steroidogenesis is controlled by LH via regulation of the intramitochondrial availability of NADPH still remains tenable.

A more direct action of ACTH or LH on G-6-P dehydrogenase has been proposed as an alternative hypothesis to that outlined above: in recent years McKerns and co-workers have presented evidence that these trophic hormones activate G-6-P dehydrogenase, probably via an allosteric binding site, reducing the apparent Km of the enzyme for $NADP^+$ (in the presence of excess G-6-P) from 3.0×10^{-5}M to 1.2×10^{-5}M, and also reducing the apparent Km for G-6-P (in the presence of excess $NADP^+$) from 12.5×10^{-4}M to 6.0×10^{-4}M. (McKerns, 1964; McKerns 1965; McKerns and Criss, 1968; Criss and McKerns, 1968; McKerns, 1969). A problem with this hypothesis lies in its failure to account for the

compartmentalization of the sites of steroidogenesis within the cell: i.e. NADPH derived from G-6-P dehydrogenase is cytoplasmic, and would presumably be available for cholesterol and fatty acid biosyntheses, reactions which occur in the cytoplasm; however, cholesterol side-chain cleavage (and several other steroid mixed function oxidations in the adrenocortical cell) takes place within the mitochondrion, an organelle thought to be relatively impermeable to NADPH (Peron et al., 1966).

A hypothesis which involved an action of the trophic hormones on mitochondrial permeability to cytoplasmic NADPH has been considered by Koritz and co-workers (Hirshfield and Koritz, 1964, 1965; Koritz, 1968). They showed that ACTH administered in vivo leads to changes in the permeability properties of mitochondria subsequently prepared from adrenal tissue. Roberts and Creange (1968) have also presented evidence which was compatible with the view that ACTH, acting via 3'5' AMP, might regulate steroidogenesis via controlling mitochondrial permeability to NADPH. It is possible that the changes in mitochondrial ultrastructure (see previous section, 1.2.iii) brought about by trophic hormone, or 3'5' AMP administration might be indicative of a change in the permeability characteristics of these mitochondria, analogous to those seen in the respiratory-dependent swelling and contraction

cycles of other mitochondrial preparations (McKerns, 1968).

(ii) via feedback inhibition of pregnenolone on cholesterol side-chain cleavage activity:

Another hypothesis involving control of mitochondrial membrane permeability is now favoured by Koritz and co-workers (Koritz and Hall, 1964a; Koritz and Hall, 1964b; Koritz, 1968; Koritz and Kumar, 1970): it is suggested that the rate of corticosteroidogenesis is controlled by the rate of efflux of pregnenolone from the mitochondrion, whose permeability to this steroid is modified by ACTH (via 3'5' AMP). Pregnenolone was shown (Koritz and Hall, 1964a) to be capable of inhibiting cholesterol side-chain cleavage activity, probably by an allosteric mechanism (Koritz and Hall, 1964b).

The theory is rather speculative: ACTH or 3'5' AMP were not shown by these investigators to effect mitochondrial permeability to pregnenolone; this suggestion was based on experiments which showed that agents which increased adrenal mitochondrial permeability, such as 11mM Ca^{++}, 100μM palmitic acid, 100μM sodium lauryl sulphate, pronase, or freezing and thawing, also stimulated pregnenolone synthesis, when the incubation medium contained a NADPH - generating system (Hirshfield and Koritz, 1964). It is more likely that the stimulation of pregnenolone synthesis which occurred in these situations was a result of enhanced penetration of

NADPH through the damaged mitochondrial membranes of the preparations.

Furthermore, it is now well established that mitochondria of luteal tissues have an active Δ 5-3β -hydroxysteroid dehydrogenase; most of the pregnenolone formed by cholesterol side-chain cleavage undergoes further metabolism to progesterone within the mitochondrion. The application of an analogous theory to that outlined above, to luteal steroidogenesis would therefore seem difficult.

(iii) via control of cholesterol availability:

The role of cholesterol as an obligatory precursor of steroid hormones seems now well established (Krum et al., 1964; Menon et al., 1965); control of the availability of this sterol as a means of regulating steroidogenesis is, therefore, an obvious possible mechanism of action of ACTH or LH.

Several groups have obtained results indicating that the cholesterol pool in ovarian and adrenal tissues is not homogenous (Hayano et al., 1956; Armstrong, 1964;) Furthermore, it has been suggested (Solod et al., 1966) that, in rabbit ovary, the cholesterol laid down most recently, whether derived from plasma cholesterol or synthesized in situ, was the first to be used for pregnenolone synthesis. This concept has been extended by Savard et al., (1965) who postulate the existence of a pool of "steroidogenic" cholesterol which is distinct

from other "structural" cholesterol pools within the cell, and whose synthesis is influenced by LH.

The corpus luteum of a number of species, including the pig (Bloor et al., 1930; Barker, 1951 and Bjersing, 1967) has long been known to contain quantities of cholesterol esters which were seen to vary during the oestrus cycle (Everett, 1945). An inverse relationship between the amount of cholesterol esters present, and progestational secretory activity of the tissue was noticed early in these studies (Bloor et al., 1930), and the concept that the sterol esters were stored precursors for ovarian steroidogenesis was proposed (Everett, 1945; Conn et al., 1950; Deane 1958); in support of this latter hypothesis it was established that luteinizing hormone induces the depletion of ovarian cholesterol stores (Levin and Jailer, 1948; Claesson et al., 1947a,b; Parlow, 1961; Bell et al., 1964 Armstrong et al., 1964; Herbst, 1967 and Armstrong, 1968). An analogous situation to that described above exists in the adrenal cortex (Sayers et al., 1944). In this tissue it was demonstrated by in vitro experiments that cholesterol esters could act as steroid hormone precursors provided that the enzyme cholesteryl esterase (sterol ester hydrolase, EC 3.1.1.13.) was present (Dailey et al., 1963). There is a considerable amount of evidence to support the occurrence of this latter enzyme in corpus luteum (Coutts and Stansfield, 1967,

1968) as well as in adrenal tissue. (Dailey et al., 1963; Brot et al., 1963; Shyamala et al., 1966)

More recently a direct effect of LH on cholesterol esterase (sterol ester hydrolase) has been demonstrated (Behrman and Armstrong, 1969). This effect of LH (or ACTH) is insensitive to protein synthesis inhibitors, in contrast to effects of LH on the rate of conversion of cholesterol to pregnenolone (Davis and Garren, 1966).

(iv) via control of oxygen availability:

Many of the reactions involved in the biosynthesis of cholesterol (Popjak and Cornforth, 1960) and in the conversion of cholesterol to steroid hormones are mixed function oxidations (Mason, 1957, 1965) and have a specific requirement for molecular oxygen (Hayano et al., 1956). At times of acute stimulation of steroid secretion an increased demand for oxygen will therefore arise. It is probable that this is related to the very rapid increase in adrenal or ovarian blood flow that follows the administration of ACTH or LH, (Sapirstein and Goldman, 1959) leading to quite marked hyperaemia in such situations (Maier and Staehlin, 1968).

An attractive, though as yet rather speculative hypothesis, concerning ACTH induced adrenal hyperaemia, has been proposed by Grant (1968, 1970): it is suggested that ACTH causes hydrolysis of adrenocortical cholesterol esters, and the fatty acids thus released, many of which are polyunsaturated in character (i.e. linoleic, linolenic,

arachidonic), are converted to prostaglandins. The prostaglandins then mediate the subsequent actions of ACTH on corticosteroidogenesis: the muscles of the sphincter-like cuffs which control blood flow through the adrenal arterioles, under the influence of these prostaglandins become rapidly dilated, leading to a greatly increased adrenocortical blood flow. It is proposed that this enhanced supply of oxygen stimulates the rate-limiting mixed function oxidase concerned with cholesterol side-chain cleavage, thus leading to increased corticosteroidogenesis.

One of the effects of gonadotrophic stimulation of ovarian tissue is an increase in mitochondrial cytochromes. In particular, the amount of cytochrome P450 is apparently almost double that seen in unstimulated preparations (Cooper and Thomas, 1970). The increased levels of this oxygen binding cytochrome, which is known to be associated with cholesterol side-chain cleavage activity (see following section 1.4), might result in greater rates of steroidogenesis being attained.

(v) via ascorbate depletion:

The concentrations of ascorbic acid in the adrenal cortex, corpus luteum, and pituitary are much greater than in other tissues of the body (Ravavenstein, 1943-1945). It was observed several years ago (Sayers et al., 1944) that the concentration of adrenal ascorbate decreased after ACTH administration, and later a similar

finding was made concerning the action of LH on the ovary (Claesson et al., 1949; Hokfelt, 1950). Although the depletion of adrenal or ovarian ascorbate formed the basis of bioassays for ACTH (Sayers et al., 1948) and LH (Parlow, 1958), the role of this process in the mechanism of action of these trophic hormones is still unclear.

Ascorbic acid was demonstrated to have an inhibitory effect on cholesterol side-chain cleavage (Hayano et al., 1956), and 21-hydroxylation (Cooper and Rosenthal, 1962) in adrenal tissue. Kitabachi (1967a) confirmed the inhibitory effect of ascorbate on adrenal 11β - and 21-hydroxylation, and the same author later (1967b) put forward a hypothesis concerning the role of ascorbate in the mechanism of action of ACTH: it was proposed that the high levels of ascorbate found in the "quiescent" adrenal inhibited corticosteroidogenesis by inhibiting the steroid hydroxylase systems; the removal of this inhibition was achieved by the ACTH-induced depletion of adrenal ascorbate. A similar inhibitory effect of ascorbic acid on cholesterol side-chain cleavage activity has been demonstrated in ovarian enzyme preparations (Sulimovici and Boyd, 1968b).

A more direct role of adrenal ascorbate in the stimulation of corticosteroidogenesis has been suggested by Harding et al., (1968). Using bovine adrenocortical mitochondria they demonstrated that ascorbate could supply

reducing power for steroid hydroxylation, providing tetramethylphenylene diamine (TMPD) was present, via an energy-dependent electron transfer pathway. They postulated that, in vivo, ACTH might stimulate steroid biosynthesis through an action which makes ascorbate available to the electron carriers of the mitochondrial respiratory chain.

In conclusion it may be stated that there are several current theories concerning the mechanism of action of the steroidogenic trophic hormones. No single theory has been accepted unequivocally; indeed, the general concept of a "unitary" mechanism of action of LH, or ACTH, from which all the observed effects of the hormone arise, now seems less likely. The trophic hormones might achieve their steroidogenic effect by modifying several intracellular processes in a concerted manner, which could involve several of the mechanisms discussed above.

1.4 The cholesterol side-chain cleavage reaction.

The cleavage of the side-chain of cholesterol, which results in the formation of a C_{21} steroid, pregnenolone, and a C_6 fragment, isocaproic aldehyde, is a reaction of great physiological importance that occurs in the steroid producing endocrine tissues - the ovary, the adrenal cortex, the testis and the placenta. Special interest has been attached to this reaction since it was demonstrated to be the rate-limiting step

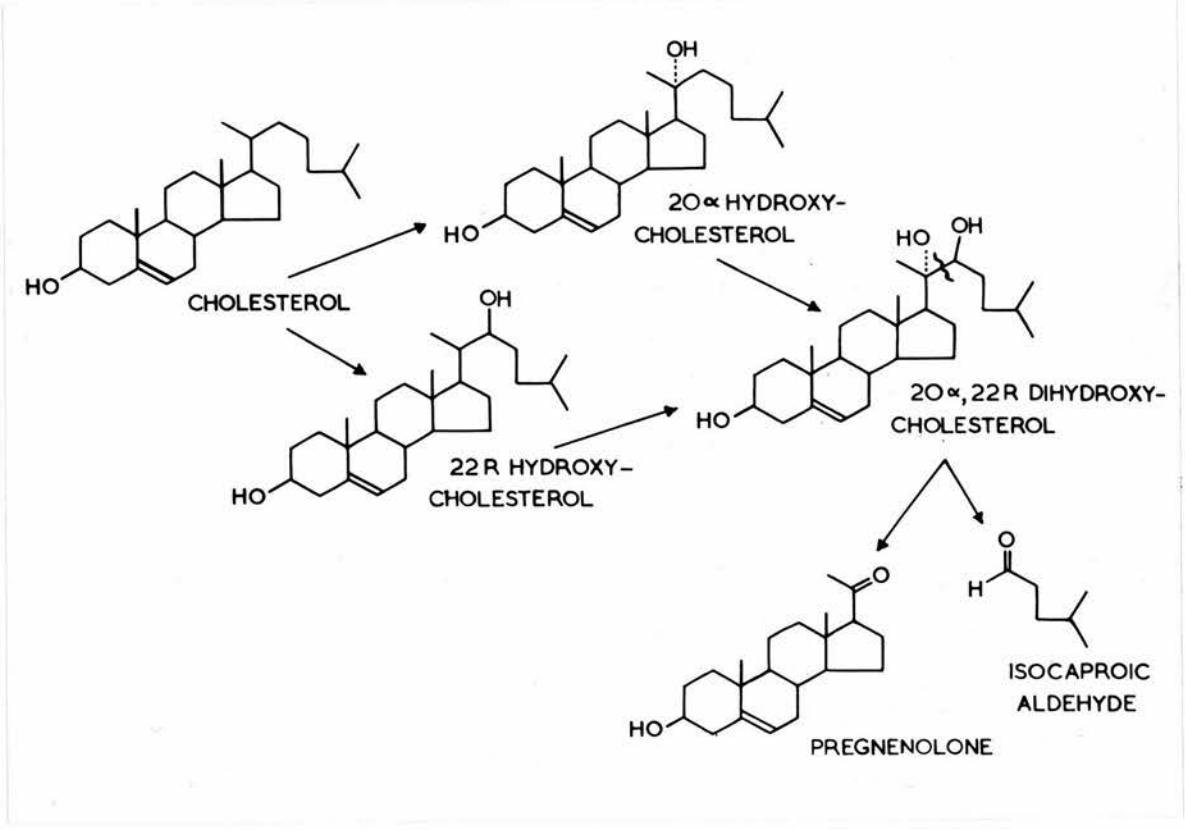


FIG. 1.3: Intermediate compounds between cholesterol and pregnenolone (after Shimizu et al., 1961, 1962 and Constantopoulos and Tchen, 1961).

in steroid biosynthesis, and one which was involved in the stimulation, by trophic hormones, of this process (Stone and Hechter, 1954).

(1.4.1) Intermediate compounds between cholesterol and pregnenolone:

The pathway most often proposed for the conversion of cholesterol to pregnenolone (Fig. 1.3) involves the formation of two hydroxylated derivatives of cholesterol, 20α-hydroxycholesterol, and 20α, 22R-dihydroxy-cholesterol (Shimizu et al., 1961; Constantopoulos and Tchen, 1961; Shimizu et al., 1962). The experimental evidence usually cited as supporting this hypothesis is that (a) the cleavage has been shown to occur between carbons 20 and 22, releasing a C_6 fragment (Staple et al., 1956; Constantopoulos et al., 1962), (b) the isolation of 20α-hydroxycholesterol, from tissue incubations supplied with (4-^{14}C) cholesterol, has been demonstrated when various trapping agents were used (Solomon et al., 1956), and (c) greater yields of pregnenolone result when 20α-hydroxycholesterol or 20α,22R-dihydroxycholesterol are used in place of cholesterol (Shimizu et al., 1961; Shimizu et al., 1962). It must be stated, however, that until recently attempts to isolate these hydroxylated intermediates of cholesterol have been unsuccessful (Koritz and Hall, 1964a; Hall and Koritz, 1964a; Simpson and Boyd, 1967a; Sulimovici and Boyd, 1968), but now Dixon et al.,

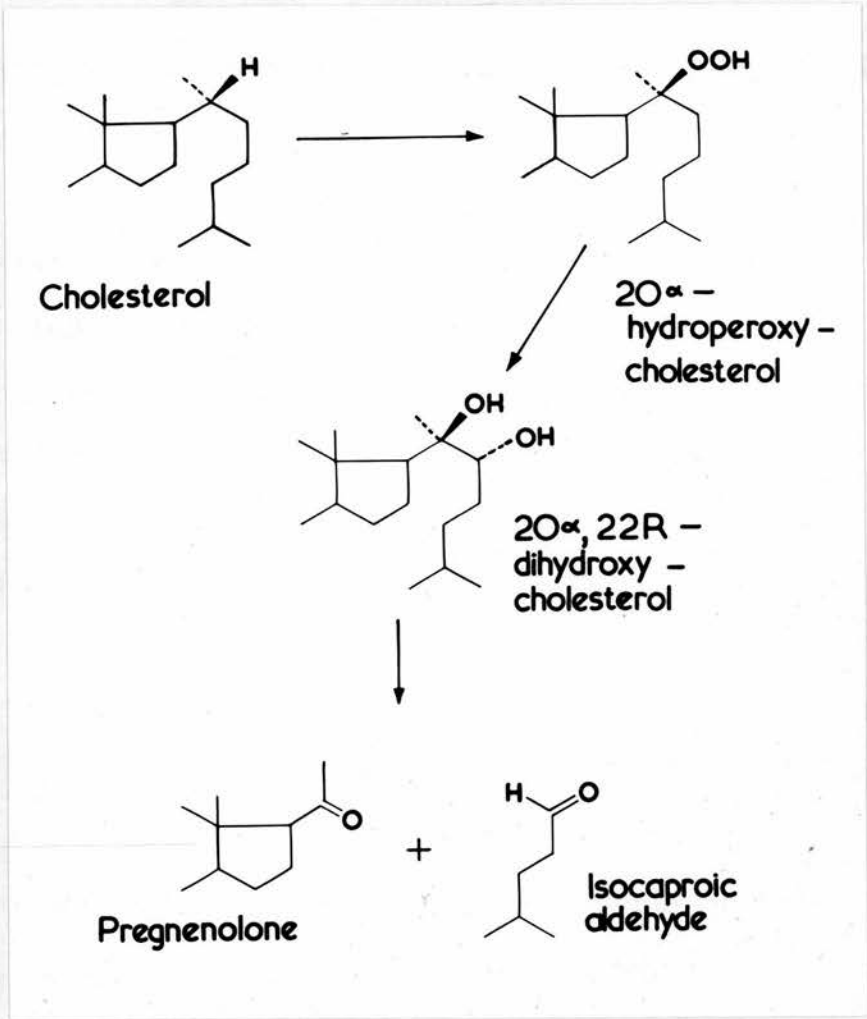


FIG. 1.4: A possible role of 20α-hydroperoxide formation in cholesterol side-chain cleavage (after van Lier and Smith, 1970a,b, and van Lier and Kan, 1971).

(1970) have isolated and crystallized 22R-hydroxycholesterol and 20α, 22R-dihydroxycholesterol from large quantities of bovine adrenals.

Recently the involvement of the 20α-hydroperoxide of cholesterol in the cholesterol side-chain cleavage process has been strongly implicated by van Lier and associates (van Lier and Smith, 1970a; van Lier and Kan, 1971): they were able to demonstrate that this compound was readily converted to 20α, 22R-dihydroxycholesterol in an anaerobic enzymic reaction by adrenal mitochondrial enzyme preparations. The triol so formed then gave rise to pregnenolone by a much slower reaction, which required oxygen and NADPH (see Fig. 1.4). The same authors have presented evidence of incorporation of ^{3}H from cholesterol -1,2-^{3}H into both 20α-hydroperoxy-cholesterol and 20α,22R-dihydroxycholesterol (van Lier and Smith, 1970b), and have shown that both these compounds could reduce the formation of [^{3}H-pregnenolone from [^{3}H-cholesterol.

(1.4.2) The involvement of cytochrome P450 in the cholesterol side-chain cleavage reaction:

Omura and Sato (1964,a,b) presented data on a carbon monoxide-binding pigment, which was a haemoprotein possessing a protohaem prosthetic group, and which they designated cytochrome P450. The studies of Estabrook et al,(1963) and Omura et al., (1965)

demonstrated that this cytochrome was involved in the NADPH requiring hydroxylation reactions of liver microsome and adrenal microsome and mitochondrial preparations.

Cholesterol side-chain cleavage activity of adrenocortical mitochondria has been shown to be inhibited by carbon monoxide (Simpson and Boyd, 1966); this inhibition could be released by light, and the photochemical action spectrum for this light reversal appeared similar to the optical spectrum of cytochrome P450 (Simpson and Boyd, 1967a), with a maximum at 450nm. The involvement of cytochrome P450 in cholesterol side-chain cleavage activity of placental mitochondrial preparations has also been demonstrated in a similar manner (Mason, 1970).

(1.4.3) Components of the cholesterol side-chain cleavage multi-enzyme:

Most mammalian steroid mixed function oxidases are associated with membranes within the cell, being located usually in the mitochondrial or microsomal subcellular compartments. The particulate nature of these enzymes made their solubilization and purification difficult to achieve. Omura et al., (1966) reported a fractionation of the adrenal mitochondrial steroid 11β-hydroxylase into three components: they comprised a flavoprotein, an iron-sulphur protein, and cytochrome P450. More recently, Boyd and co-workers have

accomplished a partial purification of the multi-enzyme associated with cholesterol side-chain cleavage in bovine adrenocortical tissue (Simpson and Boyd, 1967b) in rat ovary (Sulimovici and Boyd, 1968b) and in human placenta (Mason and Boyd, 1971): the enzyme appeared similar in all three tissues in that it could be resolved into a NADPH diaphorase, an iron-sulphur protein and cytochrome P450. Furthermore, the adrenal mitochondrial cytochrome P450 associated with cholesterol side-chain cleavage has been separated, by the same group of investigators, from the cytochrome P450 associated with 11β-hydroxylase activity (Jefcoate et al., 1970). The former fraction was isolated as a high spin form of the oxidised cytochrome (λ max. 395nm) whereas the latter was isolated as a low spin form of the oxidised cytochrome (λ max. 416nm).

1.5 Sources of electrons for mitochondrial steroid hydroxylation reactions.

The absolute requirement of most mammalian mixed function oxidases for NADPH as an electron donor is well established (Sweat and Lipscomb, 1955; Ryan and Engel, 1957; Halkerston et al., 1961). However, the physiological respiratory substrates responsible for reducing the intramitochondrial NADPH - cytochrome P450 reductase associated with these reactions are not known, and the electron transport pathways involved, are not fully understood. Most of the work done in this area

has been in relation to adrenocortical hydroxylases, especially the steroid 11β - hydroxylase reaction. That tricarboxylicacid cycle (TCA) intermediates might be involved in supporting the latter reaction in adrenocortical mitochondria was first indicated by the work of Kahnt and Wettstein (1951) and Hayano and Dorfman (1953); both groups of investigators demonstrated that fumarate, in the presence of ATP, NAD^+ and Mg^{++}, could support the 11β-hydroxylation of desoxycorticosterone. Brownie and Grant (1954) showed that several other TCA intermediates, such as citrate, α-ketoglutarate, succinate and malate were also effective in supporting this reaction. These latter authors also provided the first indications of a relationship between mitochondrial respiratory electron transport and steroid hydroxylation in the adrenal cortex: they established that when TCA intermediates were being utilized as a source of electrons for 11β-hydroxylation, the reaction could be inhibited by the respiratory uncoupling agent DNP, suggesting that the electron transfer to the steroid hydroxylase was dependent on oxidative phosphorylation. Most of the work that followed this publication was devoted to the elucidation of the route of electron flow between TCA intermediates and the mitochondrial 11β-hydroxylase. Sweat and Lipscomb (1955) suggested that the role of TCA intermediates was to reduce $NADP^+$ to NADPH, possibly via a transhydrogenase. Grant (1956) was able to

demonstrate a reduction of $NADP^+$, using acetone powder preparations of adrenocortical mitochondria, incubated in the presence of fumarate. When desoxycorticosterone was added to such incubations, the NADPH was reoxidised. Grant and Brownie (1955) showed that the oxidation of fumarate in the presence of $NADP^+$ in these adrenocortical mitochondrial preparations lead to the formation of pyruvate; they interpreted this as indicating the involvement of malic enzyme in the reduction of intramitochondrial $NADP^+$ for 11β-hydroxylation. Although at that time this possibility was considered unlikely, since malic enzyme was thought to be exclusively cytoplasmic (Rutter and Lardy, 1958), their interpretations were confirmed much later by the results of Simpson and co-workers. These authors demonstrated the existence in bovine adrenal cortex of two enzymes possessing malic enzyme activity (Simpson et al., 1968); one was present in the cytosol, the other was mitochondrial. The kinetic parameters of the cytosol and mitochondrial enzymes were shown to differ and the results suggested to the authors the possibility that a "malate shuttle" might be operative in this tissue (see equations (1) and (2)). This shuttle would enable cytoplasmic NADPH reducing equivalents to be transferred across the mitochondrial membranes via malate (Simpson and Estabrook, 1968):-

$$\text{Pyruvate} + \text{NADPH} + HCO_3^- \xrightarrow{\text{cytosol}} \text{Malate} + NADP^+ \quad (1)$$

$$\text{Malate} + NADP^+ \xrightarrow{\text{mitochondrion}} \text{pyruvate} + \text{NADPH} + HCO_3^- \quad (2)$$

The source of cytoplasmic NADPH and bicarbonate was suggested to be the pentose phosphate cycle. Although such a role of malic enzyme activity seems established for bovine adrenal cortex (Simpson et al., 1969; Simpson and Estabrook, 1969), its application to other species is less certain. It has been shown that rat adrenal mitochondria possess an active $NADP^+$-linked isocitrate dehydrogenase (Sauer and Mulrow, 1969), which can support steroid hydroxylation by a non-energy dependent pathway; in addition, an energy-dependent pathway was shown to be involved when NAD^+ reducing substrates were being utilized by these mitochondrial preparations as electron donors for steroid hydroxylases (Sauer, 1970). The presence of an energy-linked transhydrogenase which provides a connection between the respiratory electron transport chain and the 11β-hydroxylase system was demonstrated more directly in submitochondrial adrenal preparations by Oldham et al., (1968).

In these studies adrenal cortex hydroxylations, in particular the 11β-hydroxylation of deoxycorticosterone, have been examined most frequently; the supply of electrons for cholesterol side-chain cleavage, another mixed function oxidase reaction, has not been thoroughly investigated, although it has been assumed to be similar to that pertaining for the well studied 11β-hydroxylase (Simpson and Estabrook 1969; Simpson et al.,

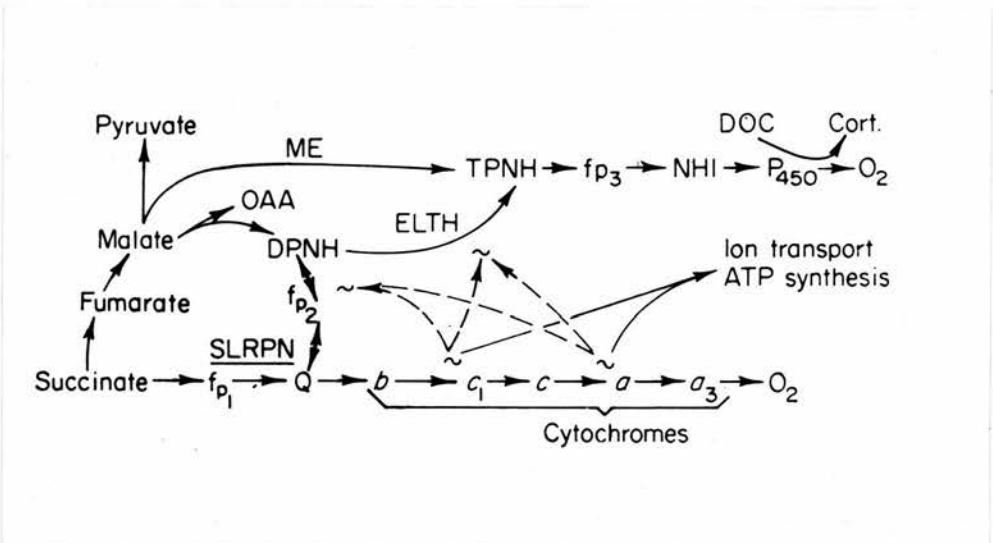


FIG. 1.5: Some postulated relationships between electron transport associated with oxidative phosphorylation and steroidogenesis in the adrenal mitochondrion. SLRPN = energy-dependent succinate-linked reduction of DPN^+; ELTH = energy-linked transhydrogenase. ME = malic enzyme. (from Cammer and Estabrook, 1967a).

1969; Harding et al., 1968). These two mitochondrial mixed function oxidase reactions do show some fundamental differences however: that of 11β- hydroxylation is relatively rapid, while the side-chain cleavage of cholesterol is at least an order of magnitude slower, and a rate limiting step in steroidogenesis. In addition, in adrenal mitochondria, it has been demonstrated that these two hydroxylases are associated with separable and specific cytochromes P450 (see section 1.4.3).

Thus in summary, the present state of knowledge suggests several sources of reducing power for the support of adrenal steroid hydroxylation, and several routes for its transfer to the mixed function oxidases concerned with this process. The scheme illustrated in Fig. 1.5 shows some of the postulated relationships between electron transport associated with oxidative phosphorylation and that associated with steroidogenesis in the adrenal mitochondrion.

1.6. Aims of the present study.

Biochemical data concerning ovarian metabolic pathways is sparse. Although some information is available which indicates that LH stimulates steroidogenesis in the corpus luteum by increasing the rate of cholesterol side-chain cleavage, little is known about how this effect might be achieved. The aim of the present study was to investigate the cholesterol side-chain cleavage reaction in relation

to the intermediary metabolism of the corpus luteum. In particular it was decided to explore sources of reducing power, and to attempt to elucidate the intramitochondrial electron transfer pathways involved in the transport of reducing equivalents from possible electron donors to the site of the cholesterol side-chain cleavage reaction. It was hoped that such an approach might provide the basis for a better understanding of the interaction of LH with luteal metabolism.

CHAPTER 2

CHAPTER 2.

EXPERIMENTAL PROCEDURE FOR ESTIMATION OF CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY.

In this chapter methods used for tissue preparation, and for the assay of cholesterol side-chain cleavage will be described; these techniques were the basis for much of the work presented in the subsequent chapters.

2.1 Tissue source.

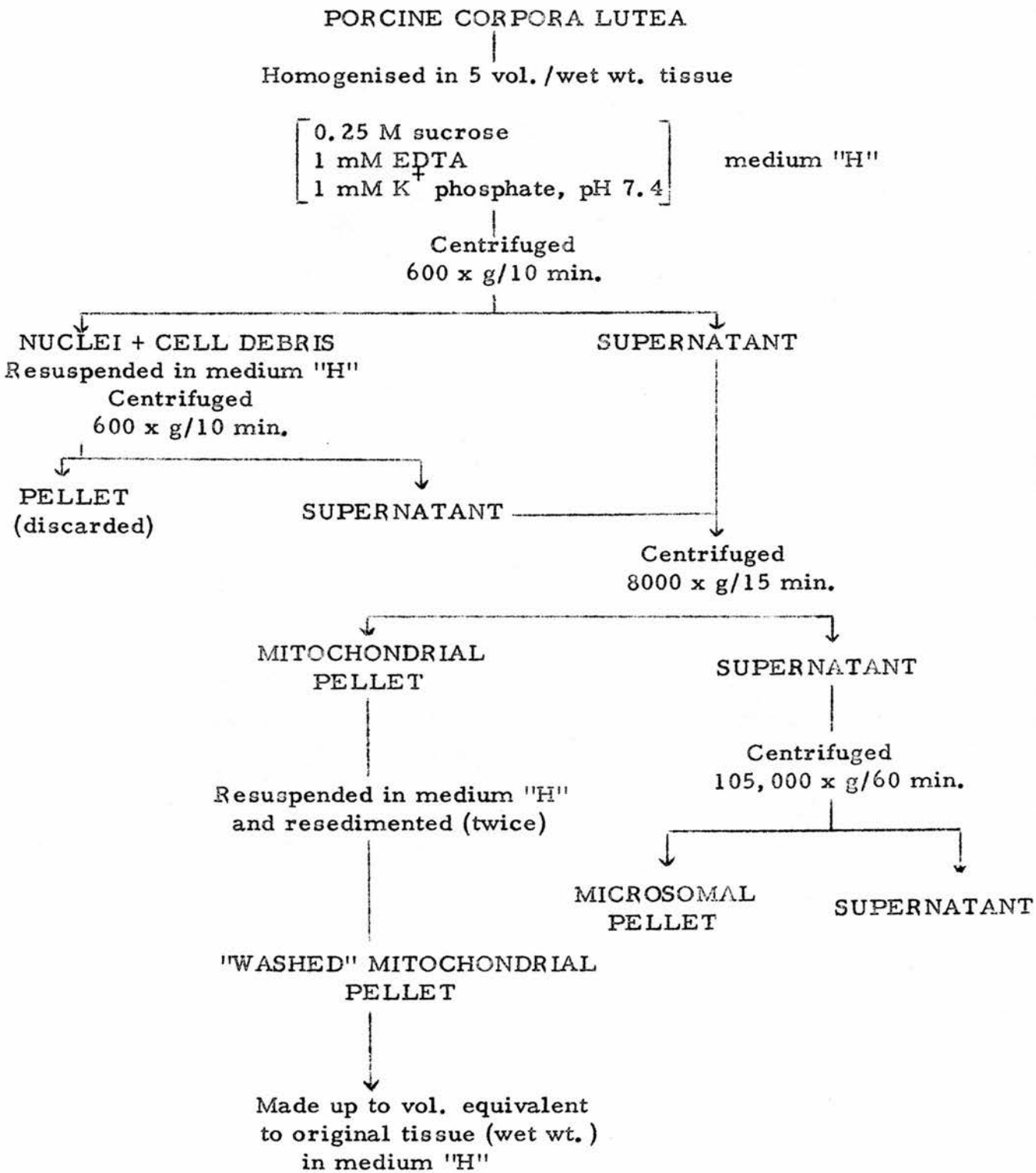
Ovarian tissue was obtained from mature female domestic pigs at slaughter. Ovaries bearing visible corpora lutea were collected within 15 minutes of death, and brought to the laboratory on ice; those which were predominantly follicular, or from pregnant animals were rejected. Whole corpora lutea were dissected out and the residual ovarian material discarded.

2.2 Preparation of luteal sub-cellular fractions.

Corpora lutea in the active secretory phase were selected on a morphological basis: ingrowth of luteinized tissue into the post-ovulatory antrum was complete, rich vascularization was evident with no signs of yellow fibrous connective tissue development characteristic of corpora albicata present. When dissected free from the ovary such corpora lutea were spherical, about 1 cm. in diameter, weighing about 500 mg. (wet wt.). Usually about 15 g. of luteal tissue was worked up for

TABLE 2.1

PREPARATION OF LUTEAL SUBCELLULAR FRACTIONS



All procedures carried out at 0 to 5°C.

one experiment, within 60 minutes of the death of the animal, the whole procedure being carried out below 5°C.

The tissue was homogenized in five volumes of 0.25 M sucrose per gm. wet wt., containing 1 mM EDTA, buffered to pH 7.4 with 1 mM potassium phosphate (medium "H"). It was homogenized by an initial gentle blending with an MSE blendor (5 secs) followed by two slow passes of a teflon pestle along a loose fitting tube into which the tissue had been transferred. This homogenate was subject to a centrifugation scheme as outlined in Table 2.1. The homogenate was centrifuged at 600 x g for 10 minutes to sediment the nuclei and cell debris. The supernatant was removed carefully with a Pasteur pipette and retained, while the pellet was gently resuspended in 5 volumes of medium "H" and centrifuged at 600 x g for a further 10 minutes. The resulting pellet was discarded, and the supernatant was combined with that of the first centrifugation, and re-centrifuged at 8000 x g for 15 minutes to sediment the mitochondrial fraction. The supernatant was removed and further centrifuged at 105,000 x g for 1 hr., yielding the microsomal (pellet) and cytosol (supernatant) fractions. The mitochondrial pellet was "washed" twice by resuspending in fresh mddium "H", resedimented, and was finally made up to a volume equivalent to the original gm wet wt. of luteal tissue in fresh medium "H". The subcellular fractionation procedure was carried out between 0 to 5°C.

TABLE 2.2

COMPOSITION OF ROUTINELY USED INCUBATION MEDIA

Medium A:

	Final molarity (mM)
Potassium phosphate buffer, pH 7.4	50
Magnesium chloride	10
*NADPH-generating system	
$NADP^+$	5
D-Glucose-6-phosphate	40
D-Glucose-6-phosphate dehydrogenase	0.2iu

*this generating system was omitted when other electron donor systems were being investigated.

Medium B:

	Final molarity (mM)
Sucrose	200
Tris-HCl buffer, pH 7.4	25
Potassium chloride	20
Potassium phosphate buffer, pH 7.4	10
Magnesium chloride	5
Tetra-sodium E.D.T.A.	0.2
Bovine serum albumin (fatty-acid free)	1% (by wt.)

2.3 Determination of cholesterol side-chain cleavage activity.

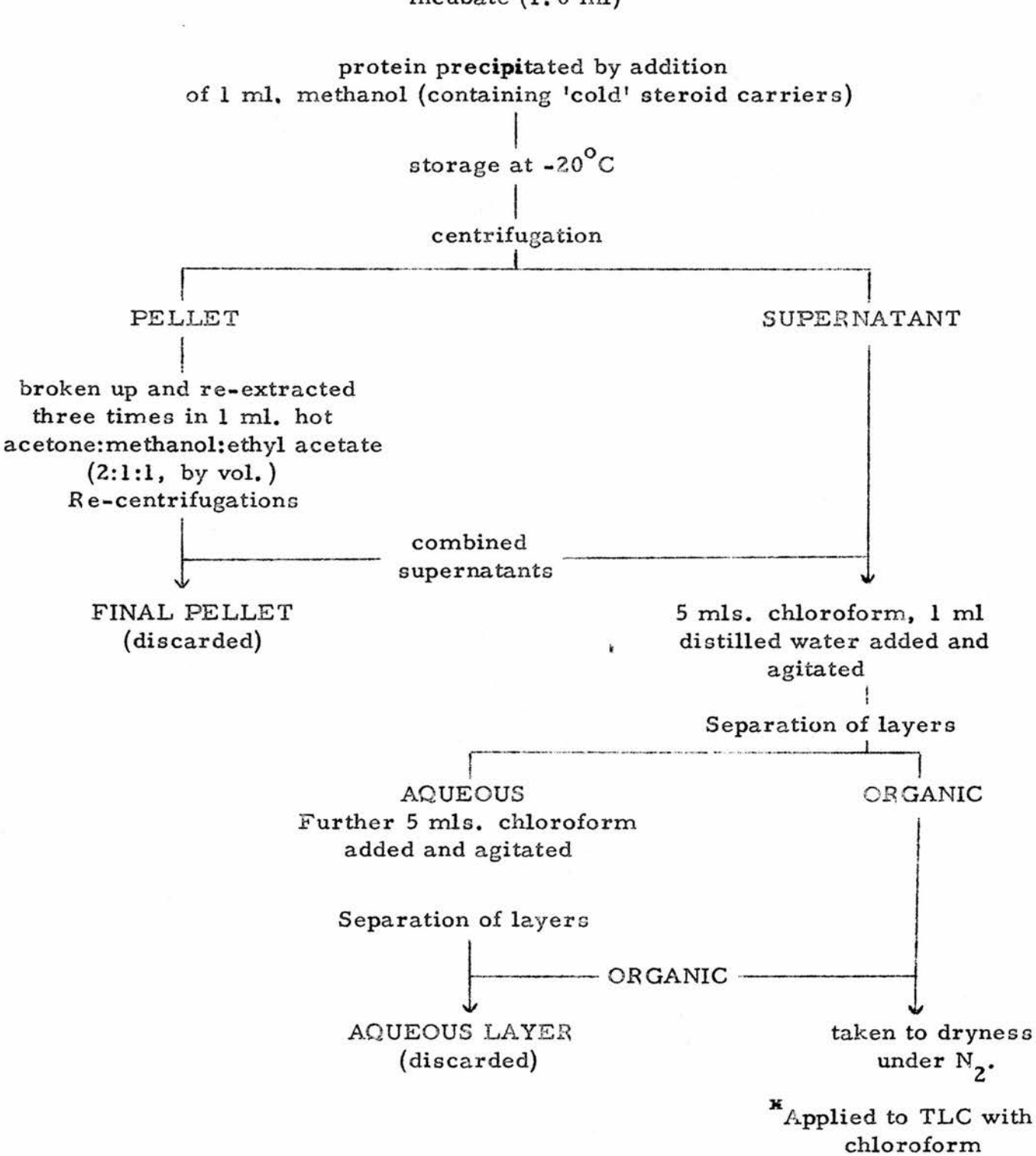
The method was based on that of Sulimovici and Boyd (1968a). The basis of the assay was the conversion of [4-^{14}C] cholesterol to [4-^{14}C] pregnenolone and [4-^{14}C] progesterone, enzyme activity being expressed as the percentage conversion of radioactive substrate to these products.

[4-^{14}C] cholesterol (58 μCi/mg.) was purchased from the Radiochemical Centre (Amersham) and aliquots were chromatographed by thin-layer chromatography on silica gel G using di-isopropyl ether:petroleum spirit (60°-80°):acetic acid (70:30:2, by vol.) together with authentic reference cholesterol. The area corresponding to cholesterol was eluted from the plate with acetone. Each batch of [4-^{14}C] cholesterol was "cleaned" in such a way immediately before use.

Incubations were carried out in medium "A" or "B" whose compositions are shown in Table 2.2, at a final volume of 1.0 ml. and a mitochondrial protein content of about 5 mg. The reaction was begun by the addition of 100,000 cpm. of [4-^{14}C] cholesterol (about 1μg) in 5 μl acetone, and carried out at 37° for 1 hr. in an atmosphere of air using a shaking water bath. Termination of the reaction was achieved by addition of 1 ml. of methanol containing 4 μg. cholesterol, 2 μg. preg-

TABLE 2.3

EXTRACTION PROCEDURE



Loss of $[^{14}C]$ steroids throughout the procedure was <4%, and occurred principally at the step shown*.

nenolone, 2 µg. progesterone, to each tube. Tubes were kept at -20°C until extraction.

The extraction procedure is outlined in Table 2.3. The dense precipitate was sedimented using a bench centrifuge (MSE minor) and the clear supernatant removed and retained. The pellet was broken up and extracted three times in 3 mls. of hot solvent (about 60°) which contained acetone:methanol:ethyl acetate (2:1:1 v/v). The final pellet was discarded, and all the supernatants were combined. 5 mls. of chloroform, and 1 ml. of distilled water were added to the combined supernatant, and the tube was agitated mechanically. The layers were separated by centrifugation, and the lower (organic) layer was removed by Pasteur pipette and retained. The remaining aqueous layer was re-extracted with 5 mls. chloroform, and centrifuged. The organic fractions were combined and taken to dryness under nitrogen.

2.4 Thin layer chromatography of incubation extract.

Thin layer plates (5 x 20 cm) precoated with silica gel G, layer thickness 0.25 mm., were purchased from E. Merck AG, Darmstadt, Germany. The extracts of the incubation were applied to the plates in chloroform (3 x 50 µl). Non-radioactive steroid standards were also applied at the origin. The thin-layer plates were developed in di-isopropyl ether:petroleum spirit

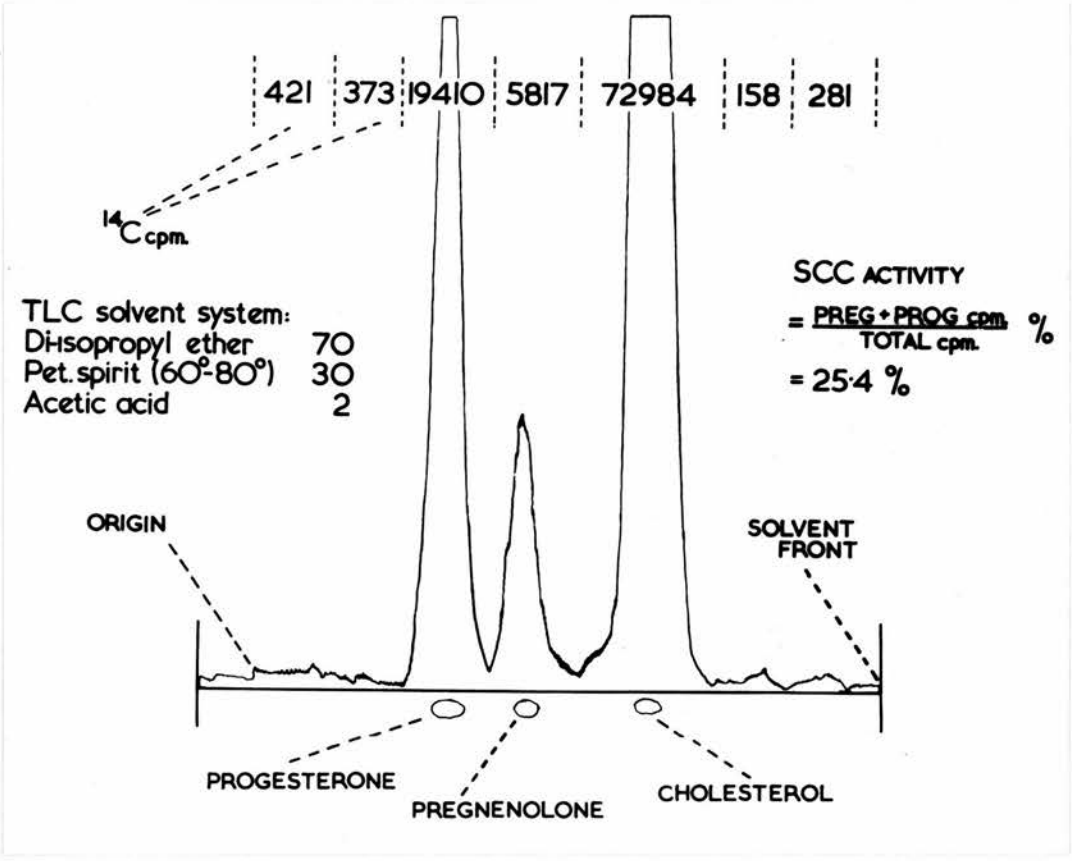


FIG. 2.1: Separation of steroids on completion of [4-^{14}C] cholesterol side-chain cleavage reaction. TLC was carried out on plates precoated with silica gel (Merck): 3 developments in the solvent system shown were carried out.

(60^{o}-80^{o}):acetic acid (70:30:2 v/v); three developments in the same direction were carried out. After chromatography the standards were located by spraying with conc. H_2SO_4/ethanol (1:1 v/v). The spray was confined to the non-radioactive areas of the plate by masking. The plates were scanned in a radiochromatogram scanner (Panax Equipment Ltd., system E.0111/P7900A) of the gas flow, windowless, Geiger-Muller type, the carrier gas mixture employed being 98% argon:2% propane. The radioactive peaks of the products corresponded to pregnenolone and progesterone (Fig. 2.1) (see also section 3.1)

2.5 Estimation of radioactivity.

Areas of the thin-layer plates corresponding to the radioactive peaks and located standard steroids, were scraped directly into scintillation vials. Scintillation fluid was prepared by dissolving 4g. 2,5-di-phenyloxazole and 30 mg. 1,4-bis-(5-phenyloxazoly-2-)-benzene in 1 litre of dry toluene containing 4% methanol. 10 mls. of scintillation fluid were added to each vial, and the radioactivity of the samples was assayed in a Packard Tri-carb liquid scintillation spectrometer, model 3375. Samples were counted until the standard deviation of the counts obtained was below 1% of the mean. The counting efficiency for the ^{14}C isotope under the conditions employed was 81%.

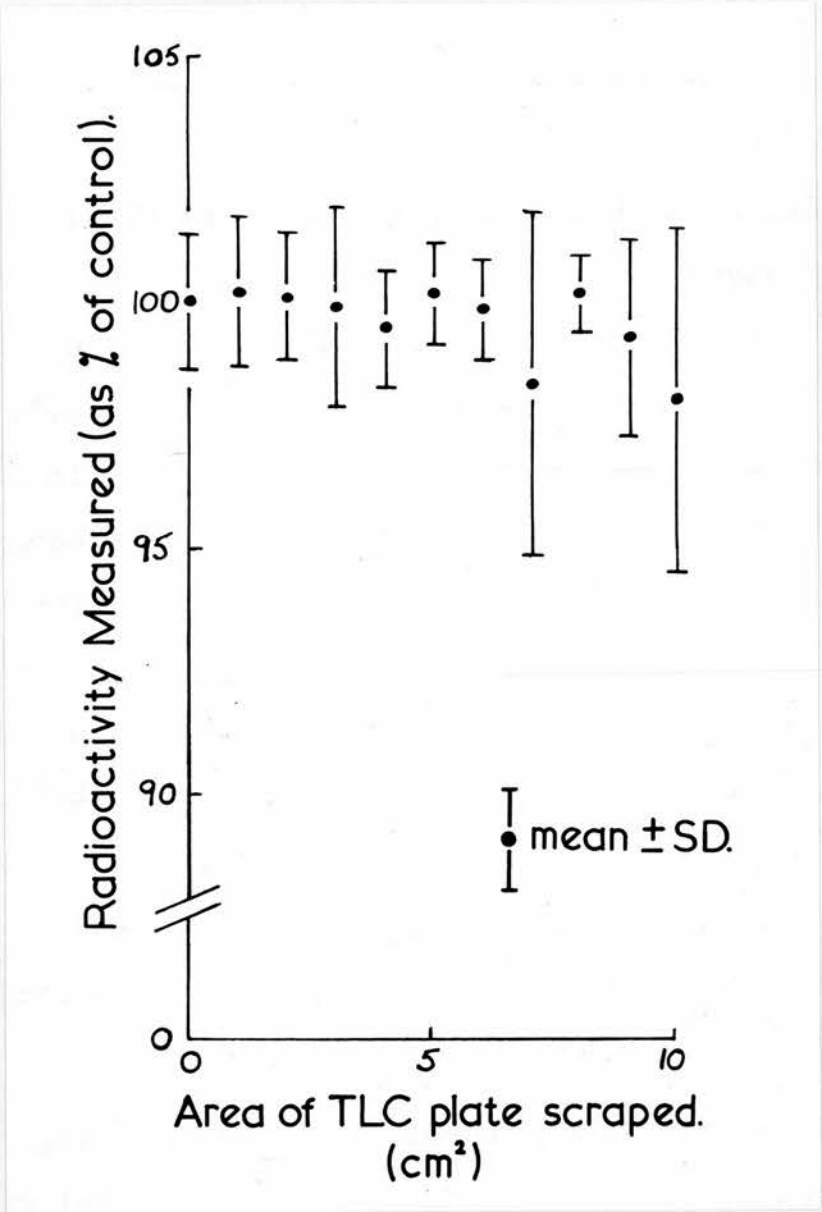


FIG. 2.2: The effect of scraping silica gel directly into scintillation vials on the estimation of radioactivity. A constant amount (about 100,000cpm) of [4-^{14}C] cholesterol was present on each area of silica gel; these areas were varied in size between 1 and 10cm^2. The values plotted (as a percentage of controls in which no silica gel was present) are the means of 5 observations for each point.

2.6. Effect of silica gel on counting efficiency.

The effect of silica gel on the estimation of radioactivity by scintillation spectrometry was investigated in two ways:

(i) Thin-layer plates were marked out in areas of increasing size, from 1cm.^{2} to 10cm.^{2}. 100,000 cpm of [4-^{14}C] cholesterol was transferred in acetone to each area. The marked areas were then scraped into separate scintillation vials and radioactivity estimated as described above. The results are shown in Fig. 2.2.

(ii) Areas of 5 cm^2 were marked out on silica gel TLC plates, and increasing amounts of [4-^{14}C] cholesterol were transferred to them. The separate areas were scraped directly into scintillation vials and radioactivity estimated as above. The results are given in Table 2.4.

In routine cholesterol side-chain cleavage assays, the area of silica gel scraped into each vial was less than 5 cm^2, and under the conditions employed this did not interfere with counting efficiency.

For estimation of radioactivity in aqueous samples, a dioxan-based scintillation fluid (type NE220, Nuclear Enterprises LTD) was used. Aqueous samples of up to 10% of the final volume of fluid in the scintillation vial could be made without appreciable quenching occurring.

2.7 Recovery of [4-^{14}C] steroids throughout experimental procedure.

TABLE 2.4

EFFECT OF SILICA GEL G ON RADIOACTIVITY COUNTED

5 cm^2 SILICA GEL G	NO SILICA GEL
c.p.m. mean ± S.D. 24 ± 4	c.p.m. mean ± S.D. 22 ± 2
7677 ± 145	7681 ± 41
11729 ± 119	11983 ±109
22966 ± 81	23193 ±321
45181 ± 375	45536 ±179
90396 ± 111	90571 ±440

T.L.C. plates were obtained from Merck (Darmstadt) pre-coated (layer thickness 0.25 mm) with silica gel G. $_2$ [4-^{14}C] cholesterol was transferred to marked areas (5 cm^2) on the plates, which were then scraped directly into scintillation vials. The mean values represent the average of 5 experiments, ± standard deviation of this mean.

To check for losses of labelled steroids throughout the experimental steps described in the previous sections, a series of three incubations was carried out. To each tube was added 80,000 cpm [4-^{14}C] cholesterol, 15,000 cpm [4-^{14}C] progesterone, and 5,000 cpm [4-^{14}C] pregnenolone. The electron donor system was omitted from the incubation to prevent side-chain cleavage taking place, but otherwise the experimental procedure was the same as that described previously. The ratio of authentic labelled steroids added approximated that routinely obtained after normal experimental incubations of [4-^{14}C] cholesterol.

Sample aliquots were taken at each extraction stage and their radioactivity estimated by scintillation counting. The final extracts were applied to TLC plates, chromatographed, scanned, scraped and counted as described previously. The results are shown in Table 2.5. 99.26% of the [4-^{14}C] steroids added to the tubes originally was accounted for; the major loss, 1.86%, was as residue in the vessels from which the final extracts were applied to TLC plates. The ratio of [4-^{14}C] steroids estimated after TLC and scintillation counting did not differ significantly from that of the steroids added at the beginning of the experiment (see Table 2.5 (b)), which indicates that when losses did occur they would not affect the calculation of the cholesterol side-chain cleavage activity.

TABLE 2.5

RECOVERY OF RADIOACTIVITY THROUGHOUT EXPERIMENTAL PROCEDURE

(a)

Radioactivity added (cpm)		% of total added cpm
[4-^{14}C] cholesterol:	88,278	79.21
[4-^{14}C] progesterone:	16,882	15.15
[4-^{14}C] pregnenolone:	6,289	5.64
Total:	111,499	100.00

Radioactivity recovered (cpm)		% of total added cpm.
In extracted pellet	109	0.10
In 1st aqueous layer	288	0.26
In 2nd aqueous layer	86	0.08
In vessel after spotting	2078	1.86
After TLC, scraping and	108052	96.95
scintillation counting	110613	99.25

(b) Radioactivity recovered from TLC plates

	cpm	% of total counts recovered from T.L.C.
Counts isolated as [4-^{14}C] cholesterol	85803	79.41
" " " [4-^{14}C] progesterone	16247	15.04
" " " [4-^{14}C] pregnenolone	6002	5.55
Total	108052	100.00

SUMMARY

1. A method for the isolation of sub-cellular fractions of luteal tissue taken from porcine ovaries was described.

2. An assay of cholesterol side-chain cleavage activity based on the conversion of [4-^{14}C] cholesterol to [4-^{14}C] steroid products was outlined.

3. The procedure for incubation of the enzyme source with the radioactive substrate, the extraction of the resulting [4-^{14}C] steroids, and their subsequent thin layer chromatogrpahy was given.

4. Methods of estimating the radioactivity involved scanningon a gas-flow thin layer radiochromatogram scanner, and scintillation spectrometry.

5. Recovery of [4-^{14}C] steroids throughout the experimental procedure was estimated: losses of ^{14}C were shown to be small (<4%) and to be unlikely to effect the estimated cholesterol side-chain cleavage activity wnen this was expressed as the percentage of [4-^{14}C] cholesterol converted to [4-^{14}C] steroid products.

CHAPTER 3

CHAPTER 3.

PRELIMINARY STUDIES ON CHOLESTEROL SIDE-CHAIN CLEAVAGE.

In this chapter, preliminary studies related to porcine luteal cholesterol side-chain cleavage are described. Some basic characteristics of this reaction were established: these included identification of the major steroid products, the effect of varying the radioactive substrate concentration, the pH profile and a time course of [4-^{14}C] metabolite formation. In addition some initial findings were made which stimulated the further studies described in this thesis: (a) it was demonstrated that succinate and malate were efficient electron donors for porcine luteal cholesterol side-chain cleavage activity, and (b) studies with some BSA preparations indicated that fatty acids might be involved in supporting this reaction.

3.1 Identification of products of the cholesterol side-chain cleavage reaction.

(3.1.1.) by thin layer chromatography (TLC):

Porcine luteal mitochondrial preparations were assayed for cholesterol side-chain cleavage activity as described in Chapter 2. The extract of an incubation was chromatographed in solvent system di-isopropyl ether:petroleum spirit (60^{o}-80^{o}):acetic acid (70:30:2 v/v) along with standard cholesterol, progesterone and pregnenolone. Subsequent scanning of

the plates in the radiochromatogram scanner showed three radioactive peaks which corresponded to the cholesterol, progesterone and pregnenolone standards (see Fig. 2.1). The area of the plate corresponding to progesterone and pregnenolone was eluted with acetone and the extract concentrated under nitrogen. Aliquots of this extract and progesterone and pregnenolone standards were applied to fresh TLC plates; development was carried out in either (a) chloroform:methanol (99:1, v/v) or (b) benzene:ethanol (19:1, v/v) or (c) chloroform: ethyl acetate (8:2, v/v). In all cases the radioactive material behaved as two fractions which corresponded, in terms of chromatographic mobility, with progesterone and pregnenolone.

(3.1.2) by combined gas chromatography-mass spectrometry:*

Cholesterol side-chain cleavage activity of porcine luteal mitochondria preparations was assayed as described in Chapter 2; in this case, however, 1 µg of standard cholesterol was used in place of the usual radioactive substrate. The final chloroform extract of each incubation was taken to dryness under nitrogen and

* The combined gas chromatograph-mass spectrometer was operated by Dr. Kelly (C.E.U.), who also instructed me in the interpretation of the data obtained.

re-dissolved 100 µl of chloroform. 20 µl aliquots were transferred to haemocrit tubes and taken to dryness in a vacuum oven (60°C). 20 µl of a 20% solution of bis-trimethyl-silyl acetamide in acetone, was added and the tubes sealed and left overnight to allow formation of trimethylsilyl ether (TMS-) derivatives. A tube containing standard cholesterol, progesterone and pregnenolone was subject to the same procedure.

Small aliquots (2 to 4µl) of the TMS-derivatives together with 1 µg of eicosane and tetracosane (C_{20} and C_{24} paraffins, respectively, for use as internal standards) were analysed by gas chromatography. A pye gas chromatograph, model 106, which included an automatic solid injecting system, was used. The glass column (150 cm. x 0.4 cm. internal diameter) contained 1% dexsil on Gas Chrom. Q (Field Instrument Co., Richmond). The operation temperature was 265°C with the flash heater at 270°C. The carrier gas (helium) flow rate was 20 mls./min. Detection was by means of a coupled mass spectrometer (A.E.I. Ltd. model MS12, fitted with a Watson B separator) which was set up for "tuned ion" analysis: output was via a multi-pen chart recorder, which monitored (i) total ion current (ii) ion mass 124 and (iii) ion mass 129. These latter two ions were selected because they are prominent in the mass spectra of cholesterol-TMS, pregnenolone-TMS (both give strong 129 peaks) (see Fig. 3.2 and 3.4) and progesterone

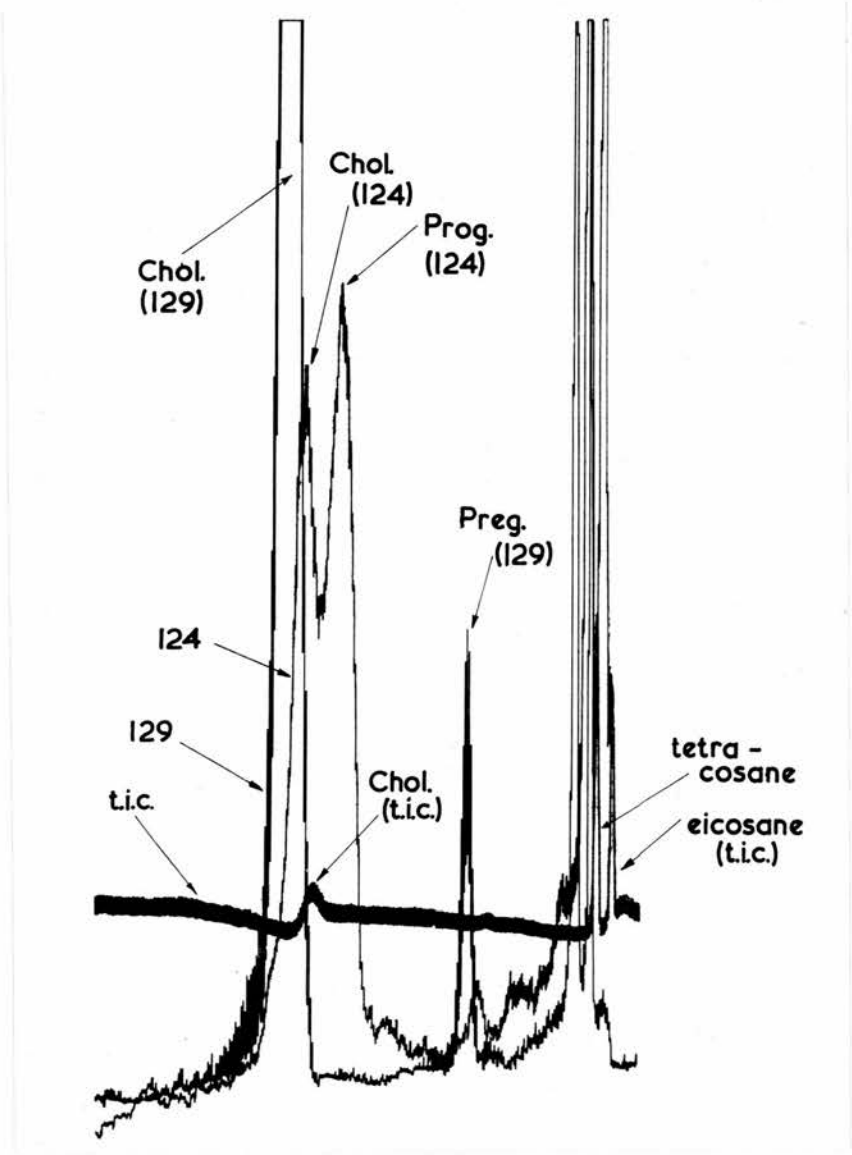


FIG. 3.1: The identification of steroid metabolites by gas chromatography-mass spectrometry. The trace is taken from a 3-pen chart recorder. (t.i.c) = total ion current; (124) is the ion chosen to detect progesterone, (129) is the ion chosen to detect pregnenolone and cholesterol. Time in this figure procedes from right to left.

(124 peak) (see Fig 3.3). The traces obtained from such a procedure are shown in Fig. 3.1. Retention times of the compounds were measured from the first paraffin (eicosane) peak, and were compared with those obtained for the steroid-TMS standards under the same conditions. The thnee principle peaks in extracts of the incubations corresponded, in terms of relative retention time, to cholesterol (8.9 mins), progesterone (7.6 mins) and pregnenolone (4.1 mins).

(3.1.3) by mass spectra characterization:*

Mitochondrial incubations, in which 1 μg of standard cholesterol replaced the usual [4-^{14}C] isotope, were carried out as described above (3.1.2) and in the previous chapter.

The final chloroform extracts from 10 x 1 ml. incubations were combined, taken to dryness under nitrogen, and applied to a thin-layer plate (see Chapter 2) in small aliquots of chloroform; an aliquot of an extract from an analogous incubation, in which [4-^{14}C] cholesterol had been used, was also applied to the TLC plate. One development in the solvent system di-isopropyl ether: petroleum spirit (60^{o}-80^{o}):acetic acid (70:30:2, v/v) was carried out, and the plate was then scanned in the

* The combined gas chromatograph-mass spectrometer was operated by Dr. Kelly (C.E.U.), who also instructed me in the interpretation of the data obtained.

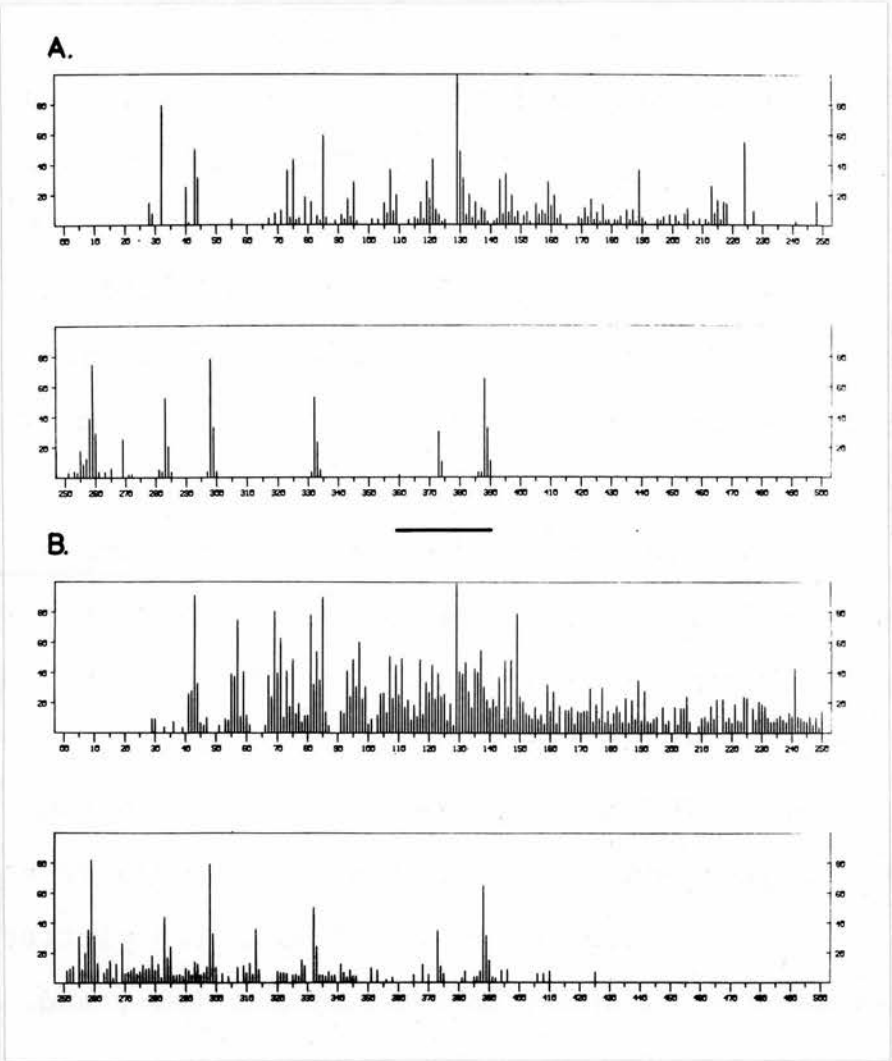


FIG. 3.2: Mass spectra of pregnenolone-TMS standard, (A) and the first major peak in the effluent from the gas chromatograph (B); in both spectra, the most abundant ion is at mass 129, and other prominent peaks common to both are at 43,85,189,259,269,283,298,332,373 and 388 (molecular ion of pregnenolone-TMS).

radiochromatograph scanner. The area of the plate that corresponded to the three radioactive peaks (e.g. see Fig 2.1) was eluted with acetone, and the extract concentrated, transferred to a haemocrit tube, and taken to dryness in a vacuum oven (60^{o}). Tri-methyl silyl-(TMS-) ether derivatives of this extract were formed, and subsequent gas chromatography performed as described above, in (3.1.2). The effluent from the gas chromatograph was applied to the adjoining mass spectrometer, which was in this case set up for four second scans, ion mass range 0-500. The total ion current monitor of the mass spectrometer was used as a means of detecting compounds emerging from the gas chromatograph column, and mass spectra were taken of each peak in this effluent. The mass spectral data for each compound was analysed and stored in a digital computer attached to the mass spectrometer, and eventually plotted out as mass spectra as shown in Figs. 3.2 , 3.3 , and 3.4. Standard cholesterol, progesterone and pregnenolone was subject to the same procedure as that outlined above, and the corresponding mass spectra obtained are also shown. The first major peak to emerge from the "unknown" extract had the same retention time as, and a mass spectrum very similar to, that of the pregnenolone-TMS standard (see Fig. 3.2.): in both spectra, the most abundant ion was at mass 129; other prominent peaks common to both were at 43,85,189,259,269,283,298,332,

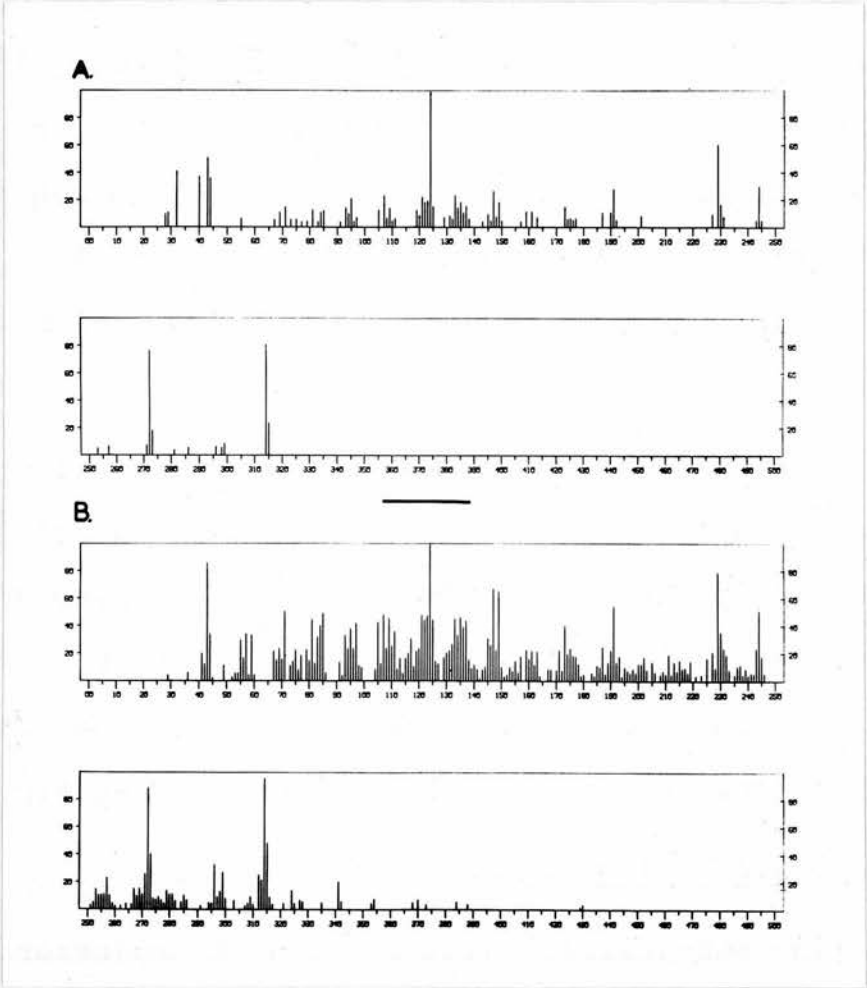


FIG. 3.3: Mass spectra of progesterone standard (A) and the second peak in the GC effluent (B); peaks common to both spectra are at 43,124 (most abundant ion), 133, 147,173,191,229,244,272 and 314 (molecular ion of progesterone).

373, and 388, this latter peak corresponding to the molecular ion of pregnenolone-TMS (molecular wt. 388).

The second "unknown" compound in the gas chromatograph effluent had similar characteristics, in terms of retention time and mass spectrum, as the progesterone standard (see Fig. 3.3): the common peaks of both spectra were at 43,124 (most abundant ion), 133,147,173,191,229,244,272 and 314 (molecular ion of progesterone).

The final, and most abundant compound in the incubation extract, behaved as the cholesterol-TMS standard in terms of chromatographic mobility and molecular fragmentation. The shared major peaks in the mass spectrum were at 32,129,329,353,368 and 458 (molecular ion of cholesterol-TMS) (see Fig. 3.4).

3.2 Distribution of the enzyme.

The sub-cellular distribution of cholesterol side-chain cleavage activity was determined in the following manner. Freshly isolated luteal tissue was homogenized and subject to differential centrifugation (see Table 2.1). Aliquots equivalent to 0.2 gm (wet weight) of original luteal tissue were taken from three sub-cellular fractions:-

(i) material sedimenting between 600-8000 x g/15 min. (twice washed) ("mitochondrial" fraction)

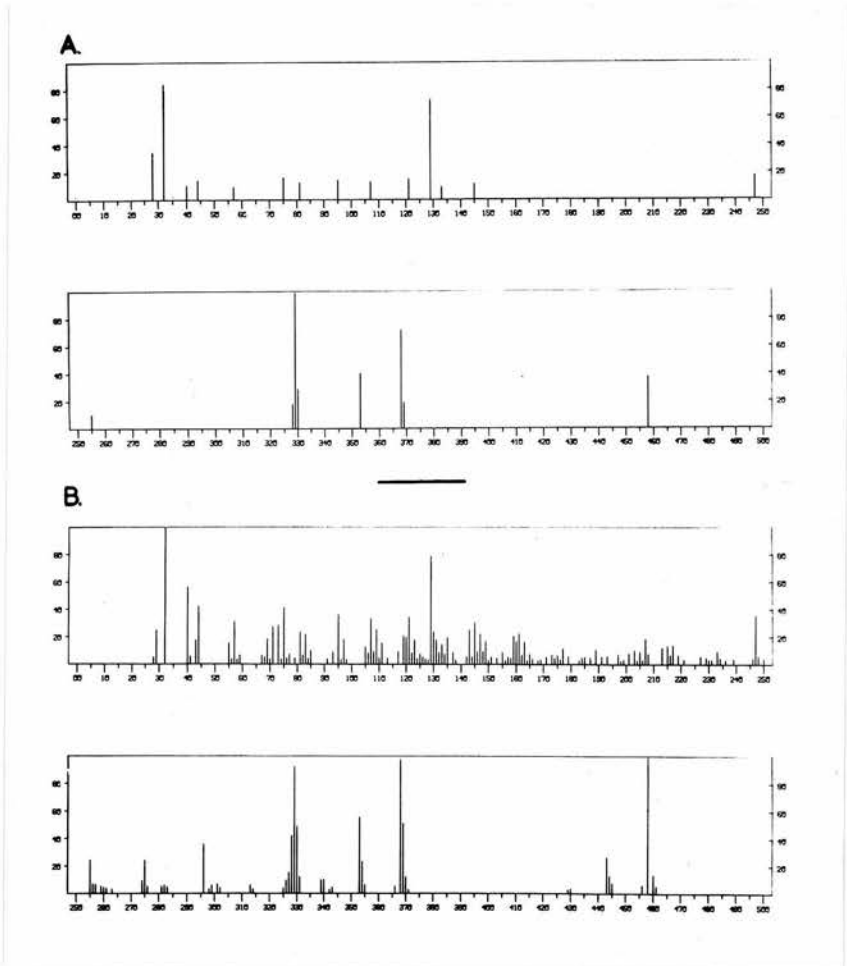


FIG. 3.4: Mass spectra of cholesterol-TMS standard (A) and the last and most abundant compound to emerge in the GC effluent (B). The common major peaks in these spectra are at 32,129,329,353,368 and 458 (molecular ion of cholesterol-TMS).

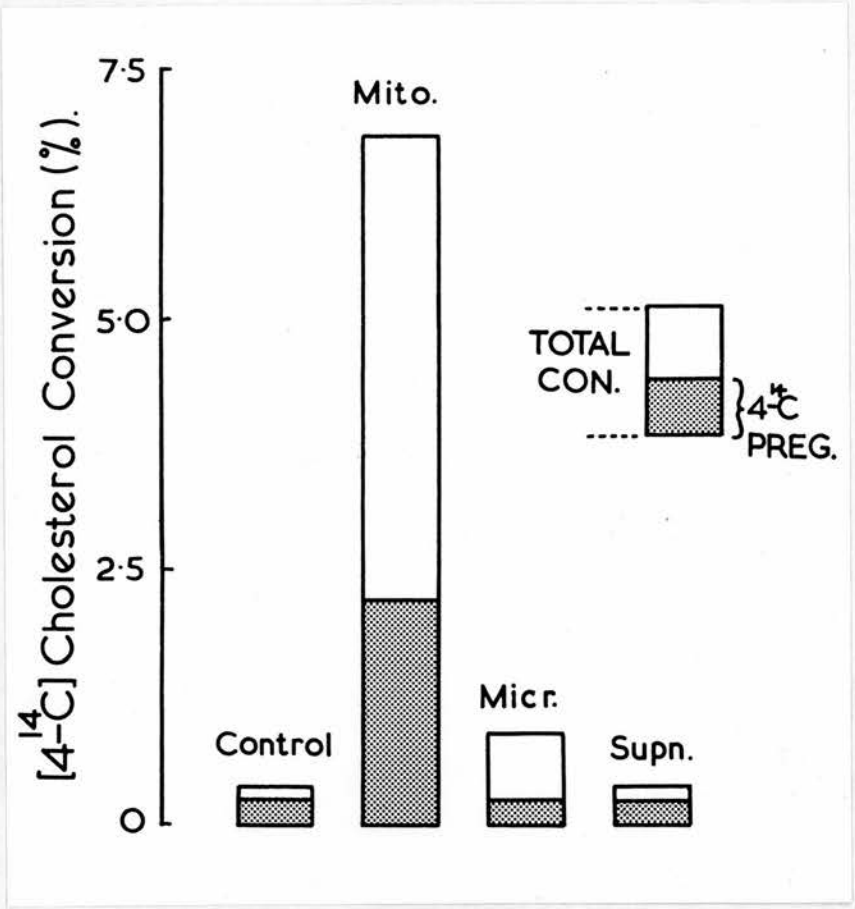


FIG. 3.5: The subcellular distribution of cholesterol side-chain cleavage activity. "Mito" = a mitochondrial fraction sedimenting between 600 to 8000xg/15min; "Micro" = a microsomal fraction, sedimenting between 8000 to 105,000xg/60 min; "Supn" is the supernatant fraction remaining after 105,000xg/60min. The aliquots from each fraction were all equivalent to 0.2gm(wet wt.) of original porcine corpus luteum.

Note: the data presented in this, and in subsequent figures, represents the results (mean of duplicates) of a typical experiment taken from a series of at least 3 in which comparable values were obtained.

(ii) material sedimenting between 8000 - 105,000 x g/1 hr. (twice washed). ("microsomal" fraction)

(iii) supernatant remaining after 105,000 x g/1 hr. ("supernatant" fraction)

These aliquots were separately incubated at 37° for an hour in medium "A" (see Table 2.2) which contained an NADPH generating system, and assayed for cholesterol side-chain cleavage activity. The results are shown in Fig. 3.5. The cholesterol side-chain cleavage activity resided predominantly in the mitochondrial fraction. A similar pattern of sub-cellular distribution has been reported for bovine corpora lutea by Hall and Koritz (1964b) and Yago et al., (1967), and for luteinized rat ovary (Sulimovici and Boyd, 1968a). Results which conflict with these, in that cholesterol side-chain cleavage activity of luteal tissue was found to be substantially present in microsomal as well as mitochondrial sub-cellular fractions, have recently been reported (Flint and Armstrong, 1971 a,b). It should be mentioned however that these authors regarded their detection of cytochrome P450 in microsomal fractions as part of the evidence for the presence of side-chain cleavage activity in this sub-cellular compartment: this does not necessarily follow, since this cytochrome is associated with several other steroid mixed function oxidase reactions,

such as the C_{20} - C_{17} desmolase and the aromatizing reactions leading to oestrogen formation.

The major [4-^{14}C] steroid product of the reaction was progesterone; pregnenolone accounted for less than 33% of the cholesterol cleaved (Fig. 3.5). In adrenal cortex mitochondria the product of cholesterol side-chain cleavage is said to be pregnenolone (Koritz, 1962): it should be noted however, that the assay method used by these authors for estimation of cholesterol side-chain cleavage activity (e.g. a modified Pettenkoffer reaction) would not detect progesterone. (see also p. 117, chapter 7.) The presence in luteal tissue of an active Δ5-3β-hydroxysteroid dehydrogenase in mitochondrial, as well as microsomal subcellular compartments, which was not due to cross-contamination of these fractions, has been demonstrated by Jackanicz and Armstrong, 1968; Sulimovici and Boyd, 1968a; Armstrong et al., 1969; Flint and Armstrong, 1971a, b.

3.3 Identification and estimation of cytochrome P450 in luteal mitochondrial preparations.

Since cholesterol side-chain cleavage activity was shown to be confined largely to mitochondrial subcellular fractions, it was of interest to examine these preparations for the presence of cytochrome P450. This cytochrome was demonstrated in

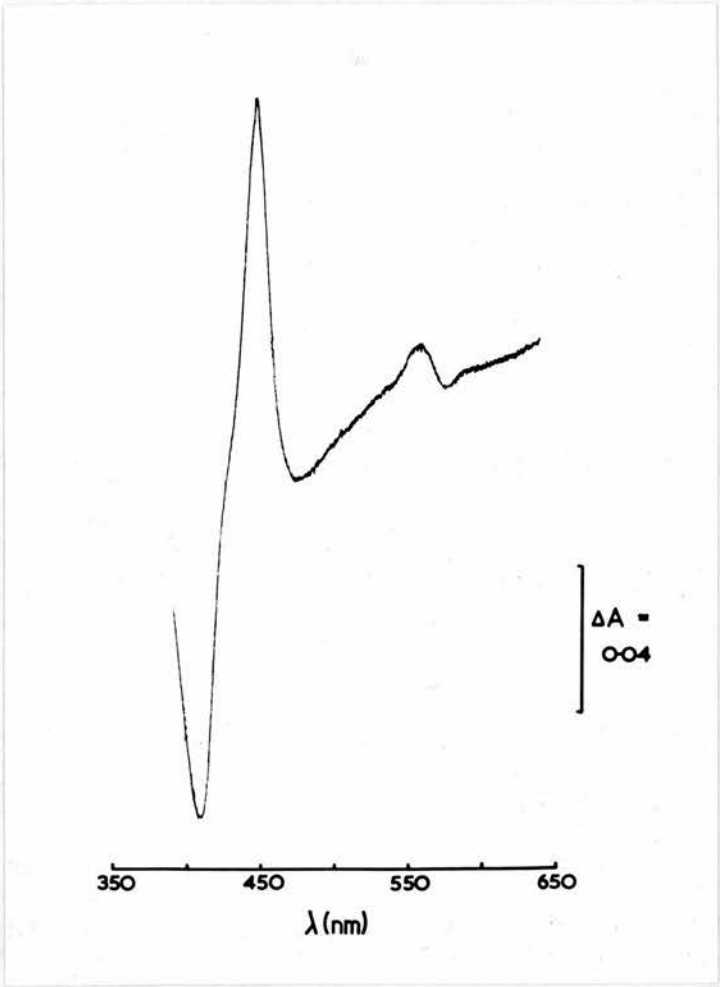


FIG. 3.6: A difference spectrum of a luteal mitochondrial preparation. Cuvette A (dithionite, CO) minus cuvette B (dithionite, anaerobic). The intense absorption at 449nm is characteristic of cytochrome P450.

preparations of intact porcine luteal mitochondria by a spectrophotometric procedure similar to that described for adrenal cortex preparations by Omura and Sato, 1964a, b.

Intact porcine luteal mitochondria were prepared as described in Chapter 2, and a mitochondrial solution containing about 3 mg protein/ml made up in medium B. Equal volumes (1.3 ml) of this solution were added to two silica cuvettes (1 cm. light path, total capacity 1.4 ml), which will be referred to as A and B, and the subsequent procedure was carried out in a Shimadzu split-beam recording spectrophotometer, model MPS-50L.

A few grains of sodium dithionite were introduced into cuvettes A and B which were then sealed and allowed to become anaerobic. Cuvette A was then gassed briefly (10 secs) with carbon monoxide. A difference spectrum was taken of A minus B. (Fig. 3.6). Since the mitochondrial respiratory cytochrome chain was reduced in both cuvettes, its components did not contribute to the difference spectrum, which was one of reduced cytochrome P450:CO complex (A) minus reduced cytochrome P450 (B). The intense absorption at about 449 nm is the characteristic from which the designation cytochrome P450 is derived. The estimation of its concentration per mg. of mitochondrial protein was based on an extinction coefficient

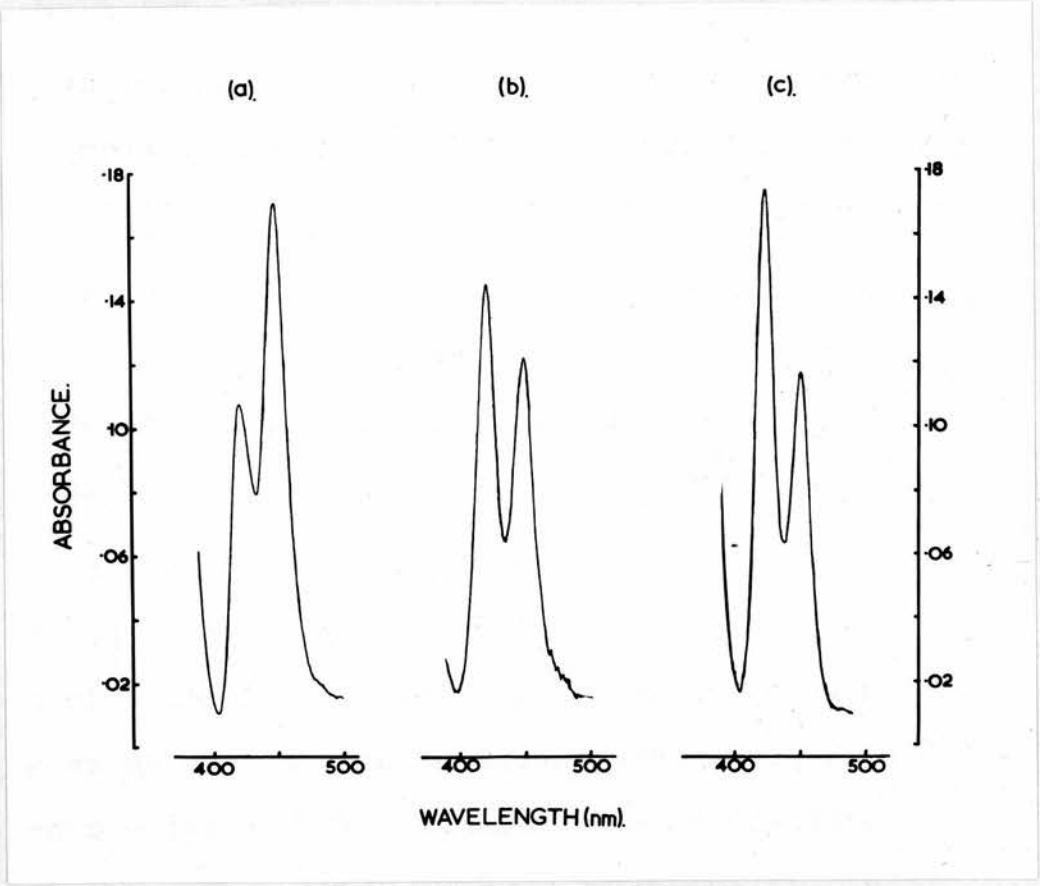


FIG. 3.7: The transformation of cytochrome P450 into cytochrome P420 in an aging mitochondrial preparation; (b) and (c) are spectra taken 4 and 8 hours respectively after that shown in (a).

determined by Omura and Sato, 1964a, b; the optical density difference between 450 and 490 nm is measured from a difference spectrum like that shown in Fig. 3.6., and the extinction coefficient is taken to be 91 (mM^{-1} cm^{-1}). The mean value derived in such experiments with porcine luteal mitochondrial preparations was found to be 0.32 n moles cytochrome P450 per mg. of mitochondrial protein.

Cytochrome P450 has also been demonstrated in microsomal preparations of rat ovary (P.M. Stevenson, personal communication); its abundance in these fractions was shown to vary with the hormonal status of the organ.

Occasionally, after following the procedures described above, carbon monoxide difference spectra like those shown in Fig. 3.7 were obtained. In these spectra a prominent absorption band at 420 nm as well as that at 450 nm was evident. The relative contribution of these two absorption peaks was seen to change as the mitochondrial preparation aged, that at 420 nm gradually becoming more prominent while that at 450 nm declined. It is probable that this change represented the modification of cytochrome P450 into another form whose occurrence was first reported by Omura and Sato (1964a, b) and which was designated cytochrome P420. A large number of agents are now known to elicit the conversion of P450 to P420: these include

lysolecithin, neutral salts (Imai and Sato, 1967), trypsin, urea (Mason et al., 1965), p-chloromercuribenzoate (Cooper et al., 1965) and organic solvents (Imai and Sato, 1967; Ichikawa and Yamano, 1967). It was noticeable that porcine luteal mitochondrial preparations in which cytochrome P420 could be demonstrated seemed to have poor integrity in terms of lower respiratory control (see section 4.8) and an inability of succinate to reduce cytochrome P450 (see section 5.6.2).

The relationship between the respiratory electron transport pathway (i.e. NADH-cytochrome a reductase) and the NADPH-cytochrome P450 reductase chain in these mitochondria is discussed in detail in Chapters 5, 6 and 7.

3.4 Some characteristics of the cholesterol side-chain cleavage reaction assayed in intact mitochondrial preparations.

(3.4.1) effect of varying the concentration of radioactive substrate, [4-^{14}C] cholesterol:

Intact luteal mitochondria were incubated in medium A, in the presence of an NADPH generating system (see Table 2.2.). The incubations were started by the addition of varying amounts of [4-^{14}C] cholesterol, in 5 µl acetone, and continued for 1 hour, at 37^{o} under an atmosphere of air. Cholesterol side-chain cleavage activity was estimated at the end of this time,

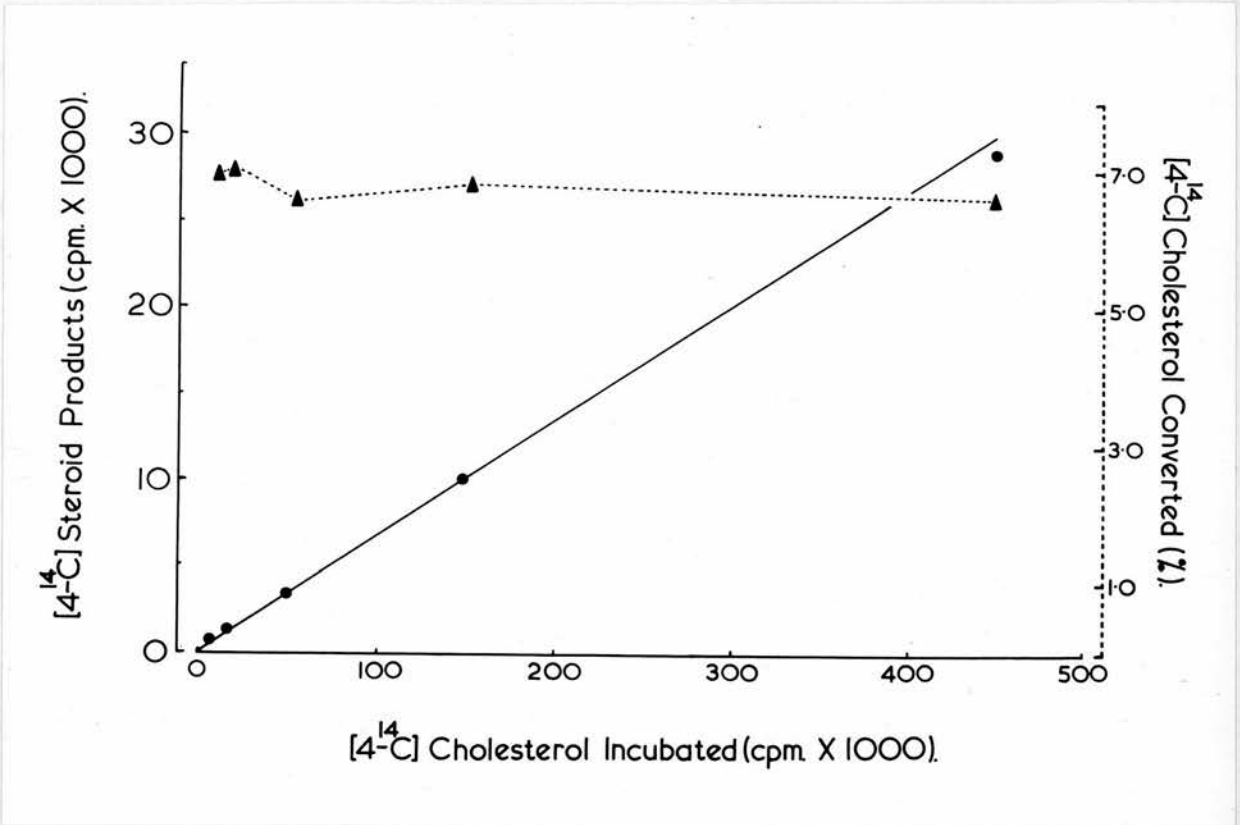


FIG. 3.8: The effect of increasing the concentration of [4-^{14}C] cholesterol on cholesterol side-chain cleavage activity. The solid line shows a linear increase in [4-^{14}C] steroid metabolites formed; the dotted line is activity expressed as the percent conversion of [4-^{14}C] cholesterol, and is seen to remain constant.

as described in Chapter 2 (sections 2.3., 2.4 and 2.5). The results of such an experiment are shown in Fig. 3.8. It can be seen that the amount of [4-^{14}C] steroid products formed was directly proportional to the radioactive substrate added over the range investigated: i.e. when the cholesterol side-chain cleavage activity was expressed as the percent conversion of [4-^{14}C] cholesterol, its value was constant under these experimental conditions. This relationship is probably a reflection of the abundant endogenous cholesterol present in these preparations: the size of the cholesterol pool was not appreciably affected by additions of up to 4 µg of exogenous [4-^{14}C] cholesterol.

(3.4.2) effect of varying pH:

Cholesterol side-chain cleavage estimations were carried out in medium A (Table 2.2) as described previously. The pH of the phosphate buffer in the incubation medium was varied between 6.4 and 8.0. The activity profile over this range, shown in Fig. 3.9., can be seen to have a fairly broad maximum, the optimum pH being about pH 7.4.

(3.4.3) time course of the reaction:

Mitochondrial incubations were carried out as described previously (section 2.3), in medium "A", being initiated by the addition of [4-^{14}C] cholesterol,

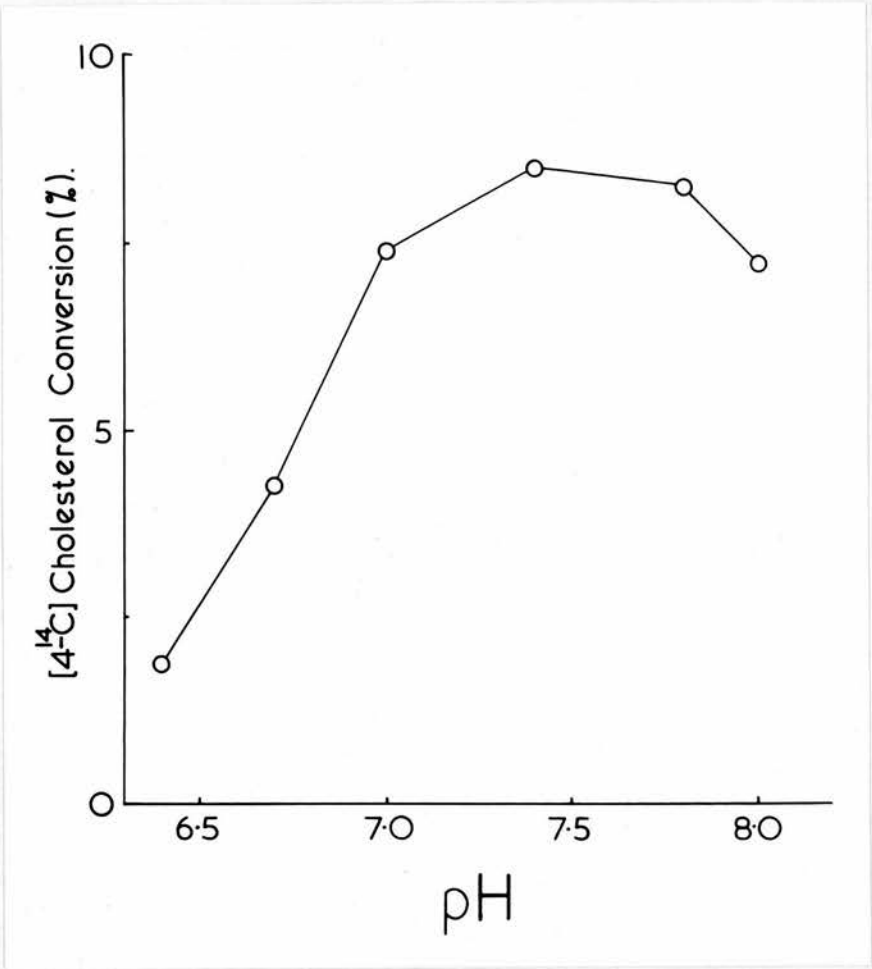


FIG. 3.9: The pH profile of cholesterol side-chain cleavage activity, in potassium phosphate buffer (50mM).

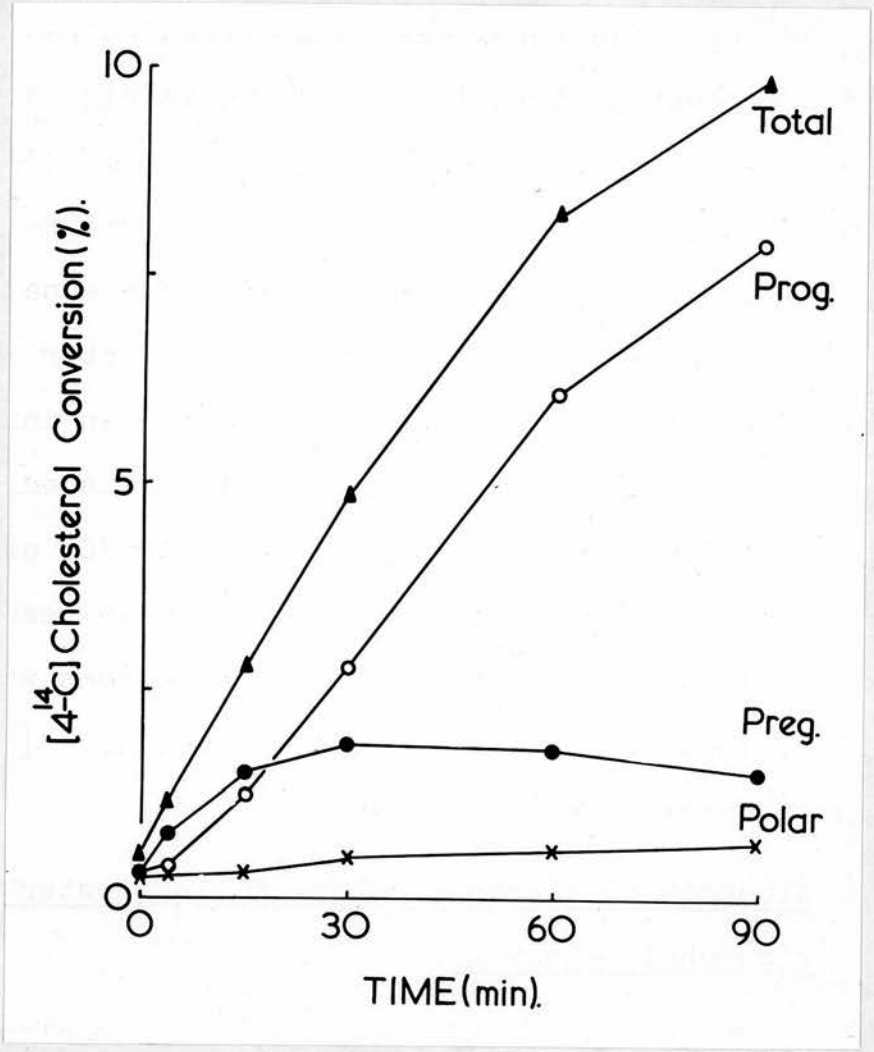


FIG. 3.10: The time course of [4-^{14}C] steroid metabolite formation.

and terminated with methanol, after incubation periods of gradually increasing time. The extent to which the [4-^{14}C] cholesterol was metabolized to radioactive products, with time, is shown in Fig. 3.10. It can be seen that the rate of cholesterol side-chain cleavage was almost linear for about 30 minutes, and thereafter gradually declined. The predominant [4-^{14}C] metabolite isolated after the shorter incubation periods was [4-^{14}C] pregnenolone; after an initial lag, however, [4-^{14}C] progesterone accumulated, and after 60 minutes accounted for more than 70% of the total [4-^{14}C] cholesterol cleaved. These results indicated that the likely order of reactions was: [4-^{14}C] cholesterol → [4-^{14}C] pregnenolone → [4-^{14}C] progesterone→→→[4-^{14}C] "polar" steroids.

3.5 Alternative electron donors for cholesterol side-chain cleavage.

The ovarian cholesterol side-chain cleavage reaction, like that in other steroidogenic tissues, is $NADP^+$ specific (Sulimovici and Boyd, 1969). The effect of NADPH in supporting cholesterol side-chain cleavage in intact porcine luteal mitochondria is shown in Fig. 3.11. Mitochondria (5 mg. protein) were incubated in medium A (see Table 2.2) at 37° for 60 minutes. The concentration of $NADP^+$ was varied, while that of G-6-P and G-6-P dehydrogenase was kept constant, at

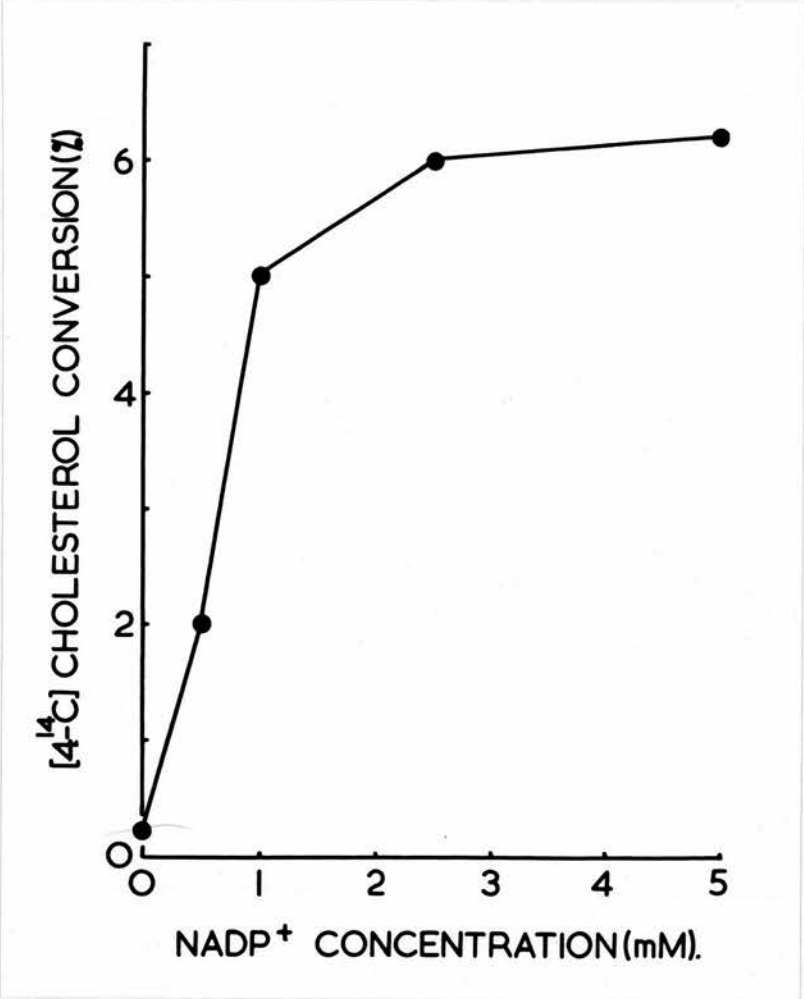


FIG. 3.11: The effect of increasing the concentration of $NADP^+$ on cholesterol side-chain cleavage activity.

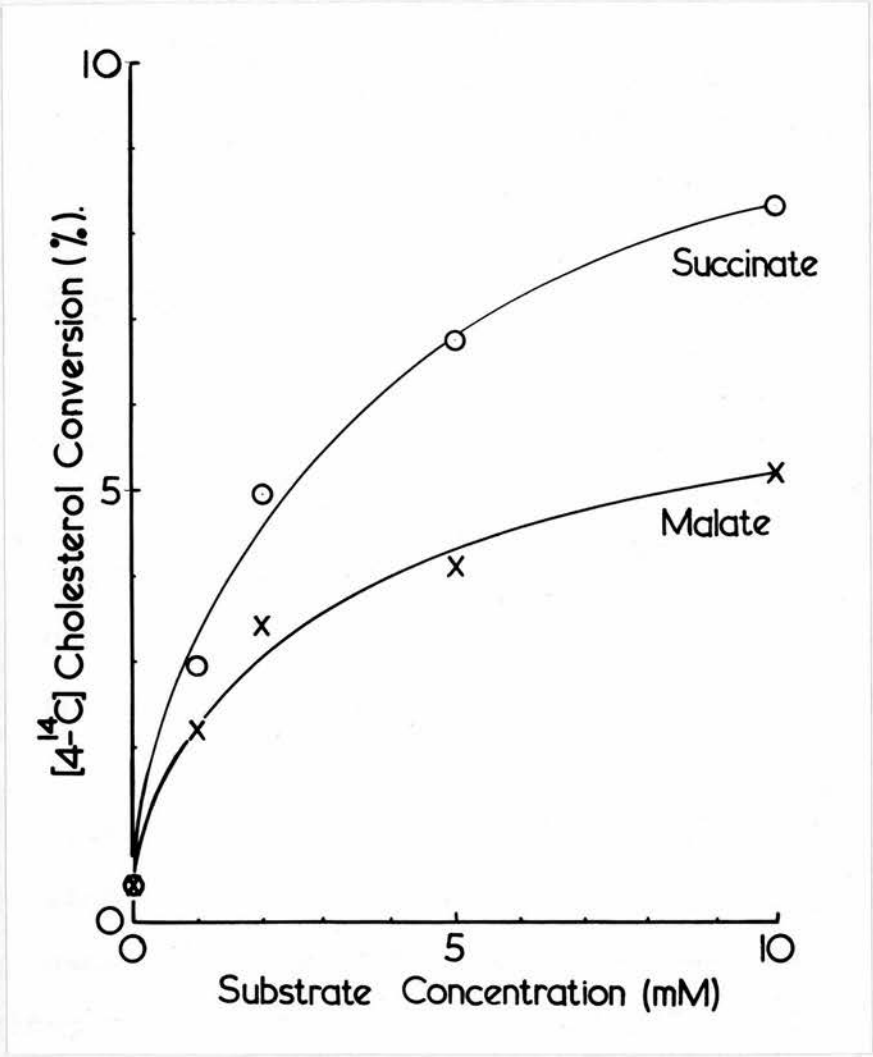


FIG. 3.12: The use of succinate or L-malate as electron donors for cholesterol side-chain cleavage activity.

40 mM and 0.2 iu respectively. Cholesterol side-chain cleavage activity increased sharply with increasing $NADP^+$ concentration up to 2.5 mM; further increase of the $NADP^+$ to 5 mM had little additional effect on cholesterol side-chain cleavage. Under these conditions [4-^{14}C] progesterone accounted for about 70% of the total [4-^{14}C] cholesterol cleaved the remainder being [4-^{14}C] pregnenolone.

The ability of substrates other than NADPH, namely, intermediates of the tricarboxylic acid cycle (TCA) to donate electrons to this system and support steroid hydroxylation in intact adrenal cortex mitochondria has been observed by several groups (Kahnt and Wettstein, 1951; Brownie and Grant, 1954; Hayano and Dorfman 1953). The effect of two TCA intermediates, succinate and malate in supporting cholesterol side-chain cleavage in intact porcine luteal mitochondria is shown in Fig. 3.12. Mitochondria were incubated in medium A (Table 2.2) in the absence of the NADPH generating system, at 37° for an hour; under these conditions succinate was a more effective electron donor than malate, and when present at 10 mM supported the cleavage of 8.2% of the [4-^{14}C] cholesterol. The efficiency of TCA intermediates in supporting cholesterol side-chain cleavage in intact porcine luteal mitochondrial preparations is examined in greater detail in Chapter 5.

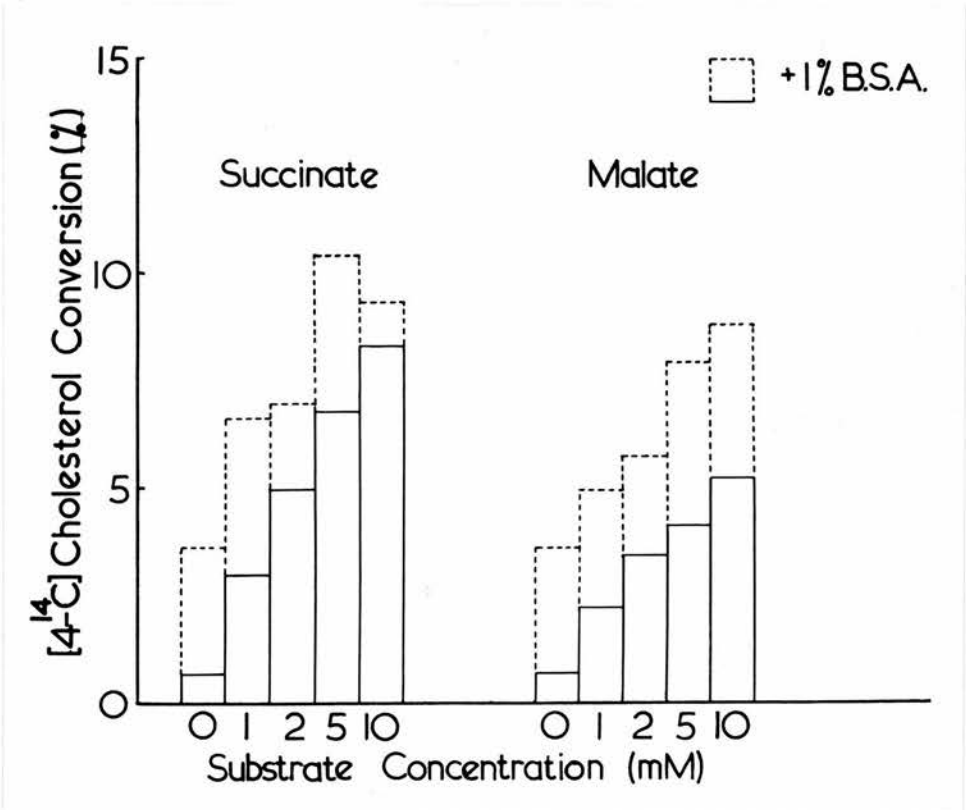


FIG. 3.13: The effect of including BSA (1%, by wt) in incubations in which succinate, or L-malate are supporting cholesterol side-chain cleavage activity. The dotted extension to the histograms shows the extent of the stimulation of the reaction in the presence of BSA.

3.6 Effect of bovine serum albumin on the reaction.

Since there have been some suggestions that TCA support for steroid hydroxylation is energy dependent (Brownie and Grant, 1954; Guerra et al., 1966; Peron et al., 1966 and Harding et al., 1965) it was decided to investigate the effect of including BSA in incubation media when estimating TCA-supported cholesterol side-chain cleavage. Davis (1967) reported that substances which can affect mitochondrial integrity and exert an uncoupling effect on oxidative phosphorylation are released when the cell is disrupted, and that these substances are absorbed by BSA. The compounds involved may be fatty acids, which are known to be potent uncoupling agents and to cause rapid mitochondrial swelling (Van den Bergh, 1967).

Intact porcine luteal mitochondria (5 mg) were incubated in medium A at 37° for 60 min; the concentration of the electron donor, succinate or malate, was varied from 0 to 10 mM, and 1% BSA (by wt) was included in the medium. A parallel series of incubations, omitting the BSA, was carried out at the same time. The results are shown in Fig. 3.13. The presence of BSA was seen to have a marked stimulatory effect on the [4-^{14}C] cholesterol conversion, giving rise in some incubations to a two-fold increase in

TABLE 3.1

EFFECT OF BSA (1% BY WT.) ON CHOLESTEROL SIDE-CHAIN CLEAVAGE SUPPORTED BY NADPH

[4-^{14}C] CHOLESTEROL CLEAVED (%)

	BSA OMITTED	BSA PRESENT
NADPH (5 mM)	6.4	4.6
$NADP^+$ (5 mM) G6P (40 mM) G6P-D (0.2 iu)	7.3	5.6
SUCCINATE (5 mM)	6.4	9.8
L-MALATE (5 mM)	3.9	7.3
NO SUBSTRATE	0.3	3.4

Incubations were carried out in buffer "A", and cholesterol side-chain cleavage activity determined as described in Chapter 2. 5 mg. of a preparation of intact porcine luteal mitochondria were present in each incubation.

cholesterol side-chain cleavage activity. BSA has been reported (Péron and McCarthy, 1968) to stimulate 11β-hydroxylation of deoxycorticosterone when succinate, malate or isocitrate were present in preparations of intact rat adrenal mitochondria; it was suggested by these authors that this was achieved by virtue of a "protective effect of BSA on the mitochondrial system, or of binding of free fatty acids which were released from the mitochondria".

In contrast to the stimulatory effect of BSA on succinate- or malate-supported cholesterol side-chain cleavage, the presence of BSA was slightly inhibitory when NADPH or an NADPH generating system was used as an electron donor for this reaction. (see Table 3.1.). Intact luteal mitochondria were incubated in medium A, with or without BSA. The electron donor was either NADPH (5 mM), $NADP^+$ (5 mM) with the usual NADPH generating system (Table 2.2.) succinate (5 mM), malate (5 mM) or omitted entirely. The results showed that cholesterol side-chain cleavage supported by either of the first two substrates was reduced in the presence of BSA, while that supported by succinate or malate was stimulated.

These results can be explained if BSA were having a protective effect on mitochondrial integrity: this might be expected to prevent ingress of NADPH, since intact mitochondria are relatively impermeable to this

cofactor, and so would result in a reduction of cholesterol side-chain cleavage activity when NADPH was the electron donor for the reaction; the penetration by succinate or malate would not be affected, since mitochondria are readily permeable to these substrates, and their utilization for cholesterol side-chain cleavage might have been enhanced if mitochondrial energy conservation reactions were protected by BSA from uncoupling.

Surprisingly an appreciable amount of [4-^{14}C] cholesterol was cleaved when BSA was incubated with the mitochondria in the absence of an added electron donor (see also Fig. 3.13.).
Two possible explanations for this finding were considered:

(1) improved mitochondrial integrity resulting from the presence of BSA might have prevented leakage of endogenous substrates capable of acting as electron donors for side-chain cleavage; or the utilization of these endogenous substrates might have been enhanced by the BSA due to better coupling of mitochondrial oxidative phosphorylation.

(2) the BSA itself was acting as an electron donor.

The mitochondria were incubated in medium A, containing BSA but no NADPH-generating system, for 15 minutes to allow depletion of endogenous substrates to occur; [4-^{14}C] cholesterol was then added and the

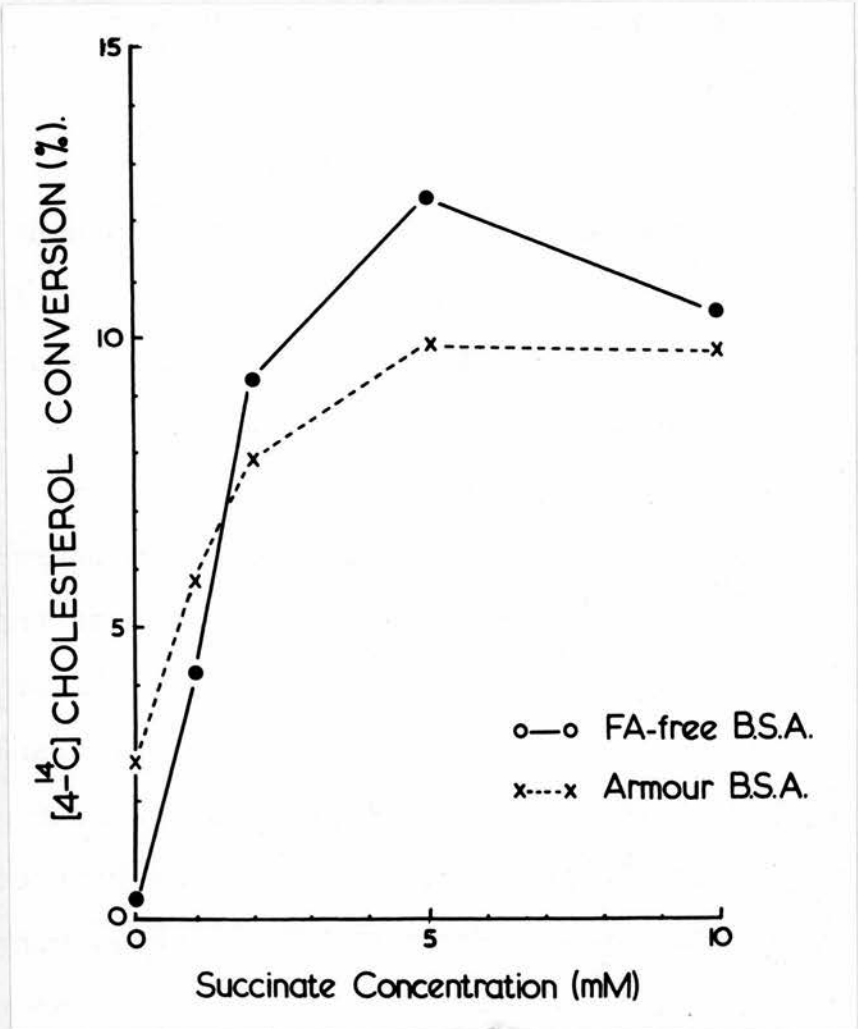


FIG. 3.14: A comparison of two grades of BSA. Cholesterol side-chain cleavage was supported by varying concentrations of succinate, in the presence of either Armour BSA (broken line) or a highly purified, fatty acid-free BSA preparation (solid line). The concentration of the BSA, in both cases, was 1% (by wt.).

incubation continued for a further hour. The amount of [4-^{14}C] cholesterol cleaved after this procedure was no less than when the "pre-incubation" was omitted.

Commercial BSA usually contains some bound fatty acids (Van den Bergh, 1967) and it was thought that these may have been a possible source of electrons for side-chain cleavage, perhaps via mitochondrial β-oxidation. This possibility was tested by comparing the BSA used previously (Armour Pharmaceutical Co., Ltd. Eastbourne) with a highly purified BSA preparation which was essentially fatty acid free (less than 0.01%, obtained from Sigma, London). The results of such a comparison, are shown in Fig. 3.14. It can be seen that when no succinate was present the Armour BSA supported appreciable cholesterol side-chain cleavage, whereas the fatty acid-free BSA supported none (0.4%). It thus seemed that of the two hypotheses considered above the second was more probable. (However, the results do not rule out the possibility that some other contaminant was responsible.) For this reason fatty acid-free BSA was routinely used in all subsequent BSA-containing media.

The results of the experiments with BSA were the first indications that fatty acids might be involved in supporting ovarian cholesterol side-chain cleavage

activity. In view of the abundance of fatty acids in this tissue, present as cytoplasmic cholesterol esters (see chapter 1, section 1.2.(iv), such a role for fatty acids has interesting physiological implications. These initial observations were later confirmed, when it was shown directly that fatty acids, or acyl carnitines could support high levels of cholesterol side-chain cleavage activity (see Chapter 6).

SUMMARY

1. The products of the cholesterol side-chain cleavage reaction assayed in intact luteal mitochondrial preparations have been identified by their chromatographic behaviour in TLC and GC systems, and further characterized by their mass spectra. Pregnenolone and progesterone were found to comprise about 95% of the metabolites of the reaction; the remaining 5%, which consisted of more polar [4-^{14}C] steroids, was not further identified.

2. The mitochondrial subcellular fraction was shown to be the principal site of cholesterol side-chain cleavage in porcine luteal tissue. This fraction was also shown to contain cytochrome P45o.

3. The effect of varying initial [4-^{14}C]substrate concentration, and pH on the reaction was investigated. A study of the time course of the reaction indicated that the relative order of formation of [4-^{14}C]steroid

metabolites was probably [4-^{14}C] cholesterol → [4-^{14}C] pregnenolone → [4-^{14}C] progesterone → → [4-^{14}C] "polar" steroids. An incubation period of 60 minutes was adopted for routine use; the rate of formation of [4-^{14}C] steroid metabolites declined with longer incubation times.

4. It was demonstrated that the TCA intermediates succinate and malate, in addition to NADPH, could act as electron donors for cholesterol side-chain cleavage in intact mitochondrial preparations.

5. The presence of BSA in incubation media was found to enhance the rate of reaction under some conditions. The relationship between this and cholesterol side-chain cleavage was investigated. The results of comparing commercial and fatty acid free preparations of BSA indicated that fatty acids might be capable of supporting the cholesterol side-chain cleavage reaction.

CHAPTER 4

CHAPTER 4.

CALIBRATION OF THE OXYGEN ELECTRODE AND ITS USE IN THE POLAROGRAPHIC DETERMINATION OF ADP:O RATIOS.

In the previous chapter results were reported which showed that some TCA intermediates could support cholesterol side-chain cleavage activity in preparations of intact porcine luteal mitochondria. It was thought possible that the donation of electrons from these substrates for the support of the reaction might involve concomitant oxidative phosphorylation; indirect support for this hypothesis had been obtained by the demonstration that BSA, a compound known to enhance the coupling of oxidative phosphorylation to mitochondrial respiration, had a stimulatory effect on cholesterol side-chain cleavage activity occurring in the presence of TCA intermediates. In this chapter mitochondrial respiration and oxidative phosphorylation supported by the same TCA intermediates are investigated directly, using a polarographic technique; the aim was to obtain further information on the relationship between two oxidases present in these mitochondrial preparations - namely the mixed function oxidase associated with cholesterol side-chain cleavage, and cytochrome oxidase concerned with mitochondrial respiration. As a prerequisite to this study, a polarographic method for measuring the oxygen content of incubation media was

developed.

4.1 Existing calibration methods and their limitations.

The oxygen electrode is now widely used in biochemistry and physiology to measure oxygen consumption rates (Lessler and Brierly, 1969). The physiochemical theory underlying the development of this instrument has been reviewed by Davis (1962); the reactions which occur at the platinum cathode are given below:

$$O_2 + 2H_2O + 2e^- \longrightarrow H_2O_2 + 2OH^-$$

$$H_2O_2 + 2e^- \longrightarrow 2OH^-$$

In order to obtain absolute values for oxygen uptake it is necessary to calibrate the instrument for each medium used in order to find the oxygen concentration corresponding to the 100% setting on the recorder. It is not always appreciated (a) that the oxygen electrode measures the activity, not the concentration of oxygen in solution and (b) that the presence of both electrolytes and non-electrolytes influences the solubility and the activity coefficient of oxygen in aqueous solutions (Chappell, 1964; Dixon and Kleppe, 1965). For these reasons equilibration of solutions with gas mixtures of known oxygen tension can only give accurate calibration if directly determined values already exist for the solubility of oxygen in the particular medium under consideration.

These solubility data are available for water, NaCl solutions and some classical Ringer solutions

(e.g. Umbriet et al., 1964; National Research Council, 1928) but are not readily available for many commonly used buffer systems. Direct measurements of oxygen solubility are therefore necessary for these solutions.

Of the existing methods for such determinations, the classical method of Winkler, (1914) is extremely tedious for routine use. The calibration method most often applied to oxygen electrode studies is that of Estabrook and Mackler (1957) where oxygen is consumed in the stoichiometric oxidation of a specified amount of NADH, catalysed by lysed mitochondria; the change in electrode current is thus equivalent to a known uptake of oxygen from solution. In this method the preparation of the mitochondria has the disadvantage of being unduly lengthy for routine use.

4.2 The development of a new calibration method.

A direct calibration method similar to that of Estabrook and Mackler (1957) was devised, in which the electron carrier N- methyl phenazonium methosulphate (PMS) was used in place of the mitochondrial preparation employed in the earlier method. The reactions involved are:-

$$PMS + NADH + H^+ \longrightarrow PMSH_2 + NAD^+ \quad (i)$$

$$PMSH_2 + O_2 \longrightarrow PMS + H_2O_2 \quad (ii)$$

Spontaneous decomposition of some hydrogen peroxide

formed in reaction (ii) releases variable amounts of oxygen back into solution, making calculations based on the stoichiometry of reactions (i) and (ii) impossible. Therefore all the hydrogen peroxide formed in reaction (ii) was broken down by including catalase in the reaction mixture.

$$H_2O_2 \xrightarrow{\text{catalase}} H_2O + \tfrac{1}{2}O_2 \qquad \text{(iii)}$$

The net reaction then became:-

$$NADH + H^+ + \tfrac{1}{2}O_2 \longrightarrow NAD^+ + H_2O \qquad \text{(iv)}$$

The apparatus used was the YSI Model 53 biological oxygen monitor (Yellow Springs Instrument Co.) which consisted essentially of a Clark electrode covered with an FEP teflon membrane and housed in a Lucite plunger. This probe was capable of insertion into a glass reaction chamber, making a closed system but for a narrow groove in the plunger, through which materials could be introduced into the chamber. The contents of the chamber were agitated continuously by magnetic stirring and maintained at a precise temperature ($\pm$ 0.1°) by means of a surrounding water bath and thermostat (Haake). The anode current from the probe was monitored using a Servoscribe recorder.

4.3 Experimental procedure.

The incubation medium, the oxygen concentration of which was to be determined, was introduced into a reaction chamber of the oxygen electrode apparatus. 20 µg PMS and 400 µg catalase (800 units)

were added, as aqueous solutions in small volumes, the final reaction volume being 3.0 ml. The contents of the water-jacketed reaction chamber were stirred for 20 min. to allow temperature equilibration and saturation with air.

The Clark electrode was then inserted, care being taken to exclude all air bubbles. After stirring for a further 2 min. to allow electrode stabilization, the recorder was adjusted to zero, and then set at 100% for the reading of the electrode current of the air-saturated medium. NADH (0.1 to 0.3µmole) was added to the stirred solution in a volume of 10 or 20µl from an Agla syringe fitted with a 3½" long needle. The concentration of the NADH was determined immediately prior to its use, spectrophotometrically.

The current was allowed to reach a steady value and then further stepwise additions of NADH were made until the oxygen tension was zero. Rapid stirring and accurate temperature control were essential and were maintained throughout. It was found advisable to protect the reaction mixture from direct sunlight to minimise photodecomposition of PMS.

The oxygen concentration (c) of the air-saturated solution was then calculated from the expression

$$c = \frac{100}{2x} \cdot \frac{n}{v} \quad \mu\text{mole/ml}$$

where n µmoles of NADH added to a volume v ml of experimental medium causes a recorder deflection of x%.

The oxygen consumed by the electrode itself over an average experimental period of 15 min. was less than 0.5% of the total oxygen present in the 3 ml reaction mixture. Diffusion of oxygen from the atmosphere into the solution was negligible, and the amount of oxygen introduced in the 10 µl or 20 µl additions of NADH was small enough to be ignored. However, with stepwise additions of volumes greater than 20 µl, the introduction of oxygen must be allowed for in calculating results.

The relative concentrations of NADH, PMS and catalase were varied to find optimum conditions for calibration.

4.4 Effect of varying catalase concentration.

Using 2o µg PMS per 3 ml reaction mixture the formation of hydrogen peroxide was rapid and reaction (iii) tended to be rate limiting unless high catalase concentrations were used. Accordingly, for a rapid calibration method, relatively high concentrations of catalase (400 µg/3 ml) were used; concentrations greater than this lead to a noisy trace-recording of the electrode current, which was attributed to an effect on the electrode membrane. With catalase concentrations lower than 40 µg/3 ml a temporary accumulation of

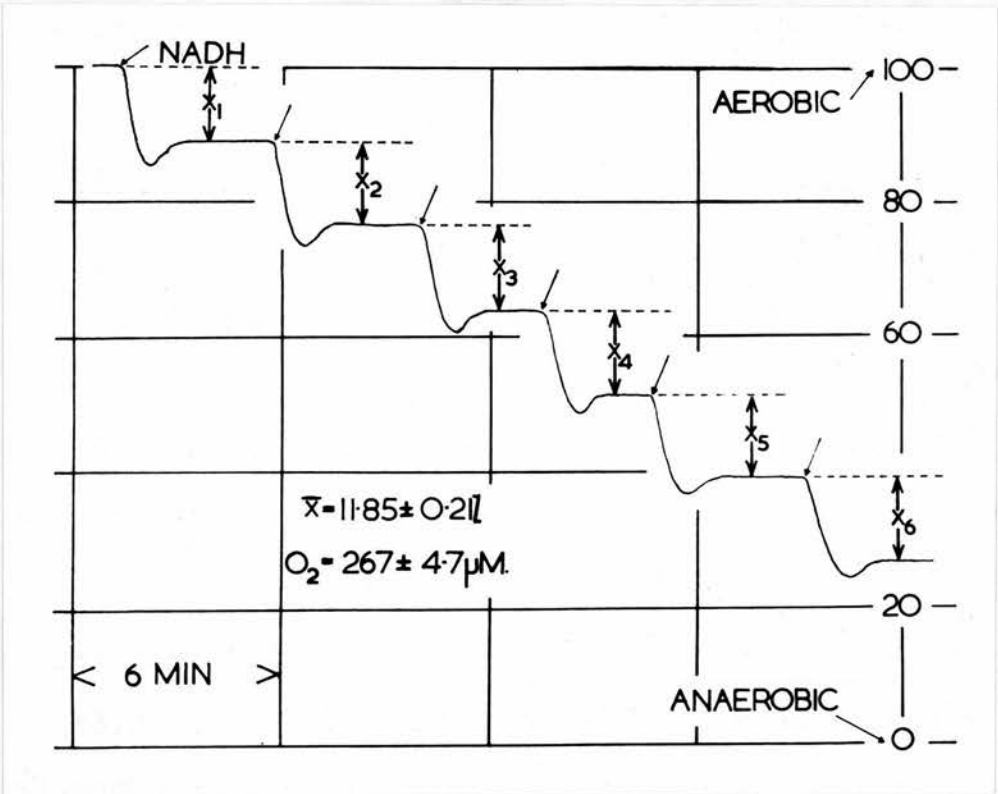


FIG. 4.1: Effect of high PMS concentrations on oxygen electrode trace recording and on calculated oxygen concentration. The reaction mixture contained 30 µg PMS and 400 µg catalase in 3 ml 0.1 M phosphate buffer, pH 7.4, at 25°C. Additions of 0.19 µmole amounts of NADH are indicated by arrows. Oxygen concentration was calculated from percentage deflections x_{1-6} obtained by measuring from each arrow to the new stable level. Mean percentage deflection x (±SD) and calculated oxygen concentration (±SD) are shown; the concentration obtained from other methods (see text) is 244±3.9 µM.

hydrogen peroxide occurred, detectable by the "overshoot" on the trace recording of the electrode current. When this occurred the oxygen concentration calculated after equilibrium was reached was identical to that obtained in the presence of greater amounts of catalase, but the reaction time was markedly increased. Hence 400 µg catalase per 3 ml reaction mixture was used routinely.

4.5 Effects of varying the concentration of NADH and PMS.

In the presence of 400 µg catalase per 3 ml medium, H_2O_2 temporarily accumulated when PMS concentrations exceeded 20 µg/3 ml, due to the increased rate of reaction (i) and perhaps some inhibition of catalase (Marcus and Feeley, 1962).

The oxygen concentration calculated from the trace recording shown in Fig. 4.1 was found to be 9.6% higher than values obtained with lower PMS concentrations, or values obtained using the mitochondrial method of Estabrook and Mackler (1957).

Addition of large amounts of NADH (0.6 µmole) to the reaction mixture described in the section on methods also caused H_2O_2 accumulation, but unlike results obtained with large amounts of PMS it did not lead to inaccurate values (see Fig. 4.2). It was concluded that the spurious values for oxygen concentration in the presence of large amounts of PMS were not due to the build-up of H_2O_2, but to the low $\frac{NADH}{PMS}$ ratio.

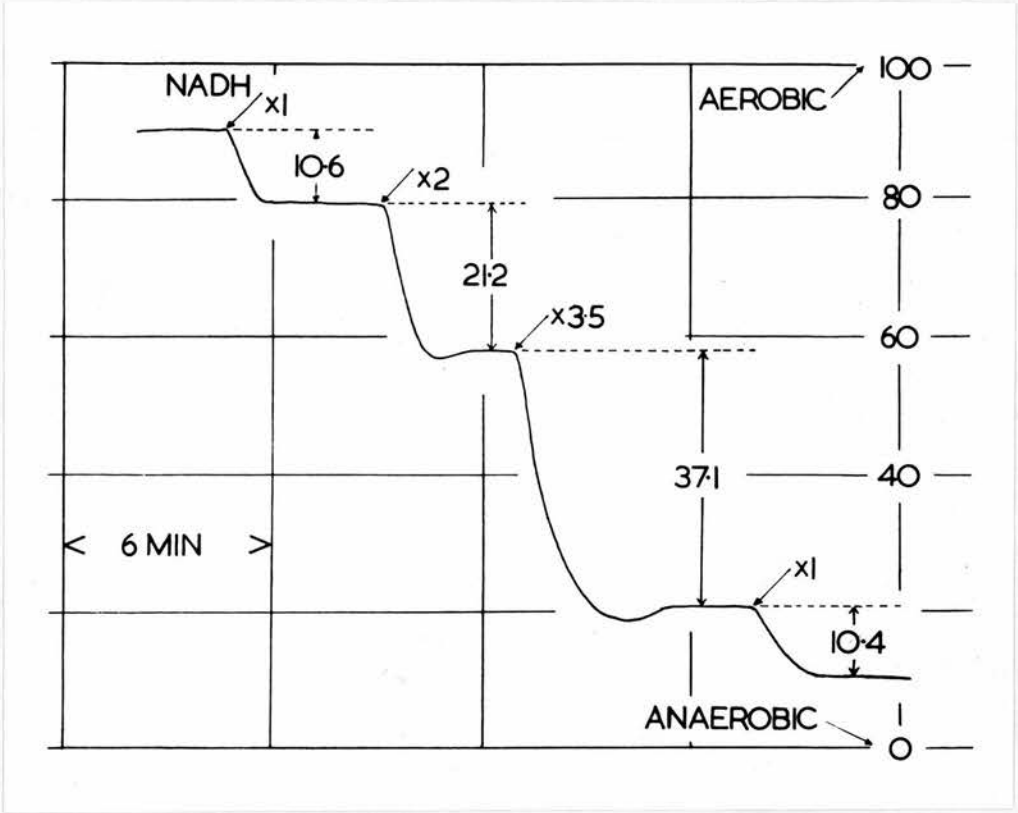


FIG. 4.2: Effect of addition of increasing amounts of NADH on oxygen electrode trace recording and on percentage deflection. The reaction mixture contained 20 µg PMS and 400 µg catalase in 3 ml 0.1 M phosphate buffer, PH 7.4, at 25°C. Additions of NADH are indicated by arrows; the numbers X1, X2 or X3.5 signify additions of 0.155, 0.310, or 0.5425 µmole NADH, respectively. Percentage deflection (x) for each addition is shown; the calculated oxygen concentrations are 243,243,243, and 248 µM, respectively.

A similar phenomenon where a fraction of $PMSH_2$ apparently remained unoxidised has been reported by Sowerby and Ottaway (1966) and Ottaway (1966). Ottaway (1966) postulated the formation of a NAD-$PMSH_2$ complex with $\frac{NADH}{PMS}$ ratios less than unity in order to explain his spectrophotometric data. Formation of such an unreactive type of $PMSH_2$ would explain the data obtained above.

4.6 Optimum conditions for calibration procedure.

As a result of the experiments described in the previous paragraphs it was decided routinely to use 400 µg of catalase and 20 µg of PMS for a final reaction volume of 3.0 ml. NADH was added in aliquots of 0.1 to 0.3 µmoles.

4.7 Validity of the method.

The method was checked by comparison with the method of Estabrook and Mackler (1957) over a range of oxygen concentrations produced by saturating the medium with air at different temperatures. A range of different oxygen concentrations was also produced by increasing the NaCl content of the medium. In the latter case the oxygen tensions found experimentally using the PMS method were compared with values cited for similar solutions in the International Critical Tables (National Research Council, 1928).

(i) Comparison with mitochondrial method. Traces obtained from an alternating recording of two electrodes

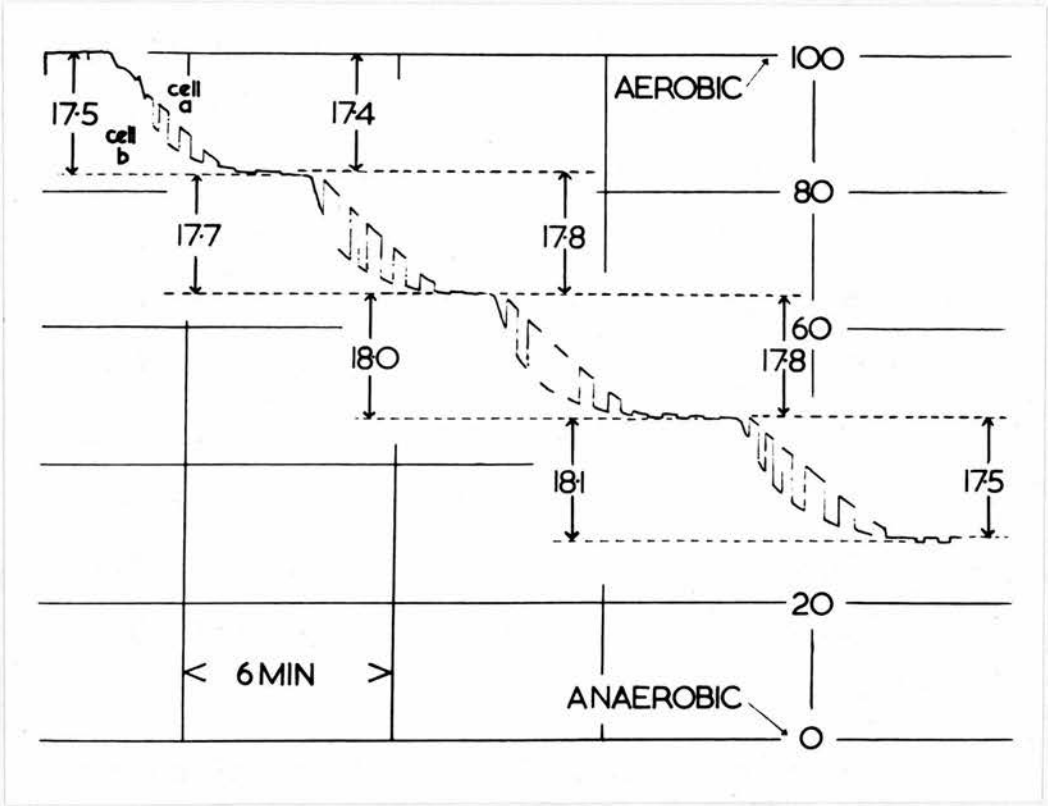


FIG. 4.3: Comparison of two oxygen electrode trace recordings using (A) calibration method of Estabrook and Mackler (1957) and (B) PMS method. Cell A contained 4.5×10^{-9} mole cytochrome c and 4 mg mitochondrial protein in 3 ml 0.1 M phosphate buffer, pH 7.4, at 25°. Addition of 0.255 µmole NADH to each cell is indicated by arrows, and percentage deflection (x) for each cell is shown. Two oxygen electrodes were used and anode currents were recorded from cell A and B alternately.

immersed in separate chambers containing the same medium (0.1M phosphate pH 7.4) at 25° are shown in Fig. 4.3. One cell (A) contained 4.5×10^{-9} moles cytochrome c and 4 mg mitochondrial protein (prepared according to Estabrook and Mackler (1957), while the other cell (B) contained PMS and catalase. NADH (0.255 µmole) was added to each chamber and the oxygen content of the medium calculated from the trace recordings of the electrode currents. Values of oxygen concentrations obtained from similar experiments over a range of temperatures are shown in Fig. 4.4. Both methods gave the same results over the entire range tested. Values for air saturated pure water taken from the International Critical Tables are also shown in Fig. 4.4. As expected the oxygen content of pure water is slightly higher than that of 0.1M phosphate.

(ii) Oxygen content of 0.01M phosphate pH 7.4 containing 0.5M, 1.0M, 2.0M, 3.0M, or 5.43M NaCl was determined using the PMS method described above. For the purpose of determining the actual oxygen content, the recorder was reset for each air saturated NaCl solution to give 100% deflection. The superimposed trace recordings of the electrode current after addition of NADH to each of these solutions is shown in Fig. 4.5. The decreased amount of oxygen in solution caused by the increasing concentration of NaCl is demonstrated by the

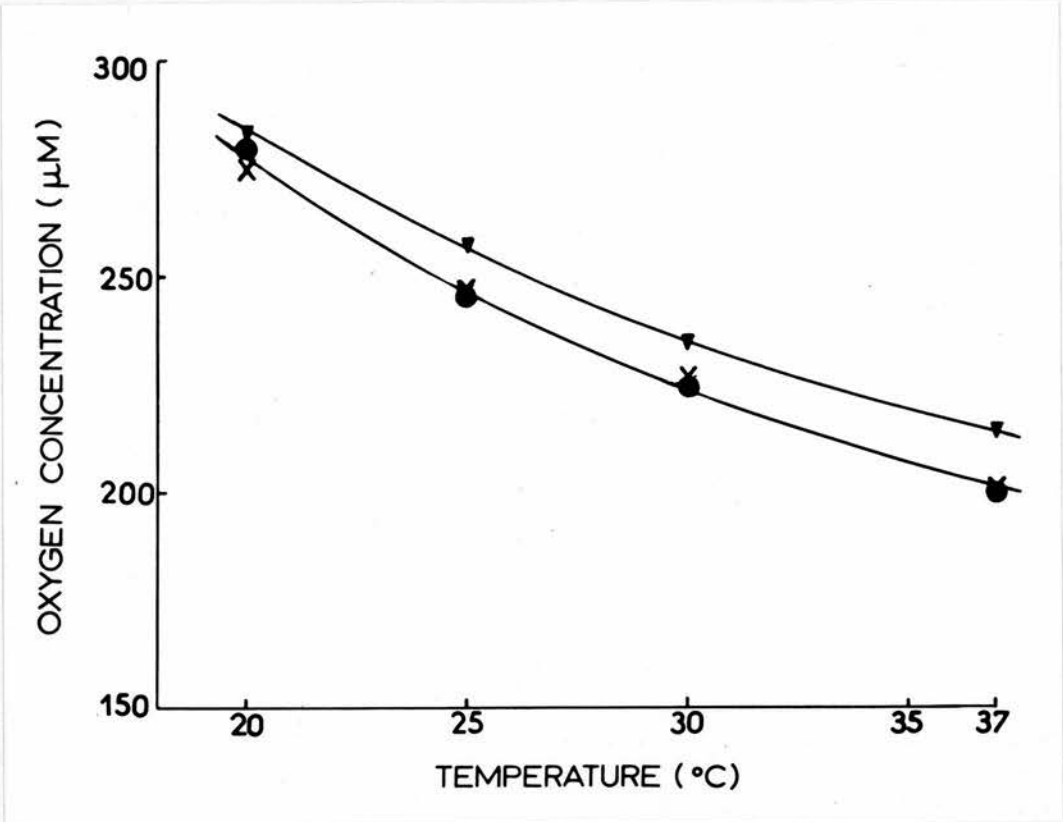


FIG. 4.4: Oxygen concentrations for media at different temperatures obtained from tables and experimentally: (Δ) data for pure water obtained from International Critical Tables, (O) oxygen concentrations for 0.1 M phosphate buffer, pH 7.4, obtained using calibration method of Estabrook and Mackler (1957), (X) oxygen concentrations for 0.1 M phosphate buffer, pH 7.4, obtained using PMS method.

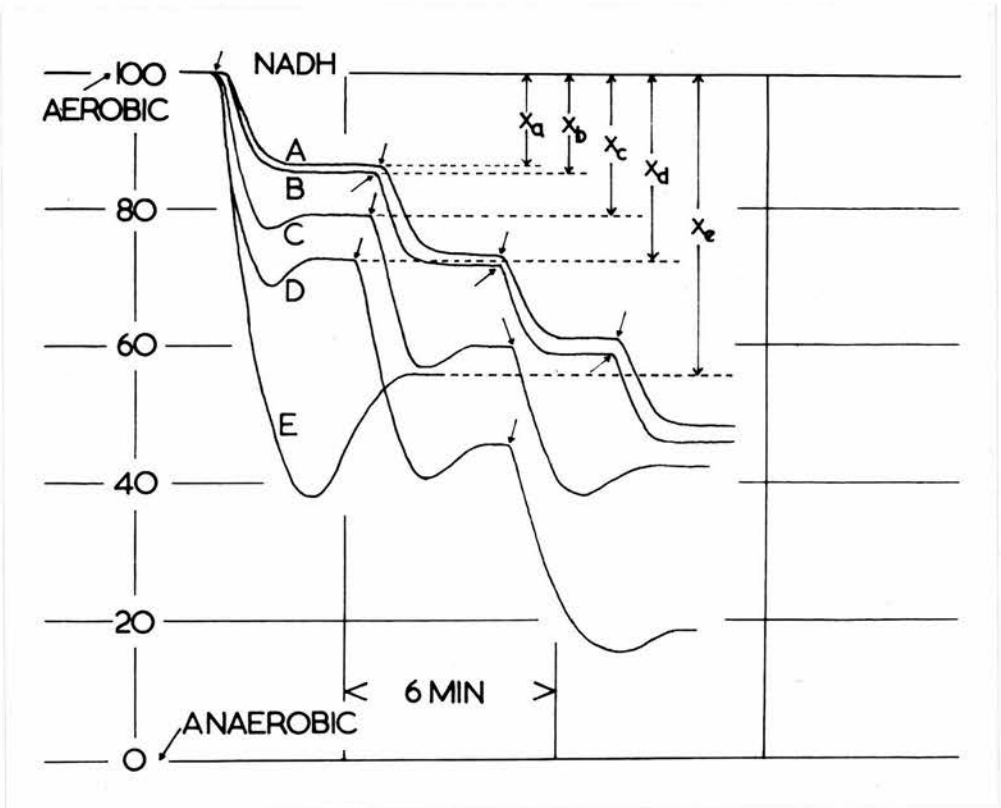


FIG. 4.5: Effect of varying NaCl concentrations on oxygen electrode trace recording. The reaction mixtures were 20 µg PMS and 400 µg calalase in 3.0 ml 0.01 M phosphate buffer containing different amounts of NaCl, and were equilibrated with air at 25°C. NaCl concentrations were (A) 0.5 M; (B) 1.0 M; (C) 2.0 M; (D) 3.0 M, and (E) 5.43 M (saturated). The recorder was reset for each air-saturated NaCl solution to give 100% deflection and trace recordings were superimposed. Additions of about 0.14 µ mole NADH are indicated by arrows, the exact amount in each case being determined by extinction at 340 nm. x_a to x_e indicate initial percentage deflections for each solution

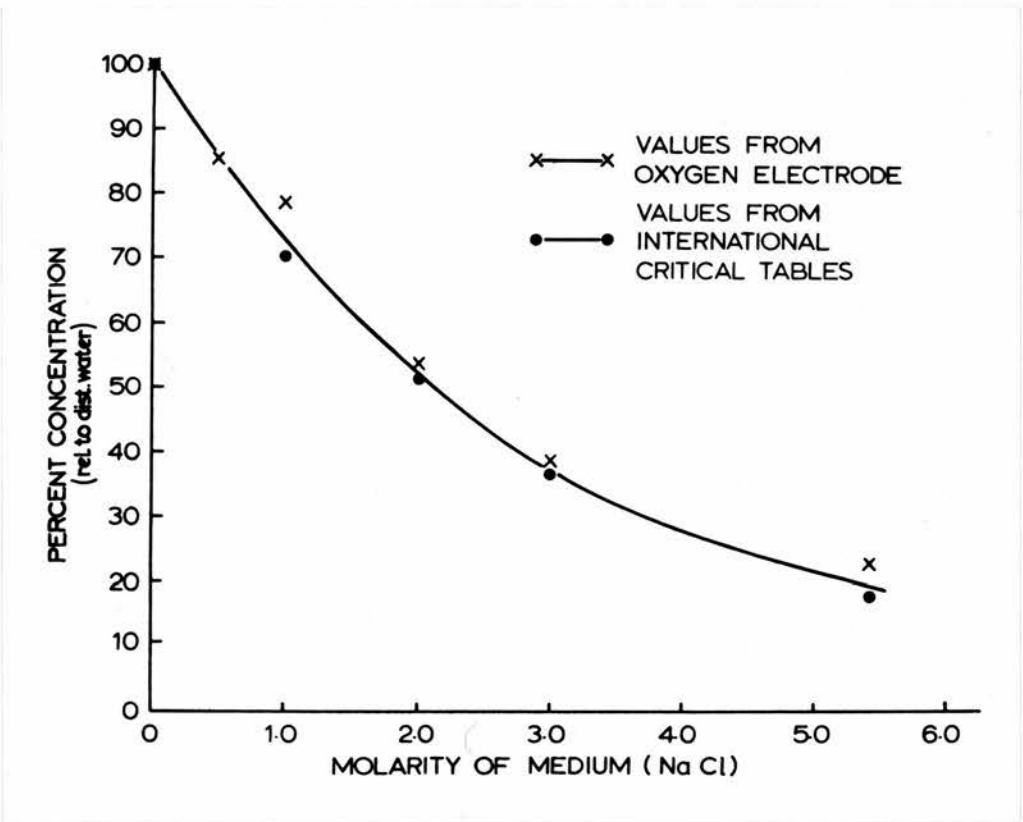


FIG. 4.6: Oxygen concentrations relative to distilled water for NaCl solutions of varying molarity (oxygen concentration of distilled water is taken as 100%): (O) data taken from International Critical Tables for NaCl in pure water, at 25°C, (X) data obtained using PMS calibration method as described in Figure 4.5 for NaCl in 0.01 M phosphate buffer, pH 7.4, at 25°.

progressively larger trace deflections on addition of the same amount of NADH (see Fig. 4.5). The actual oxygen concentrations calculated from these results are shown in Fig. 4.6 together with data taken from the International Critical Tables. The values confirm that the PMS method described gave true values for oxygen concentrations of solutions.

4.8 Determination of ADP:O ratios.

The procedure for the polarographic determination of ADP:O ratios was essentially that of Chance and Williams (1955a). A Clark oxygen electrode with an attached Servoscribe chart recorder was used as described above (section 4.2). Incubations were carried out in 3.0 mls of medium "B" (see Table 2.2). The oxygen concentration of this medium was determined as described above (section 4.3) and found to be 220 μM at 30°C. i.e 660 nmoles O_2 were present in the reaction chamber when it was sealed from the atmosphere by insertion of the electrode. A copy of a typical trace obtained from this apparatus during the course of an experiment is shown in Fig. 4.7.

Samples of liver and luteal tissue were taken from the same sow, and mitochondria were prepared as described in Chapter 2. These two different mitochondrial preparations were compared simultaneously by monitoring two reaction chambers with separate electrodes, at the same time. Mitochondria were introduced into the chambers

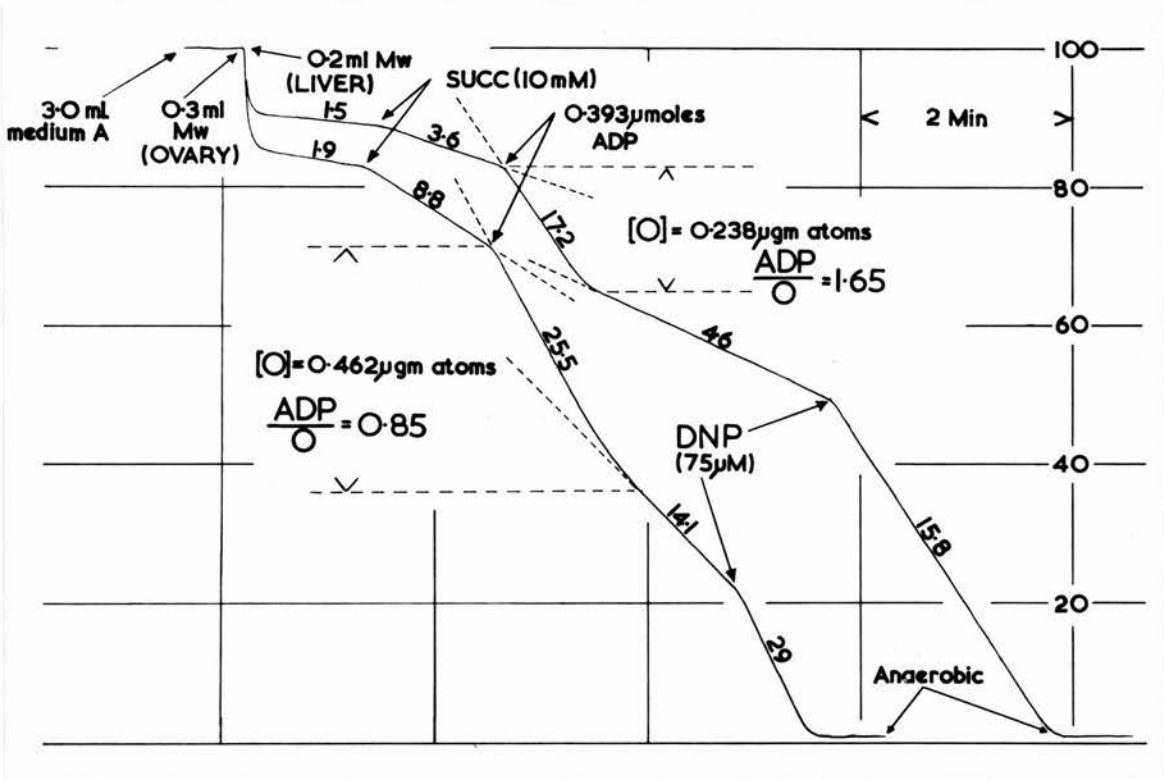


FIG. 4.7: Comparison of oxygen uptake, and ADP:O ratios for succinate in porcine liver and luteal mitochondria preparations, measured simultaneously. Reaction mixture (medium "B") contained 200 mM sucrose, 25 mM tris-HCl (pH 7.4), 10 mM potassium phosphate (pH 7.4), 20 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 1% BSA. Initial volume was 3.0 mls and the reaction chamber was maintained at 30°C. Succinate was added to a final concentration of 10 mM and 0.393 μ moles of ADP were added as shown. Respiratory control ratios were 3.7 and 1.8 for liver and luteal mitochondria respectively. Numbers on the traces refer to rate of oxygen uptake (nmoles/min/mg.).

causing an immediate fall in total oxygen tension recorded, because such solutions are essentially anaerobic. The subsequent traces show that endogenous respiratory rates were 1.3 and 1.8 n moles O_2/min/mg for liver and luteal mitochondria respectively. On addition of succinate to a final concentration of 10 mM to each chamber a difference in the two mitochondrial preparations became apparent: luteal mitochondria in State 4* (ADP-deficient) used oxygen at a much greater rate (8.8 n moles O_2/min/mg) than liver (3.6 n moles O_2/min/mg). In State 3*, obtained when an equal quantity of ADP was added to each reaction mixture, the difference in oxygen uptake between the two mitochondrial preparations was less marked; luteal mitochondria consumed O_2 at a rate of 25.5 n moles/min/mg, while liver mitochondria used 17.2 n moles O_2/min/mg. When ADP was depleted, respiratory rates were again limited, but could be stimulated to the same extent by further additions of ADP. 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 Fig. 4.7. The value was 0.85 for the luteal mitochondria and 1.65 for the liver mitochondria;

* the characteristics of these respiratory states are defined by Chance and Williams, 1955b.

TABLE 4.1

RESULTS OF ADP:O DETERMINATIONS IN LUTEAL AND LIVER MITOCHONDRIAL PREPARATIONS

Respiratory substrate (10mM)	Luteal mitochondria ADP:O ratios	Liver mitochondria ADP:O ratios
Succinate	0.85 ± 0.07	1.75 ± 0.14
L-malate	1.67 ± 0.25	2.82 ± 0.12
Pyruvate (+ L-malate, 500 μM)	1.67 ± 0.13	2.81 ± 0.18
D, L-isocitrate	1.85 ± 0.23	2.79 ± 0.15

ADP:O ratios were determined as described in the text. Substrates were added to a final concentration of 10 mM and ADP to 123 μM. 10 mg mitochondrial protein was added and the reaction carried out at 30°C, in buffer "B".

respiratory control ratios (defined by Chance, 1959) showed a similar difference, that for the luteal preparation being 1.8, while that for the liver was 3.7. Addition of the uncoupling agent, 2,4-dinitrophenol (DNP) released the preparations from dependence on ADP and their respiratory rates again became maximal.

This experimental procedure for comparing luteal and liver mitochondrial preparations from the same animal was repeated using other respiratory substrates, and the ADP:O ratio values obtained are shown in Table 4.1. In all cases, the luteal mitochondria showed significantly lower values, these being most marked in the case of succinate (49.1% of corresponding value for liver "control"), and somewhat less so for isocitrate (66% of liver "control"). Respiratory control ratios for liver were usually about 4 for succinate, and higher with NAD^+-linked substrates; in contrast the corresponding values in luteal mitochondrial preparations were about 2 for succinate, and 2-3 for NAD^+-linked substrates.

Similar respiratory characteristics of another steroidogenic tissue, the adrenal cortex, have been reported: Cammer and Estabrook (1967a) using a bovine preparation found ADP:O ratios for succinate and malate to be 1.2 and 1.0 to 2.0 respectively. Respiratory control ratios greater than 3 in their preparations were never

observed. Brownie et al., (1968) obtained similar results with rat adrenal preparations, and found that both respiratory control, and ADP:O ratio values were increased when the rats were pretreated with methylandrostenediol; adrenal mitochondria isolated from such animals were deficient in cytochrome P450, and showed greatly reduced 11β- and 18-hydroxylation capabilities. Purvis et al. (1968) and Sauer and Mulrow (1969) using rat adrenal mitochondria also reported low ADP:O and respiratory control ratios which were in each case sensitive to Mg^{++} ions: omission of Mg^{++} from the incubation media increased both values. Sauer (1970) demonstrated that ATP formation was inhibited more than 50% when 11β-hydroxylation of deoxycorticosterone was occurring; addition of metapirone relieved this inhibition.

That the low ADP:O and respiratory control ratios found in luteal mitochondria were not simply the result of poor preparation was indicated by their fulfilling various criteria of "biochemical integrity" which have been defined by Chance et al. (1968) and Chappell and Hansford (1969):

(i) endogenous respiratory rate was low (Fig. 4.7).

(ii) mitochondrial matrix enzyme activities were "latent" i.e. malate dehydrogenase and isocitrate dehydrogenase activity was not detectable in the intact preparations (see Table 5.1)

(iii) oxygen uptake was not stimulated by exogenous NADH.

(iv) the rate of oxygen utilization when malate was present was not stimulated by addition of NAD^+, or cytochrome c.

Most of these criteria ((ii) to (iv)) are related to the integrity of the inner mitochondrial membrane, which when intact is relatively impermeable to NAD (Lehninger, 1951). Demonstration of respiratory control (expressed as a ratio of the maximum rate of oxygen uptake in the presence of ADP to that obtained when the ADP has been used up (Chance, 1959) is usually regarded as the most sensitive indicator of mitochondrial damage. Acceptable ratios for liver mitochondria are 4 or more for succinate oxidation, 6 or more for NAD^+-linked oxidations (Chappell and Hansford, 1969).

Several possible explanations were considered to account for the low ADP:O and respiratory control ratios observed with the luteal mitochondrial preparations. It now seems probable that no single explanation for all the substrates used is tenable: the pathway of electron transport between the different TCA intermediates and the NADPH - cytochrome P450 reductase was later shown to vary with each substrate, and also the energy requirements of the different pathways were found to be dissimilar. These findings are presented in the subsequent chapters of the thesis, and explanations of the results of this

chapter are discussed in chapter 7 (see section 7.5 and Fig. 7.1).

SUMMARY

1. A method of calibrating the oxygen electrode for incubation media of unknown oxygen content has been described. It is based on the stoichiometric oxidation of NADH via the electron carrier PMS.

2. The optimum quantities of reactants for use in such calibration experiments were found to be 400 µg. catalase (i.e. 800 units) 20 µg. PMS for a reaction volume of 3 mls. NADH was added in aliquots of 0.1 to 0.3 µ moles.

3. The validity of this method was verified by comparing its performance with that of an established calibration method (Estabrook and Mackler, 1957); values obtained for oxygen concentrations determined by the PMS method were also checked with data available in the International Critical Tables.

4. The use of the calibrated oxygen electrode in the determination of ADP:O ratios in mitochondrial preparations has been described.

5. ADP:O and respiratory control ratios in luteal mitochondrial preparations were significantly lower than those measured in liver mitochondria prepared similarly.

CHAPTER 5

CHAPTER 5

TRICARBOXYLIC ACID CYCLE DEHYDROGENATIONS IN RELATION TO CHOLESTEROL SIDE-CHAIN CLEAVAGE.

The results presented in Chapters 3 and 4 showed that in porcine luteal mitochondrial preparations TCA intermediates can be oxidized by both the respiratory cytochrome chain and via the cytochrome P450 mixed function oxidase concerned with cholesterol side-chain cleavage. The aim of the work reported in this chapter was to obtain information on the connections between these two electron transport pathways present in luteal tissue.

5.1 The assay of some dehydrogenase activities in subcellular fractions of porcine luteal tissue.

(i) Assay of isocitrate and malate dehydrogenases:

These enzymes were assayed spectrophotometrically at 30°C, following the reduction of NAD^+ or $NADP^+$ at 340 nm using a Unicam SP800 recording spectrophotometer. Suitable quantities of porcine luteal subcellular fractions, which in the case of mitochondria had been previously sonicated or frozen at -20°C and thawed, were used and the activity calculated from the initial velocities of the reactions.

For the assay of $NADP^+$-dependent malate dehydrogenase activity ("malic enzyme") 4.5 µmole $MnCl_2$, and 1.5 µmole $NADP^+$ were added to 3 mls. of buffer "B"

TABLE 5.1

SPECIFIC ACTIVITIES OF ISOCITRATE AND MALATE DEHYDROGENASES IN SUBCELLULAR FRACTIONS ISOLATED FROM PORCINE CORPORA LUTEA

SUBSTRATE and CO-FACTORS present	SUBCELLULAR FRACTION Specific activities (nmoles $NAD(P)^+$ reduced/min/mg protein)	
	"Mitochondrial" (600-8000 x g. /15 min)	"Supernatant" (above 8000 x g. /15 min)
D, L-ISOCITRATE NAD^+ and ADP	not detectable	not detectable
D, L-ISOCITRATE, $NADP^+$	253*	162
L-MALATE NAD^+	3700*	341
L-MALATE, $NADP^+$	2-25†	81

* These activities were completely "latent" in intact mitochondrial preparations: assays were done after sonication.

† "malic enzyme" activity was detectable in very low amounts in some fresh intact mitochondrial preparations only; sonication did not enhance this activity, and after freezing and thawing all activity was lost.

(see Table 2.2) containing no $MgCl_2$ or phosphate, and the reaction was initiated by the addition of 25 µmole L-malate (di-sodium salt).

NAD^+-dependent malate dehydrogenase was assayed in 0.1 M Tris-NaOH, pH 9.5, or 0.1M methyl-amino propanol, pH 10.0 containing 1.5 µmole NAD^+. The reaction was started by adding 25 µmole L-malate.

The incubation medium for measuring isocitrate dehydrogenase activities contained either 45 µmole $MgCl_2$ and 1.5 µmole $NADP^+$, or 1.5 µmole NAD^+ and 2.0 µmole ADP in 3 mls of buffer "B", (see Table 2.2), in which phosphate had been omitted. The reactions were started by adding 15 µmole DL-isocitrate (tri-sodium salt).

(ii) Specific activities of isocitrate and malate dehydrogenases:

Some of the results obtained using the assays outlined in (i) are given in Table 5.1. The only dehydrogenase activity detectable in intact fresh luteal mitochondrial preparations was an $NADP^+$-linked malate dehydrogenase ("malic enzyme"). Values obtained for this activity varied between 2 to 25 n.moles $NADP^+$ reduced/min/per mg.of mitochondrial protein. Sonication of the mitochondrial preparation did not increase these low values, and after freezing and thawing the activity was lost. This dehydrogenase activity was

however more evident in supernatant subcellular fractions. That the activity found in the mitochondrial fractions was not simply the result of contamination with supernatant was indicated by the finding that the ratio of the two enzyme activities changed when the pH of the medium was varied. In addition the two other mitochondrial dehydrogenases investigated were not detectable before sonication of this fraction (see Table 5.1), even though the supernatant contained similar enzyme activities.

In luteal mitochondrial preparations the most active $NADP^+$-reducing enzyme appeared to be that of isocitrate dehydrogenase. The NAD^+-linked enzyme was not detectable, even in the presence of ADP, which is known to activate the enzyme (Plaut, 1969). Appreciable $NADP^+$-linked isocitrate dehydrogenase activity was also measurable in supernatant subcellular fractions of luteal tissue (see Table 5.1).

The major reason for carrying out the above assays was to assess the capability of the various dehydrogenase activities as possible sources of electrons for cholesterol side-chain cleavage. However, the routine assay used for estimating cholesterol side-chain cleavage activity did not provide information about the absolute rates of cholesterol cleaved, and so the precise requirement for electrons to support this reaction under these experimental conditions was unknown. In

order to obtain such information, mitochondrial incubations were carried out as described in Chapter 2 (section 2.3), in which standard cholesterol was used in place of the usual radioactive isotope; the reaction products were extracted and estimated by gas chromatography (see Chapter 3, section 3.1.2). Data obtained from such experiments indicated that, in the presence of either D,L-isocitrate or L-malate (both 10mM), 1mg. of mitochondrial protein cleaved about 3.2 nmoles of cholesterol in an hour. This value is of the same order as that reported by Armstrong et al., (1969) for rat ovarian mitochondrial preparations. On this basis, the amount of NADPH generated by isocitrate dehydrogenase under the conditions of the cholesterol side-chain cleavage assay would seem to be more than adequate to support the latter reaction.

The distribution of malate dehydrogenases in porcine luteal cells appeared to be the same as that reported for the superovulated rat ovary, by Stevenson and Forman, (1971). The mitochondrial "malic enzyme" activity was low, although it could possibly produce enough reducing equivalents to account for the cholesterol side-chain cleavage activity observed with malate as substrate. However, this malic enzyme, unlike isocitrate dehydrogenase, was accessible to exogenous $NADP^+$ when assayed in intact mitochondria. It appeared likely, therefore, to be in a different

mitochondrial compartment from the cholesterol side-chain cleavage complex, which can not readily use exogenous NADPH (Yago et al., 1967; see also Fig. 5.1). More recently Stevenson and Taylor (1971) have shown that the porcine luteal "malic enzyme activity" demonstrable in mitochondrial preparations is not a simple $NADP^+$ dehydrogenase: an alkaline phosphatase, or phosphotransferase is present in the outer mitochondrial membrane, and is capable of forming NAD^+ from $NADP^+$ and transferring this nuclectide to the inner mitochondrial membrane where it is reduced, in the presence of malate, via malate dehydrogenase. It thus seems unlikely that "malic enzyme" (i.e. L-malate:NADP oxidoreductase, decarboxylating, E.C.1.1.1.40.) plays any role in the supply of intramitochondrial NADPH for porcine luteal steroidogenesis. The contribution of $NADP^+$ dependent malic enzyme activity to steroid hydroxylations in various tissues appears to be species specific: this has been discussed by Simpson and Estabrook, (1969).

5.2 Relative efficiency of TCA intermediates in supporting side-chain cleavage, compared with NADPH.

Intact luteal mitochondria (about 5 mg.) were incubated in medium "B" at 37° for 1 hour in the presence of [4-^{14}C] cholesterol, and a variety of possible electron donors (all at 5 mM). The efficiency of these different substrates in supporting the cholesterol

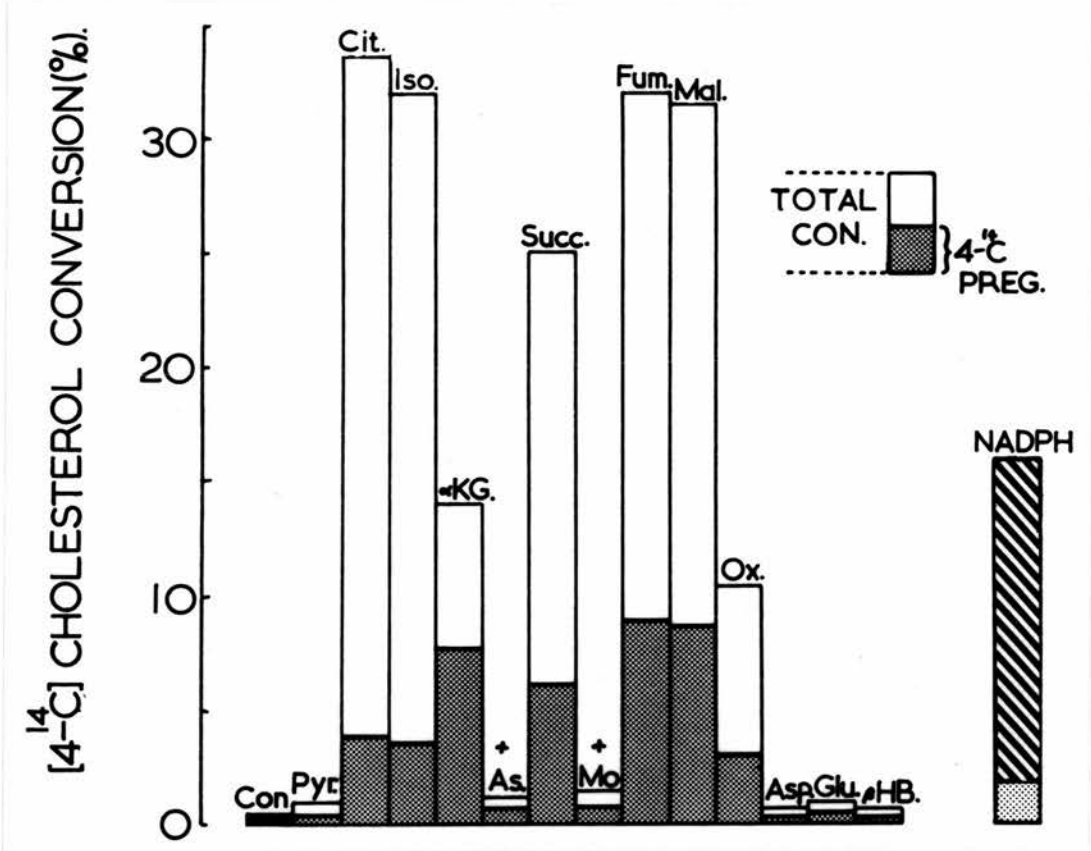


FIG. 5.1: Comparative efficiency of respiratory substrates in supporting the cholesterol side-chain cleavage reaction. Incubations were carried out in 1 ml. of medium "B" and contained 5 mg mitochondrial protein. The control tube contained no respiratory substrate. Pyruvate (Pyr), citrate (Cit), isocitrate (Iso), α-ketoglutarate (αKG.), succinate (Succ), fumarate (Fum), malate (Mal), oxaloacetate (Ox.) aspartate (Asp.), glutamate (Glu), β - hydroxy butyrate (βHB) and NADPH were all added to a final concentration of 5 mM. Arsenite (As) to a concentration of 2 mM was added to α-ketoglutarate, and malonate (Mo) to 10 mM was added to a succinate supported reaction mixture. The percent conversion of [4-^{14}C] cholesterol was determined as described in Chapter 2.

side-chain cleavage reaction is shown in Fig. 5.1. The TCA intermediates citrate, isocitrate, succinate, fumarate and malate were all more effective in supporting the cleavage of [4-^{14}C] cholesterol than was NADPH. The keto-acids, α-ketoglutarate and oxaloacetate were less effective, and pyruvate did not support the reaction at all.

Inhibition of specific dehydrogenases abolished the cholesterol side-chain cleavage activity when the corresponding substrate was the sole electron donor: e.g. 2 mM arsenite (which was shown to completely inhibit the oxidation of α-ketoglutarate in this system - see Table 5.2) abolished side-chain cleavage supported by 5 mM α-ketoglutarate, and malonate prevented the reaction when 5 mM succinate was the electron source. In the absence of any added respiratory substrates the utilization of [4-^{14}C] cholesterol was always less than 0.4%. Aspartate, glutamate and β-hydroxybutyrate did not support cholesterol side-chain cleavage, nor stimulate oxygen uptake in these mitochondrial preparations (see Table 5.2).

These results are comparable with those concerning steroid hydroxylation in adrenal cortex mitochondria in that TCA intermediates are generally more efficient electron donors than exogenous NADPH for such reactions. The reported relative efficiency of the various TCA intermediates varies with different groups: Brownie and Grant (1954); Sweat and Lipscomb (1955), Harding

TABLE 5.2

RATES OF OXYGEN UTILIZATION FOR INTACT LUTEAL MITOCHONDRIA IN THE PRESENCE OF RESPIRATORY SUBSTRATES AND SOME INHIBITORS

RESPIRATORY SUBSTRATE (5 mM)	OXYGEN UPTAKE RATE* (nmoles/mg/min.)
α-KETOGLUTARATE	9.8
α-KETOGLUTARATE + ARSENITE (2mM)	1.6
SUCCINATE	27.6
SUCCINATE + MALONATE (10 mM)	2.4
ASPARTATE	1.8
GLUTAMATE	1.6
β-HYDROXYBUTYRATE	1.6
NONE (i.e. endogenous)	1.8

* Oxygen uptake rates given are those measured polarographically, in the presence of 1 mM ADP (i.e. "state 3" respiration); the basal level of oxygen uptake in these preparations varied between 1-3 nmoles O_2/mg/min.

TABLE 5.3

RELATIVE EFFICIENCY OF TCA INTERMEDIATES IN SUPPORTING MITOCHONDRIAL STEROID HYDROXYLATIONS

(data taken from the literature compared with that obtained with porcine luteal mitochondrial preparations)

ELECTRON DONOR	11β-HYDROXYLASE ACTIVITY (ADRENAL)					CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY (PORCINE CORPU LUTEUM)
	(a)	(b)	(c)	(d)	(e)	
CITRATE	24	9	-	72	77	100
ACONITATE	-	14	-	-	-	-
ISOCITRATE	-	20	-	79	100	96
α-KETOGLUTARATE	85	1	-	72	49	41
SUCCINATE	84	-	100	100	62	75
SUCCINATE + ATP	100	-	-	-	-	-
FUMARATE	83	100	-	79	-	96
MALATE	60	84	68	86	76	94
OXALOACETATE	42	-	-	-	-	30
PYRUVATE	3	-	-	0	-	0
β-HYDROXYBUTYRATE	-	-	0	0	-	0
GLUTAMATE	-	-		0	-	0

The relative hydroxylase activities are expressed as a percentage of that of the most effective electron donor in each series.

Sources of data:

(a) Brownie and Grant, 1954
(b) Sweat and Lipscomb, 1955
(c) Harding et al., 1965
(d) Cammer and Estabrook, 1967a
(e) Péron and McCarthy, 1968

et al., (1965); Cammer and Estabrook (1967a) and Peron and McCarthy, (1968) have presented data concerning 11β-hydroxylation in adrenal cortex mitochondria and it is compared with that obtained with intact porcine luteal mitochondria for cholesterol side-chain cleavage activity in Table 5.3.

The results presented in Fig. 5.1. show that TCA cycle activity can be related to cholesterol side-chain cleavage, but give no direct information as to which dehydrogenase steps are involved; i.e. the relatively low efficiency of α-ketoglutarate could be a consequence of limited mitochondrial permeability to this substrate rather than a quantitative reflection of the actual relationship of α-ketoglutarate dehydrogenase to the NADPH-cytochrome P450 reductase system and cholesterol side-chain cleavage. Under the conditions employed in this type of experiment, once the TCA intermediate has entered the intact mitochondrion it may undergo a series of dehydrogenations as TCA cycle activity occurs, thus making it difficult to assess which particular dehydrogenations are more closely involved in donating electrons to the cytochrome P450 reductase. To overcome this problem it is necessary to interrupt the TCA cycle at specific steps; this was carried out in the following experiments.

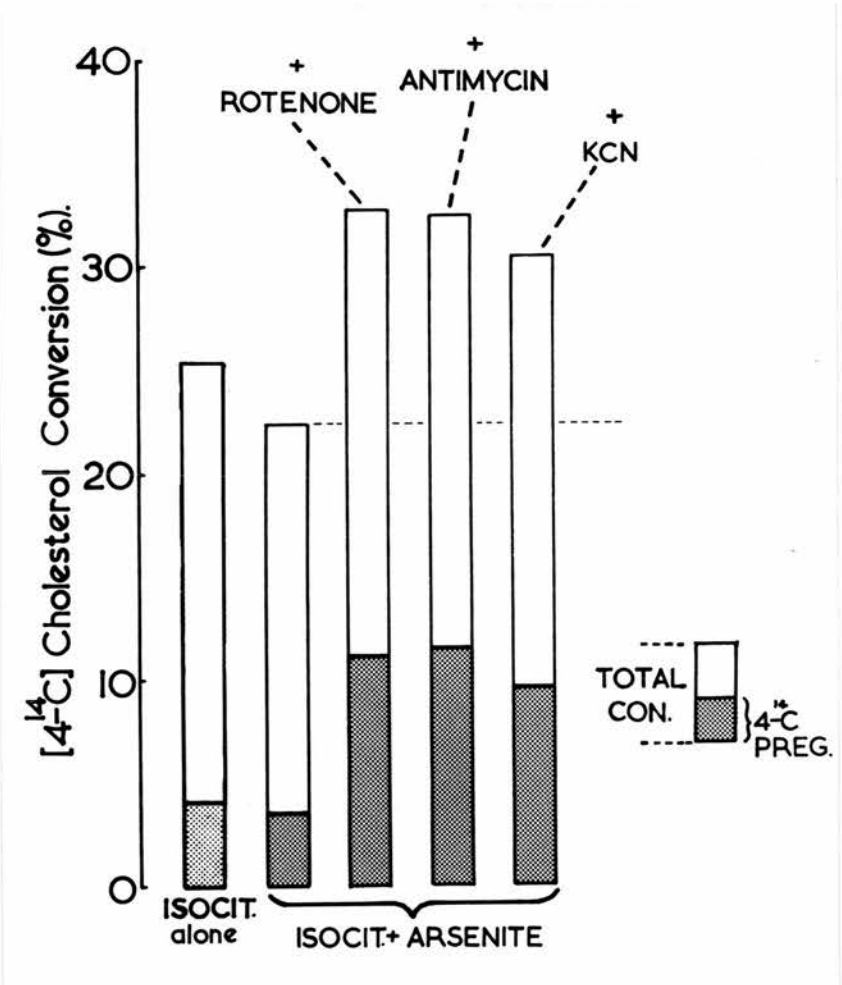


FIG. 5.2: Isocitrate-supported cholesterol side-chain cleavage. The reaction was carried out as described in Chapter 2, using medium "B". 5 mM isocitrate was used alone, and with 2 mM arsenite as shown. Rotenone, antimycin or KCN were added to a final concentration of 10 μM, 330 ng per mg of mitochondrial protein or 1 mM respectively.

5.3 The effect of respiratory inhibitors on cholesterol side-chain cleavage supported by isocitrate.

Intact luteal mitochondria were incubated in medium "B" with [4-^{14}C] cholesterol in the presence of isocitrate and some inhibitors, and the results are given in Fig. 5.2: it can be seen that in the presence of 5 mM D,L-isocitrate, 25.4% of the added [4-^{14}C] cholesterol was cleaved. In order to show that isocitrate dehydrogenation itself was capable of effectively supporting cholesterol side-chain cleavage oxidation of the subsequent respiratory product, α-ketoglutarate, was inhibited with arsenite (2 mM): this did not markedly effect the percentage conversion of the [4-^{14}C] cholesterol, demonstrating that other dicarboxylic acids formed from isocitrate were not involved.

The support for cholesterol side-chain cleavage by isocitrate was not abolished by the respiratory inhibitors rotenone, antimycin, or cyanide. Similar findings were reported by Sauer and Mulrow (1969) who studied 11β-hydroxylation in rat adrenal mitochondria, but in contrast, Purvis et al., (1968) reported a partial inhibition when rotenone, antimycin or cyanide were present. Since isocitrate dehydrogenation in porcine luteal mitochondria provides NADPH directly, (see Table 5.1) it is not surprising that cholesterol side-chain cleavage was not decreased by these inhibitors when

isocitrate was the electron donor for the reaction; on the contrary, the addition of rotenone (to 10μM), antimycin (330 ng per mg of mitochondrial protein, i.e. 3μM) or cyanide (1mM), increased the conversion of the [4-^{14}C] cholesterol by 47, 46, or 37% respectively. A similar degree of stimulation occurred when the same NADH oxidase inhibitors were included in incubations where NADPH was the sole electron donor for cholesterol side-chain cleavage (see Fig. 5.4)

An explanation of these results is that rotenone, antimycin or CN^- conserve the NADPH for cholesterol side-chain cleavage by preventing its removal via a pyridine nucleotide transhydrogenase: this hypothesis will be discussed later (section 5.5.)

5.4 The effects of antimycin and arsenate on cholesterol side-chain cleavage supported by α-ketoglutarate dehydrogenation.

Since ovarian cholesterol side-chain cleavage has been shown to require NADPH (Hall and Koritz, 1964b; Yago et al., 1967; Sulimovici and Boyd, 1968b) it was of interest to investigate a specifically NAD^+ linked dehydrogenase in relation to this reaction.

Intact porcine luteal mitochondria were incubated in medium "B" from which phosphate had been omitted, in the presence of α-ketoglutarate and [4-^{14}C] cholesterol, and cholesterol side-chain cleavage activity was estimated; the results are given in Fig. 5.3. The

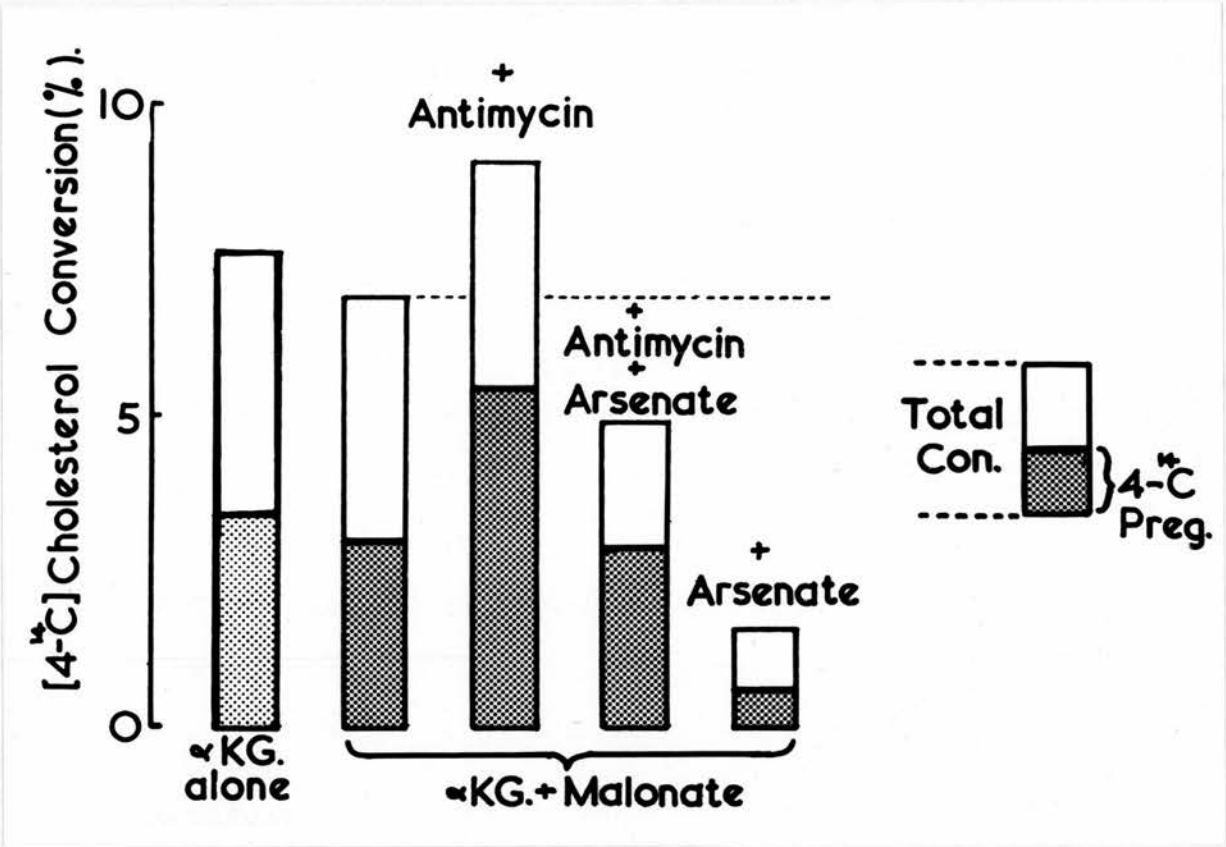


FIG. 5.3: The effect of arsenate and antimycin on the α-ketoglutarate + malonate supported cholesterol side-chain cleavage reaction. The reaction was assayed as described in Chapter 2, in phosphate-free medium "B". α-ketoglutarate, malonate, antimycin and arsenate were present at final concentrations of 5 mM, 10 mM, 3 μM and 40 mM respectively.

addition of 10 mM malonate to this system (to inhibit succinate dehydrogenase - see Table 5.2) did not abolish side-chain cleavage activity. It seems probable therefore that α-ketoglutarate dehydrogenation, a reaction which generates NADH, can supply reducing equivalents to the NADPH-cytochrome P450 reductase system in the luteal mitochondrion.

In the adrenal cortex it has been postulated that α-ketoglutarate dehydrogenation can supply electrons for 11β-hydroxylation via an intramitochondrial NADH: NADPH transhydrogenation mechanism (Sauer and Mulrow, 1969); the results obtained by these investigators indicated that the reaction occurring was probably energy dependent, akin to the energy linked pyridine nucleotide transhydrogenase of liver mitochondria described by Danielson and Ernster, (1963).

The energy requirement of the α-ketoglutarate-supported cholesterol side-chain cleavage reaction was investigated in porcine luteal mitochondria (Fig. 5.3). Addition of antimycin to such incubations, to prevent oxidative phosphorylation occurring, did not inhibit side-chain cleavage. The possibility that substrate level phosphorylation, inherent in the conversion of α-ketoglutarate to succinate, was involved was excluded by including 40 mM arsenate in the phosphate free incubation medium, to prevent phosphate esterification (Welle and Slater, 1964; Slater, 1967). Under these extreme

conditions, with antimycin also included the formation of any high energy intermediate is most unlikely and yet an appreciable amount of cholesterol side-chain cleavage occurred (Fig. 5.3). That the small reduction in activity seen when antimycin and arsenate were present together might be due to mitochondrial damage by the arsenate (Welle and Slater, 1964; Slater, 1967) was indicated by finding that this amount of arsenate without added antimycin gave rise to greater inhibition (more than 75% of control - Fig. 5.3). It thus seemed probable that preparations of porcine luteal mitochondria differ from those derived from the adrenal cortex (Sauer and Mulrow, 1969), in that the NADH:NADPH transhydrogenase is not energy dependent. Such a transhydrogenase has been described for liver and heart mitochondria (Kaplan et al., 1952, 1953); experiments designed to obtain more information about the possible relationship of such a reaction to electron transport in the porcine luteal mitochondrion are described in the following section.

5.5 The use of NADPH and NADH as electron donors for the cholesterol side-chain cleavage reaction and the effect of respiratory inhibitors in these situations.

Exogenous NADPH is a poor source of reducing equivalents for the cholesterol side-chain cleavage reaction when this is being measured in intact luteal preparations (Yago et al., 1967); it was demonstrated

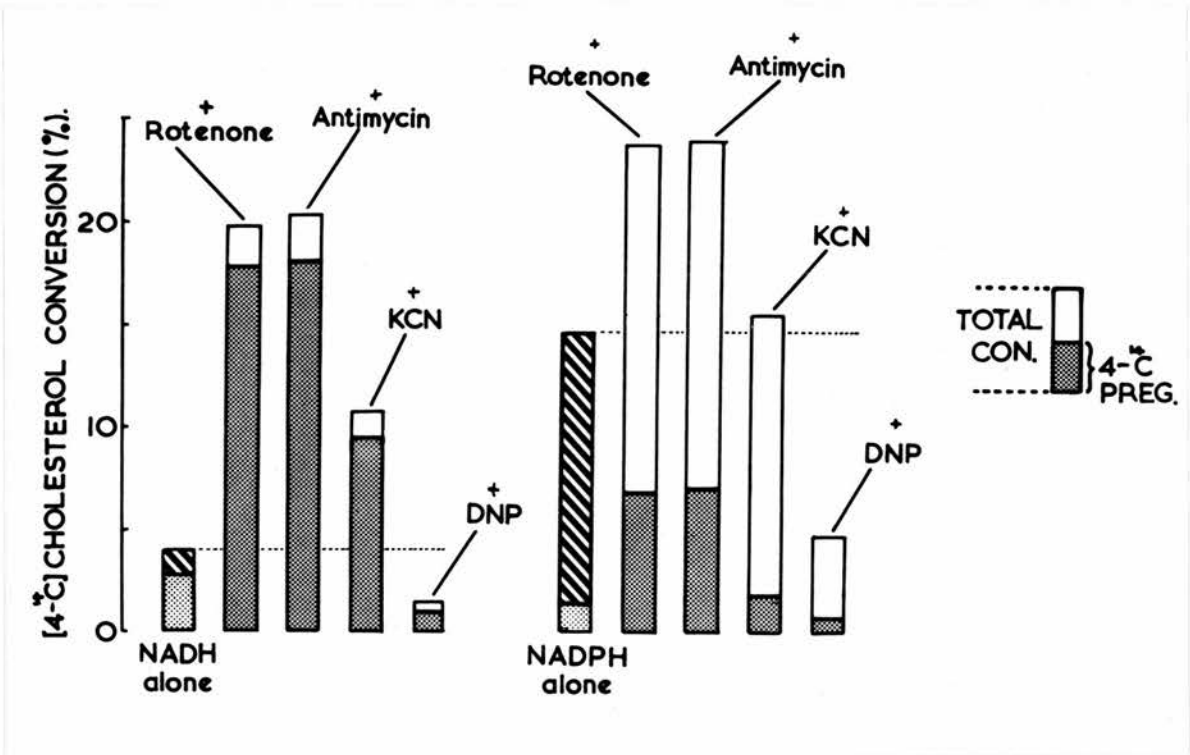


FIG. 5.4: The effect of respiratory inhibitors on NADH- and NADPH- supported cholesterol side-chain cleavage activity. NADH, and NADPH were present at a final concentration of 5 mM, and rotenone, antimycin, KCN and DNP at 10 μM, 3 μM, 1 mM or 100 μM respectively.

that several TCA intermediates would support twice as much activity as this nucleotide in porcine luteal mitochondria preparations (see Fig. 5.1). Nevertheless enough cholesterol side-chain cleavage occurred in the presence of NADPH under these circumstances to be easily measurable. It was decided therefore to investigate NADPH and NADH support for the reaction, in the presence of some of the respiratory inhibitors mentioned previously in an attempt to obtain more direct, and also additional, information about the possible role of transhydrogenation in electron transport in luteal mitochondria.

Porcine luteal mitochondria were incubated in medium B at 37° for 1 hour, in the presence of [4-^{14}C] cholesterol and either NADPH or NADH. Similar incubations, in which in addition to these compounds, either rotenone (10 μM), antimycin (3 μM), potassium cyanide (1 mM) or DNP (100 μM) were also present, were carried out at the same time. The results obtained from such an experiment are shown in Fig. 5.4. It can be seen that in the presence of NADPH (5 mM), 14.7% of the [4-^{14}C] cholesterol was cleaved; the corresponding value when NADH (5mM) replaced NADPH was 4%. The addition of inhibitors of NADH oxidase gave rise to a stimulation of cholesterol side-chain cleavage which occurred in the presence of either NADPH or NADH; the stimulation was much more marked in the case of the NADH-supported

reaction however, where a five-fold increase in side-chain cleavage activity was observed in the presence of either rotenone or antimycin. The addition of rotenone or antimycin to incubations where cholesterol side-chain cleavage was supported by NADPH brought about a stimulation of about 65% compared with inhibitor-free controls. Similar results were obtained when cyanide was added to these incubations: when NADH was the electron source, addition of cyanide caused a 170% increase in side-chain cleavage activity, whereas with NADPH the corresponding increase was only about 5%. The addition of the uncoupling agent DNP to these incubations inhibited cholesterol side-chain cleavage activity, whether NADPH or NADH was used as the electron donor for the reaction; 68% or 63% inhibition, respectively, was observed compared with DNP-free controls. NADH was a very poor source of electrons for cholesterol side-chain cleavage, compared with NADPH; however, when rotenone or antimycin was present with each coenzyme, this relative difference was almost entirely abolished, and NADH supported almost 90% of the activity measurable for NADPH.

A possible explanation for these results could be 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 luteal mitochondrial preparations, then the addition of inhibitors of NADH oxidase which would result in the accumulation of NADH, might be expected to displace the NADH:NADPH equilibrium in favour of NADPH. Furthermore if one assumes that under normal (i.e. inhibitors absent) conditions in these luteal mitochondria preparations most of the electron flux from NADPH is to the NADPH-cytochrome P450 reductase system, rather than to the transhydrogenase reaction, and that similarly, the greater part of the electron flux from NADH is to the NADH-cytochrome oxidase system, then all the results shown in Fig. 5.4 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 would cause diversion of electron flow from NADH to NADPH, and so stimulate cholesterol side-chain cleavage.

(iii) Such stimulation would be more marked when NADH, rather than NADPH was the electron donor present.

(iv) Agents which stimulate NADH-cytochrome oxidase electron flux, such as DNP (and also ADP-see later) would

displace the equilibrium of the transhydrogenase in favour of NADH and so tend to inhibit cholesterol side-chain cleavage.

(v) The amount of cholesterol side-chain cleavage supported by NADH should approach the values measured for NADPH, when inhibitors of NADH oxidase were present in both situations.

The inhibitory effect of respiratory uncoupling agents such as DNP and dicumarol on 11β-hydroxylation supported by various TCA intermediates, demonstrated originally by Brownie and Grant (1954) and later by Peron et al., (1966), Guerra et al., (1966) and Cammer et al., (1968) has led to the widely accepted conclusion that in adrenal cortex mitochondria, the transfer of hydrogen from NADH to $NADP^+$ is energy-dependent (Oldham et al., 1968). Other explanations for these results are however possible:

(i) It has been reported that malic enzyme, which has been implicated in the support of steroid hydroxylation in the bovine adrenal cortex (Simpson and Estabrook, 1968) is inhibited by DNP or dicoumarol (Simpson and Estabrook, 1969). This explanation of the inhibitory effect of DNP described above (see Fig. 5.4) on cholesterol side-chain cleavage in porcine luteal mitochondrial preparations is unlikely, since malic enzyme activity is not present in this tissue (see section 5.1).

(ii) Uncoupling agents stimulate electron flow from

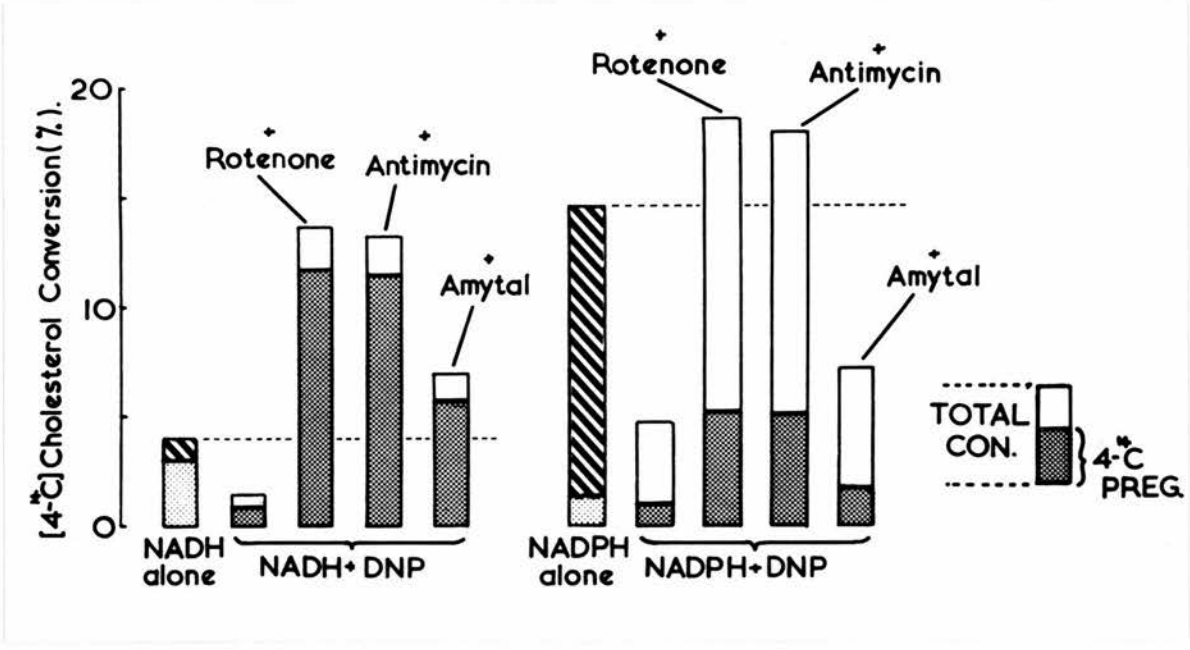


FIG. 5.5: The removal of DNP inhibition of NAD(P)H-supported cholesterol side-chain cleavage activity by some NADH oxidase inhibitors. NADH, and NADPH were present at 5 mM final concentration, and DNP, rotenone, antimycin and amytal at 100 μM, 10 μM, 3 μM and 1.8 mM respectively.

NADH to cytochrome oxidase, and so might be expected to displace the equilibrium of NADH:NADPH transhydrogenase in favour of NADH formation. Thus NADPH would be removed via the mitochondrial respiratory chain and would not be available for cholesterol side-chain cleavage (i.e. see (iv) above).

The second explanation was investigated further in relation to electron transport in porcine luteal mitochondria: cholesterol side-chain cleavage incubations were carried out in the presence of DNP and NADH, and in some tubes the respiratory inhibitors rotenone, amytal or antimycin were also included; at the same time a parallel series of incubations, in which NADPH replaced NADH, was carried out. The results of one such experiment are shown in Fig. 5.5. It can be seen that the inhibition caused by addition of DNP was relieved by inhibitors of NADH-cytochrome oxidase electron transport, such as rotenone or antimycin. It is interesting to note that amytal, an inhibitor which is thought to act at the same site as rotenone (Ernster et al., 1963), was not so effective in relieving DNP inhibition. Chance and Hollunger (1963) showed that NADH oxidase inhibition by amytal was partially relieved by DNP, and if this were so in luteal mitochondria also, it would account for this result. The results of these experiments suggested that the inhibitory effect of DNP on NADH-supported cholesterol

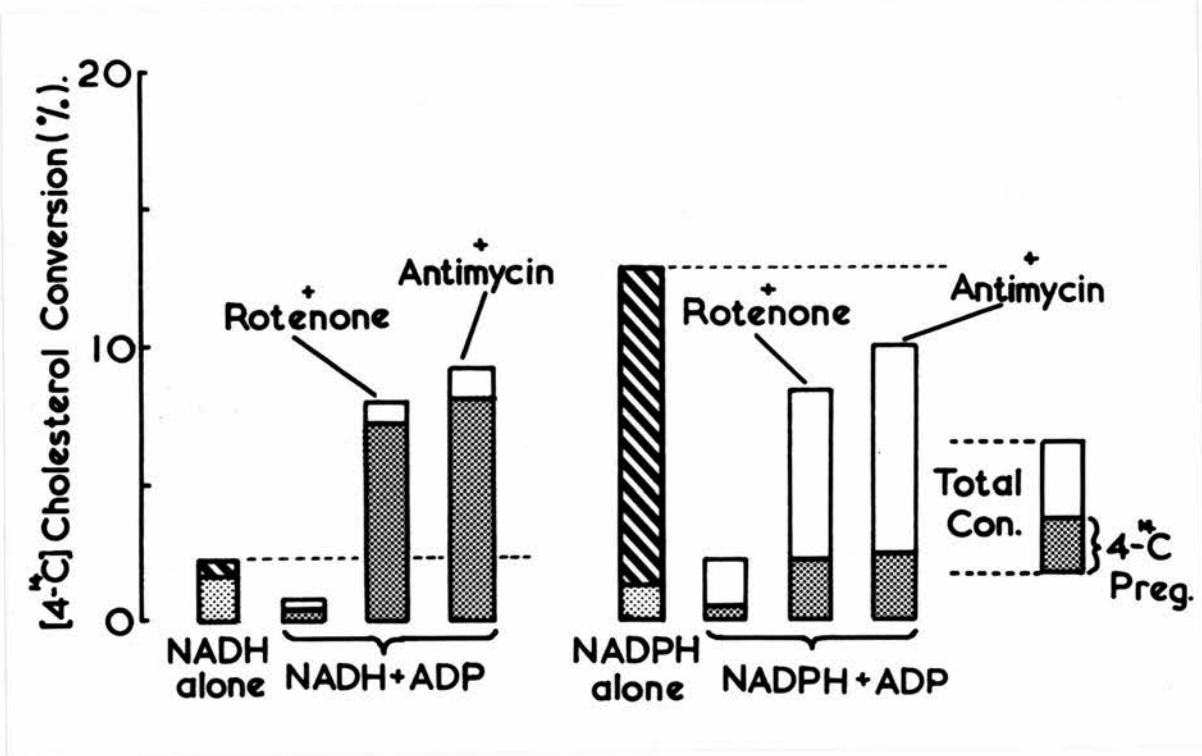


FIG. 5.6: The removal of ADP inhibition of NAD(P)H-supported cholesterol side-chain cleavage activity by rotenone and antimycin. NADH and NADPH were present at final concentrations of 5 mM, and rotenone, antimycin and ADP at 10 μM, 3 μM and 2 mM respectively.

side-chain cleavage was a consequence of its stimulating NADH-cytochrome oxidase electron flow, rather than its property of preventing oxidative phosphorylation. This possibility was also indicated by the use of another agent which stimulates NADH oxidation maximally in coupled mitochondria, namely ADP: a series of incubations similar to those described above were carried out, substituting ADP for DNP. The results are shown in Fig. 5.6. A similar level of inhibition occurred in the presence of ADP as had for DNP, and the addition of the respiratory inhibitors, rotenone or antimycin, relieved it.

The inhibitory effect of ADP on cholesterol side-chain cleavage activity supported by extra-mitochondrial NADH or NADPH might also be explained by an effect on mitochondrial permeability to these latter nucleotides. ATP, an agent which prevents the cyclical low amplitude swelling and contraction characteristic of intact mitochondria, was demonstrated to have a marked inhibitory effect on cholesterol side-chain cleavage supported by exogenous NADPH or NADH in preparations of porcine luteal mitochondria (see Table 5.4.). Mitochondria in such contracted states are known to show virtually complete impermeability to NADPH (Hirshfield and Koritz, 1964). It is conceivable therefore that the addition of ADP to preparations of porcine luteal mitochondria, and its subsequent phosphorylation to ATP, would cause

TABLE 5.4

THE EFFECT OF ATP ON CHOLESTEROL SIDE-CHAIN CLEAVAGE SUPPORTED BY NADPH OR NADH

COFACTORS PRESENT	[4-^{14}C] CHOLESTEROL CLEAVED (%)
NADPH	14.1
NADPH + ATP	1.1
NADH	4.3
NADH + ATP	0.5

NADH, NADPH, and ATP were present at final concentrations of 5 mM, 5 mM, and 1 mM respectively. Mitochondria (about 5 mg) were incubated in medium "B" at 37^{o} for 1 hour, and cholesterol side-chain cleavage activity was determined as described in Chapter 2.

a similar reduction in NAD(P)H uptake and cholesterol side-chain cleavage.

A hypothetical scheme of electron transport in the porcine luteal mitochondrion in which a role for a non-energy dependent pyridine nucleotide transhydrogenase is indicated, is given in Chapter 7 (Fig. 7.1).

5.6 The relationship between succinate dehydrogenase and cholesterol side-chain cleavage

The studies described in the previous sections of this Chapter have concerned $NAD(P)^+$ specific dehydrogenases, and they demonstrated that under the experimental conditions used there was no obligatory requirement for energy to drive electrons from pyridine nucleotides to the site of the cholesterol side-chain cleavage reaction. The following experiments were intended to provide analogous information about electron transfer from the flavoprotein associated with succinate dehydrogenase to the same mixed function oxidase.

The study of mitochondrial steroid hydroxylations in the adrenal cortex has led several groups to suggest that succinate can support cholesterol side-chain cleavage via reversed electron flow from succinate dehydrogenase to NAD^+, an endergonic process (Chance and Hollunger, 1961), followed by an energy linked transhydrogenation to NADPH and reduction of the NADPH-cytochrome P450 reductase (Koritz, 1966; Hall, 1967a,b and Purvis *et al.*,

TABLE 5.5

THE EFFECT OF RESPIRATORY INHIBITORS AND UNCOUPLING AGENTS ON SUCCINATE SUPPORTED CHOLESTEROL SIDE-CHAIN CLEAVAGE, AND OXYGEN UPTAKE

INHIBITOR ADDED	OXYGEN UPTAKE RATE (nmoles O_2/mg/min)		[4-^{14}C] CHOLESTEROL CLEAVED (%)
	State 4	State 3	
None	8.0	28.0	15.4
Antimycin	2.4	2.1	1.9
Cyanide (1 mM)	1.0	1.1	0.6
DNP (100 μM)	30.0	29.0	1.2

Luteal mitochondria (about 5 mg) were incubated in medium B; "State 4" and "State 3" oxygen uptake rates refer to those measured before, and after addition of ADP (1mM) respectively. Antimycin, when added, was present at a concentration of 330 ng. per mg. of mitochondrial protein. The concentration of succinate present in all incubations was 10 mM. Cholesterol side-chain cleavage estimations were done in the absence of exogenous ADP.

1968). It was of interest therefore to investigate this hypothesis in relation to cholesterol side-chain cleavage in the luteal mitochondrion.

(5.6.1) Cholesterol side-chain cleavage supported by succinate, and the effect of various inhibitors on this system:

Mitochondrial incubations in which cholesterol side-chain cleavage activity was determined were carried out in medium "B"; in parallel experiments the oxygen uptake of these preparations was monitored polarographically. The electron donor was in all cases 10 mM succinate. The effects of including various inhibitors in these systems are given in Table 5.5. It can be seen that the respiratory inhibitors antimycin, or cyanide abolished cholesterol side-chain cleavage, as well as inhibiting oxygen uptake, in the presence of succinate. The uncoupling agent DNP, while markedly stimulating State 4 oxygen uptake in the presence of succinate, had an inhibitory effect on cholesterol side-chain cleavage activity.

These results indicated that mitochondrial oxidative phosphorylation might be involved in the process by which succinate supplied electrons for cholesterol side-chain cleavage in the porcine corpus luteum. In some other tissues (e.g. liver, heart) reversed electron flow from succinate to NAD^+ has been demonstrated (Chance and Hollunger, 1961; Low et al.,

TABLE 5.6

THE EFFECT OF VARIOUS INHIBITORS ON SUCCINATE-SUPPORTED CHOLESTEROL SIDE-CHAIN CLEAVAGE

Substrate and other compounds present	Percent conversion of [4-^{14}C] cholesterol to [4-^{14}C] steroid products		
	(a) [4-^{14}C] Pregnenolone	(b) [4-^{14}C] Progesterone	(c) Total conversion
No additions	0.1	0.3	0.4
Succ.	4.5	14.3	18.8
Succ. + rotenone	5.6	17.9	23.5
Succ. + amytal	3.0	7.7	10.7
Succ. + antimycin	1.2	1.6	2.8
Succ. + rotenone + ATP	6.5	12.7	19.1
Succ. + amytal + ATP	4.5	6.4	10.9
Succ. + antimycin + ATP	14.1	8.6	22.7
Succ. + antimycin + ATP + rotenone	15.9	7.8	23.7
Succ. + antimycin + ATP + amytal	9.4	3.5	12.9
Succ. + antimycin + ATP + rotenone + amytal	9.5	3.5	13.0
Succ. + rotenone + amytal	4.7	8.5	13.2
Succ. + rotenone + antimycin	3.6	7.9	11.5
Succ. + antimycin + amytal	0.5	1.2	1.7
Succ. + rotenone + amytal + ATP	5.8	6.8	12.6
Succ. + ATP	6.4	14.5	20.9
ATP	1.7	4.5	6.2
ATP + rotenone	4.1	4.2	8.3
ATP + amytal	3.9	3.7	7.6
ATP + antimycin	0.3	0.8	1.1

1961); this electron transfer process is thought to require an energy expenditure equivalent to 1 high energy phosphate bond ("X~P") per $2e^-$ transferred and in some preparations can be driven by exogenous ATP (Ernster and Lee, 1967). It was decided therefore to investigate the effect of adding ATP to porcine mitochondrial incubations in which succinate and antimycin were present, in relation to cholesterol side-chain cleavage activity; in addition the action of compounds which might be expected to interfere with reversed electron flow between succinate and NAD^+, namely rotenone and amytal, was studied in these preparations. The results of such experiments are given in Table 5.6. It can be seen that antimycin virtually abolished cholesterol side-chain cleavage activity supported by succinate; this effect could be relieved by the further addition of ATP. It thus seems probable that electron transfer from succinate to the cholesterol side-chain cleavage system is strictly energy-dependent, unlike the situation described previously for NAD^+-linked substrates. The involvement in this process, however, of reversed electron flow in the NADH - cytochrome b portion of the mitochondrial respiratory electron transport chain could not be conclusively demonstrated. Rotenone and amytal, although they are both said to prevent electron transfer in this region (Horgan et al., 1968), brought about very different effects on the present incubation

system: rotenone stimulated cholesterol side-chain cleavage occurring in the presence of succinate, or succinate, antimycin and ATP, whereas amytal in all these cases inhibited the reaction. Rotenone is a more specific inhibitor of electron transport than amytal (Ernster et al., 1963), especially when used in conjunction with BSA (Horgan et al., 1968), and acts at a site on the oxygen side of the NADH dehydrogenase flavoprotein. It seemed likely therefore, that this site was not involved in the transfer of electrons from succinate for cholesterol side-chain cleavage, a finding which was made more plausible by the results of later experiments. (see section (5.6.3) below). A similar situation where rotenone and amytal were shown to have contrasting effects on cholesterol side-chain cleavage activity, is described in Chapter 6; the electron donor in this case was palmityl-carnitine (see Fig. 6.4). It was thought possible that the inhibitory characteristics of amytal in the above situations might be a reflection of its inhibitory effects on energy transfer reactions (Siekevitz et al., 1958; Low, 1959 and Ernster et al., 1963). A hypothesis which accounts for the results of these inhibitors in situations where flavoprotein-dependent dehydrogenases are supporting the side-chain cleavage of cholesterol is discussed in greater detail in Chapter 7 (see Fig. 7.1).

(5.6.2) The reduction of cytochrome P450 by succinate in the presence of some inhibitors:

The presence of cytochrome P450 in porcine luteal mitochondrial preparations, and its reduction by dithionite, was described in Chapter 3. Cammer and Estabrook, (1967b), using bovine adrenal mitochondria, demonstrated that the addition of succinate to these preparations caused a reduction not only of the cytochromes of the respiratory electron chain, but also those concerned with steroid hydroxylation in these mitochondria; when succinate was used as an electron donor, reduction of these latter electron carriers could be prevented by addition of inhibitors of mitochondrial respiration, such as cyanide. An analogous spectrophotometric study of porcine luteal mitochondria was undertaken to investigate further the relationship between succinate and cholesterol side-chain cleavage in this tissue.

Intact mitochondrial preparations were isolated from porcine corpora lutea, as described in Chapter 2, and made up in medium "B" to a mitochondrial protein concentration of about 7 mg per ml. This suspension was divided evenly between two silica micro-cuvettes, A and B (total capacity of each cuvette, 1.7 ml., light path 1 cm) and the following procedure was carried out in a Shimadzu split beam recording spectrophotometer, model MPS-50L:

(a) The spectrophotometer was set to scan wavelengths

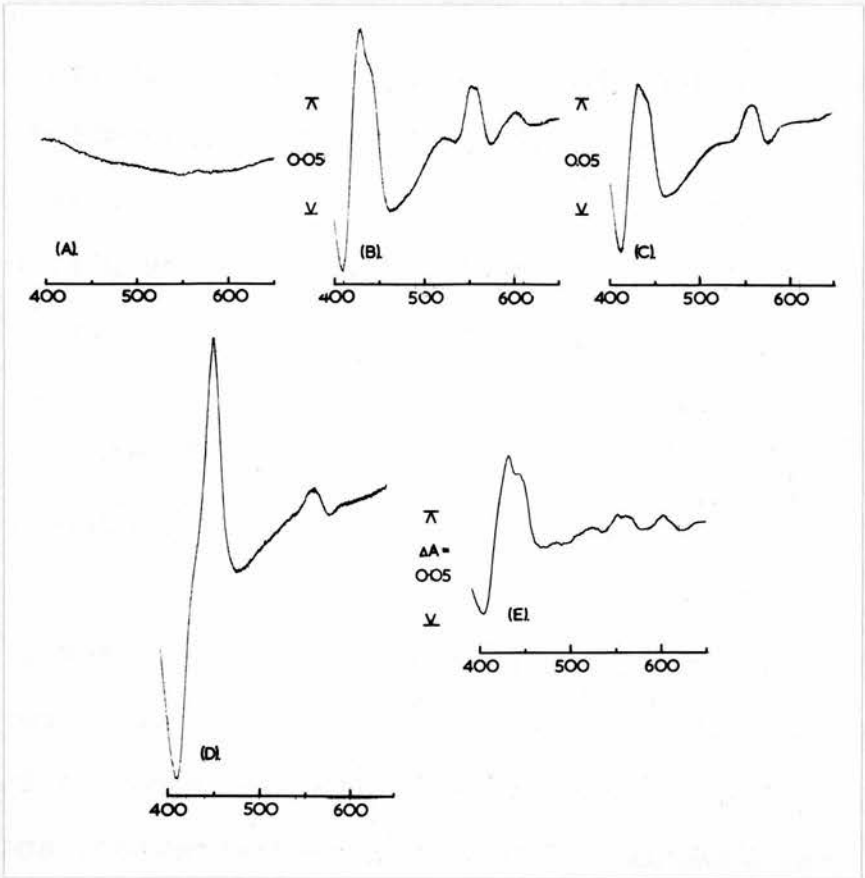


FIG. 5.7: Difference spectra of intact porcine luteal mitochondrial preparations. Both cuvettes contained 3.5 mg of mitochondrial preparation suspended in 1.5 ml of medium "B". (a) zero baseline (b) "total" cytochromes, reduced minus oxidized (c) reduced cytochrome P450 minus oxidized P450 (d) reduced cytochrome P450:CO complex minus oxidized P450 (e) "respiratory" cytochromes, reduced minus oxidized.

from 380-650 nm, and a difference spectrum of A minus B was taken; since conditions in both cuvettes were the same (i.e. oxidized, no substrate) the spectrum obtained was a zero base line (see Fig. 5.7(a)).

(b) Succinate (10 mM final concentration) was added to cuvette A which, when sealed, became anaerobic. A difference spectrum of A (succinate, anaerobic) minus B (no substrate, oxidized) was recorded and is shown in Fig. 5.7(b). This spectrum shows a very marked absorption in the Soret region, and also a prominent peak at 553 nm. It is in fact a composite spectrum, in which both the reduced cytochromes of the classical mitochondrial respiratory chain and also cytochrome P450 contribute.

(c) In order to obtain a difference spectrum of reduced cytochrome P450 only, the following procedure was carried out. Cuvette A was fully reduced as in (b). Potassium cyanide (1 mM final concentration) and then succinate (10 mM) were added to cuvette B which was open to air. Under these conditions the components of the mitochondrial respiratory electron transfer chain became reduced, while those of the NADPH-cytochrome P450 reductase concerned with cholesterol side-chain cleavage system remained oxidized (see previous section 5.6.1). By taking a difference spectrum of A (reduced "respiratory" and "steroidogenic" cytochromes) minus B (reduced "respiratory", oxidized "steroidogenic" cytochromes) a spectrum of cytochrome P450 (reduced minus oxidized)

was obtained (see Fig. 5.7(c)). This has a prominent Soret peak at 432 nm and a broad α-band with a maximum at about 557 nm. Carbon monoxide was then bubbled through both cuvettes for about 15 seconds and a further difference spectrum taken (see Fig. 5.7(d)). It shows an intense absorption at about 449 nm which is characteristic of the reduced cytochrome P450:CO complex.

In order to demonstrate the presence of the mitochondrial respiratory cytochromes in the porcine corpus luteum it is necessary to ensure that cytochrome P450 remains oxidized in both cuvettes. Cyanide was therefore added to cuvette A, followed by succinate, thus reducing only the electron carriers of the mitochondrial respiratory cytochrome chain. Cuvette B was left aerobic with no substrate added to the mitochondrial preparation. When this procedure was carried out a difference spectrum (A-B) like that shown in Fig. 5.7(e) was obtained: the absorption bands with maxima at 605 and 445 nm, 561 and 428 nm, and 552 and 419 nm are characteristic of the normal respiratory chain cytochromes ($a+a_3$), b, and ($C+C_1$), respectively. Inspection of the spectra illustrated in Fig. 5.7. confirms that spectrum (b) is the sum of spectra (c) and (e).

By repeating the procedure above it was possible to demonstrate that as well as cyanide, antimycin or carbon monoxide would prevent the reduction of cytochrome P450 in the presence of succinate; furthermore, if ATP (2 mM)

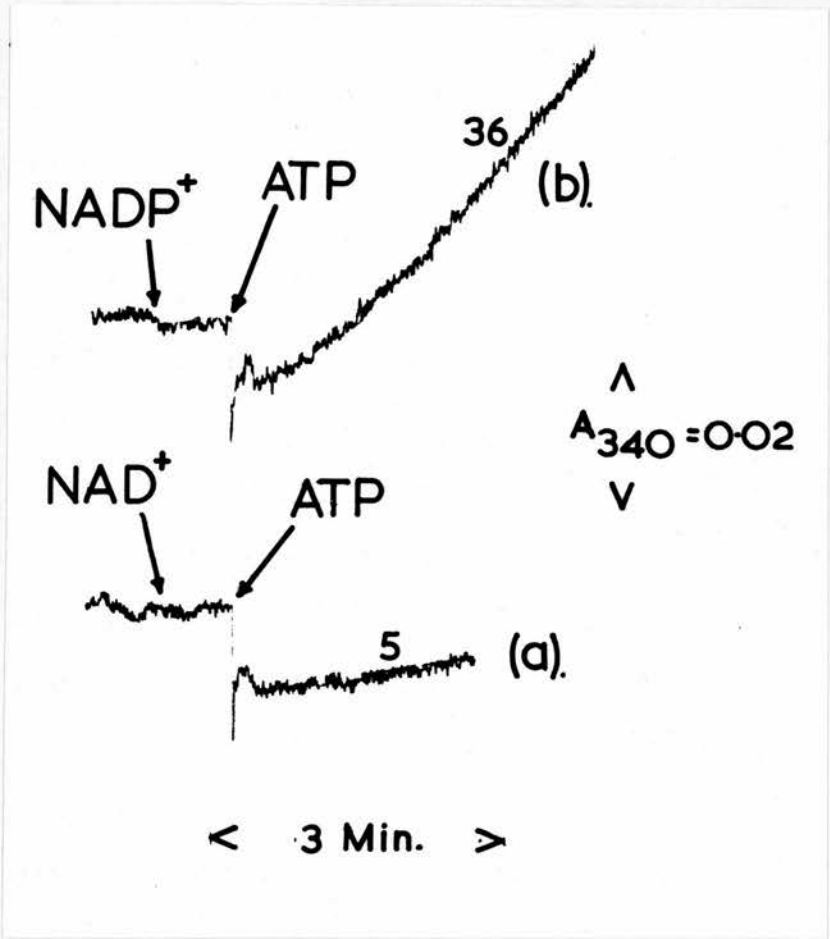


FIG. 5.8: Energy dependent reduction of $NAD(P)^+$ by succinate Both cuvettes contained 0.2 mg of sonicated preparation of porcine luteal mitochondria, in 3mls of medium "B", with succinate (10mM) also present. NAD^+ (in (a)) or $NADP^+$ (in (b)), and ATP were added to the experimental cuvettes as shown, to final concentrations of 300 µM, 300 µM and 2mM. The temperature was 23°C, and the traces show the change in absorbance at 340 nm; the numbers above the traces refer to the rate of $NAD(P)^+$ reduction, expressed as nmoles/min/mg.

was then added, this inhibition was relieved, and cytochrome P450 gradually became reduced.

(5.6.3) Energy-dependent reduction of $NAD(P)^+$ by succinate:

The experiments described in the previous two sections (5.6.1 and 5.6.2) demonstrated that succinate could donate electrons to the cytochrome P450 reductase involved in cholesterol side-chain cleavage activity in luteal mitochondrial preparations. In an attempt to further define the electron pathways involved in this process, a spectrophotometric study of the reduction of $NAD(P)^+$ by succinate was undertaken: results of such an experiment are shown in Fig. 5.8. A Shimadzu MPS-50L recording spectrophotometer, programmed for constant wavelength operation was used. The enzyme source was a sonicated preparation of porcine luteal mitochondria. Trace (a) (Fig. 5.8) shows the change in absorbance at 340 nm when additions of succinate (to a final concentration of 10 mM), NAD^+ (to 300 μM) and finally ATP (to 2 mM) were made to a cuvette containing 0.2 mg of sonicated mitochondria suspended in isotonic medium "B" (see Table 2.2); trace (b) shows an analogous experiment, in which $NADP^+$ (to 300 μM) replaced NAD^+. It can be seen that ATP was necessary to allow $NAD(P)^+$ reduction to occur in the presence of succinate, and also that $NADP^+$ was apparently reduced at a much greater rate ($>$7-fold) than NAD^+. Further experimental controls (not illustrated) established that in the absence of one of any of the

substrates succinate, $NAD(P)^+$ or ATP, no optical density change at 340 nm occurred.

These results suggested the possibility that succinate could donate electrons to $NADP^+$ by a route not necessarily involving NADH as an intermediate. The implications of this finding, along with those of the previous section (5.6.1) involving effects of rotenone and amytal, are discussed in Chapter 7 (see section 7.4.(iii))

SUMMARY

1. Isocitrate and malate dehydrogenases were assayed in porcine luteal mitochondrial preparations. Although some reduction of $NADP^+$ occurred in the presence of malate, much more was evident when isocitrate replaced this substrate.
2. Several TCA intermediates were shown to be effective electron donors for cholesterol side-chain cleavage activity; citrate isocitrate, fumarate and malate were somewhat more effective than succinate, which in turn was a better electron donor for the reaction than α-ketoglutarate or oxaloacetate.
3. Isocitrate dehydrogenation was shown to be capable of donating electrons for cholesterol side-chain cleavage in the presence of inhibitors of NADH oxidase; in fact the addition of these inhibitors stimulated the reaction in such situations.
4. α-Ketoglutarate dehydrogenation supported cholesterol

side-chain cleavage even when concomitant ATP formation was prevented.

5. NADH and NADPH were shown to be more effective electron donors for cholesterol side-chain cleavage when inhibitors of NADH oxidase were present, than when such compounds were omitted. The inhibitory effects of DNP and ADP on cholesterol side-chain cleavage activity supported by these pyridine nucleotides could be prevented by the addition of rotenone or antimycin.

6. The results with NAD(P)H, and α-ketoglutarate were interpreted to indicate that a non-energy dependent pyridine nucleotide transhydrogenase was involved in the transfer of electrons from these substrates to the mixed function oxidase associated with cholesterol side-chain cleavage.

7. Succinate was shown to reduce cytochrome P450, and support cholesterol side-chain cleavage, via an energy dependent route. The experimental evidence indicated that this electron transfer pathway might not involve the reduction of NAD+.

CHAPTER 6

CHAPTER 6

SOME RELATIONSHIPS BETWEEN CHOLESTEROL ESTERS, FFA METABOLISM AND CHOLESTEROL SIDE-CHAIN CLEAVAGE.

The results presented in Chapters 4 and 5 concerned the involvement of TCA intermediates with cholesterol side-chain cleavage activity. Although it was shown *in vitro* that these substrates can support the reaction it was not known which substrates are in fact the electron donors under physiological conditons. Some of the preliminary studies described in this thesis had indicated that fatty acids might be capable of acting in this capacity (see section 3.6); in addition measurements of the RQ in ovarian tissue had previously suggested (Cooper and Stevenson, unpublished results) that the major endogenous respiratory substrate was lipid. For these reasons it was decided to investigate further the possible role of fatty acids as electron donors for porcine luteal cholesterol side-chain cleavage.

The abundant cytoplasmic lipid droplets found in luteal tissue (see section 1.2.iv) are a major source of fatty acids, which are present in esterified form with cholesterol. Since LH *in vivo* is well known to stimulate the hydrolysis of these esters (see section 1.3.2), it seemed relevant to investigate the capability of the tissue to utilize cholesteryl esters as substrates for mitochondrial cholesterol side-chain cleavage.

Previous investigations of cholesterol ester metabolism in steroidogenic tissue have been directed principally at the cholesterol moiety of the molecule, in view of the role of free cholesterol as a precursor of the steroid hormones; the fate of the fatty acid released by cholesteryl ester hydrolysis has been almost entirely ignored, although speculations as to its further role in the metabolism of the steroidogenic cell have been made (Bjersing, 1967; Peron, 1969).

6.1 Cholesterol ester hydrolysis: the availability of the released cholesterol for mitochondrial side-chain cleavage.

The presence of an enzyme in adrenal cortex homogenates capable of hydrolysing cholesteryl esters was demonstrated by Dailey et al., (1963); one of the products of this hydrolysis, free cholesterol, was shown to be incorporated into adrenal steroids. Coutts and Stansfield, (1967 and 1968) demonstrated similar cholesterol esterase activity in rat and bovine corpora luteal preparations; this enzyme activity was apparently not confined to one intracellular location, being found throughout all the subcellular fractions, although it was maximal in the 5000 xg. pellet.

The utilization of cholesteryl esters by porcine luteal mitochondrial preparations was investigated as

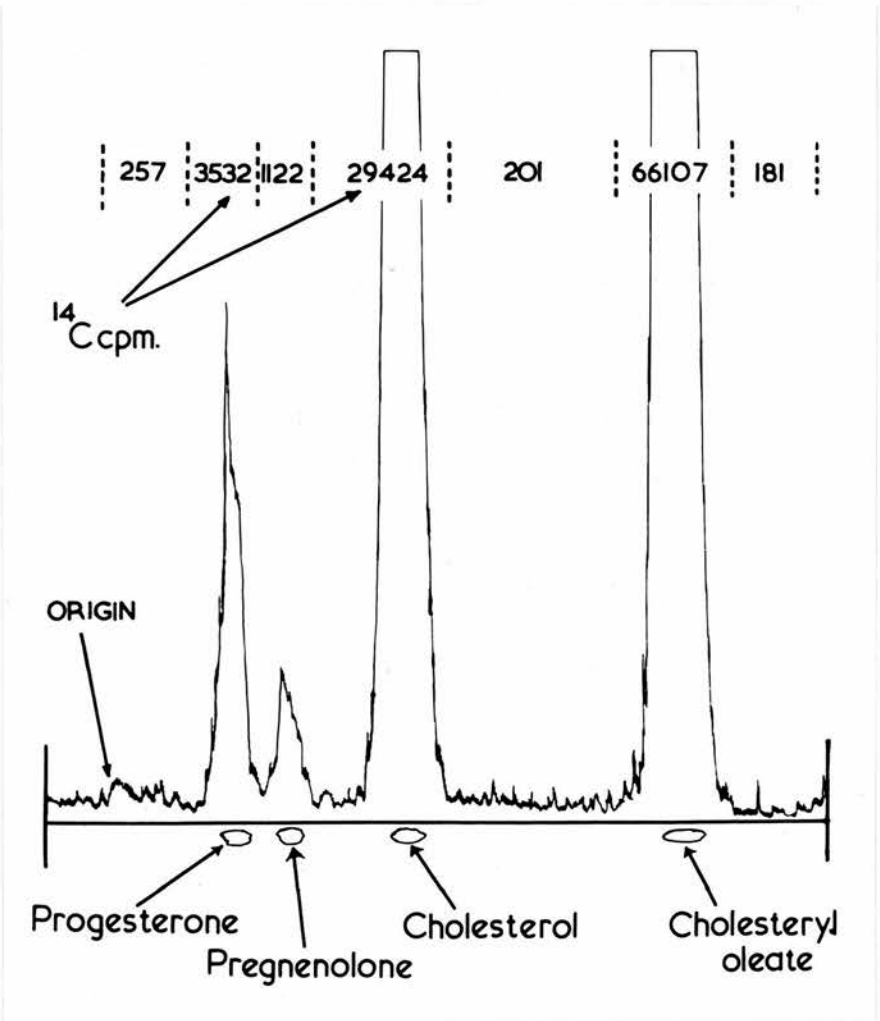


FIG. 6.1: Separation by TLC, of the metabolites resulting from incubating [4-^{14}C] cholesteryl oleate with intact porcine luteal mitochondrial preparations. The solvent system was di-isopropyl ether, petroleum spirit (60^{o}-80^{o}), acetic acid (70:30:2, v/v).

follows, using cholesteryl oleate as a representative ester: this has been shown to be the most abundant ester in other luteal tissues (Armstrong, 1969). [4-^{14}C] cholesteryl oleate of high specific activity (46 μCi/mg) was obtained from the Radiochemical Centre, Amersham. It was purified by TLC before use, in a manner similar to that described in Chapter 2 for [4-^{14}C] cholesterol. Cholesterol side-chain cleavage incubations were set up in which aliquots of 100,000 cpm (about 1μg) of [4-^{14}C] cholesteryl oleate replaced the usual radioactive substrate; preparations of intact mitochondria were incubated in medium "B" for periods of up to two hours. The electron donor used was 10 mM D,L-isocitrate. Reactions were terminated, sterol and steroids extracted, and TLC carried out as described in Chapter 2. The TLC solvent system used (section 2.4) was found adequate to separate unchanged sterol ester from the products of its hydrolysis (see Fig. 6.1). The results of such an experiment are given in Fig. 6.2, which shows a time course of the utilization of the radioactive substrate in terms of the production of [4-^{14}C] metabolites. [4-^{14}C] cholesterol is the first major product of the incubation, while the subsequent gradual accumulation of more polar steroids, [4-^{14}C] pregnenolone and [4-^{14}C] progesterone, follows a similar pattern

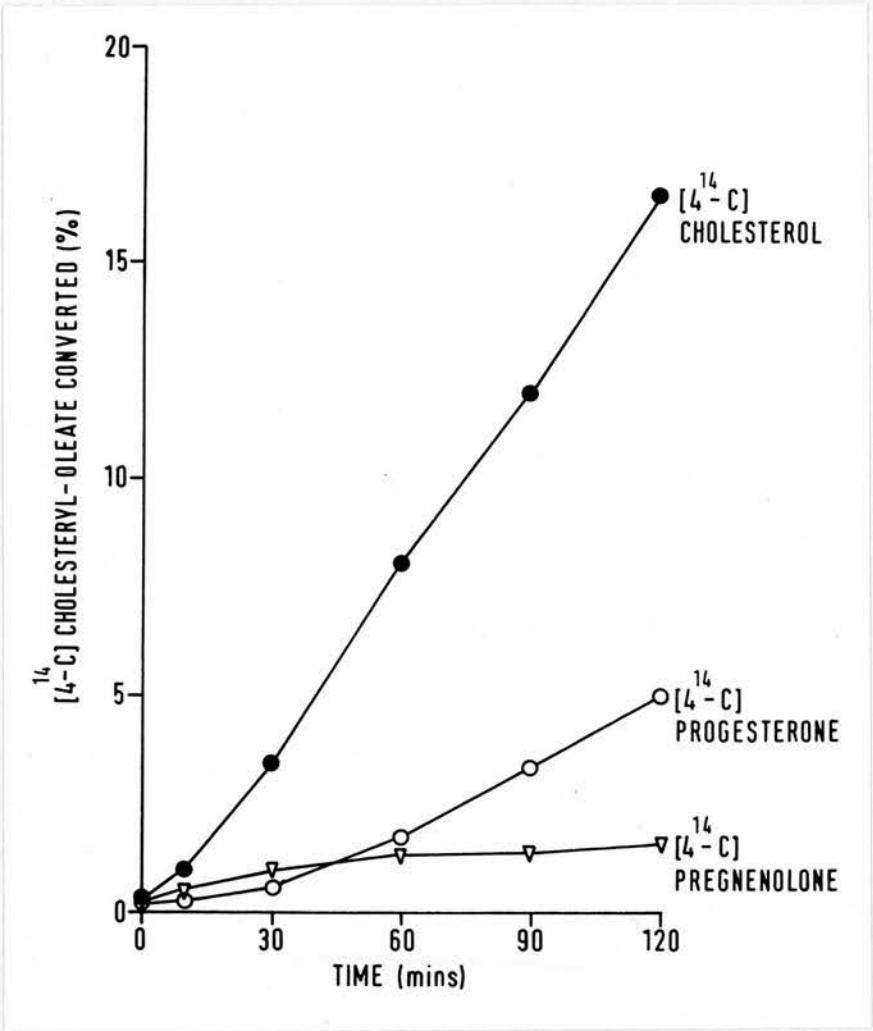


FIG. 6.2: Time course of [4-^{14}C] cholesteryl oleate utilization by preparations of porcine luteal mitochondria. 3 mg of mitochondrial protein were present, in medium "B", and the electron donor was D.L-isocitrate (10mM).

to that shown previously (Fig. 3.10) for side-chain cleavage incubations in which [4-^{14}C] cholesterol was used as substrate. No compound corresponding to pregnenolone-oleate was isolated and this finding, together with data like that shown in Fig. 6.2 suggests that hydrolysis of the ester linkage is probably obligatory before cleavage of the side-chain of cholesterol can procede. Raggatt and Whitehouse, (1966) in their study on adrenocortical cholesterol oxidation found that although cholesteryl sulphate could undergo side-chain cleavage to pregnenolone sulphate without prior hydrolysis of the ester linkage, acyl esters of cholesterol could not.

6.2 Utilization of FFA as electron donors for cholesterol side-chain cleavage.

Although the ovarian cholesterol side-chain cleavage reaction requires NADPH when a "soluble" enzyme source is used (Sulimovici and Boyd, 1968b) various TCA intermediates have been shown to act as electron donors when the reaction was studied in intact mitochondrial preparations (see Fig. 5.1). However, the actual energy source *in vivo* remains unknown. Sowerby (1968) and Channing and Villee (1966) showed that uptake or utilization of glucose by ovarian luteal tissue was increased by gonadotropic stimulation of the organ. The fate of this glucose was investigated by

Flint and Denton (1969), who found that it was metabolised mainly to lactate and pyruvate; their study suggested that it was not a principal source of either carbon atoms or ATP for ovarian steroidogenesis. Significantly, in these and further studies (1970a,b) the same authors showed that oxygen uptake was unaffected by the addition in vitro of glucose, and that the oxidation of this substrate accounted for less than one quarter of the total oxygen consumption of the LH-stimulated tissue. They suggested (1970a) that fatty acids might be the main endogenous fuel for ovarian respiration; a similar conclusion was reached by Cooper and Stevenson (personal communication) as a result of respiratory quotient determinations on such tissue.

In view of these latter studies, and the results in the previous section (6.1), it was decided to investigate the role of fatty acids as a potential source of electrons for cholesterol side-chain cleavage.

Preparations of intact porcine luteal mitochondria were incubated in medium "B", and cholesterol side-chain cleavage activity estimated as described in Chapter 2. Fatty acids were added to these incubations as free acids, to a final concentration of 100μM, in small volumes of ethanol. Palmitic, stearic, oleic, linoleic and linolenic acids were all found to be

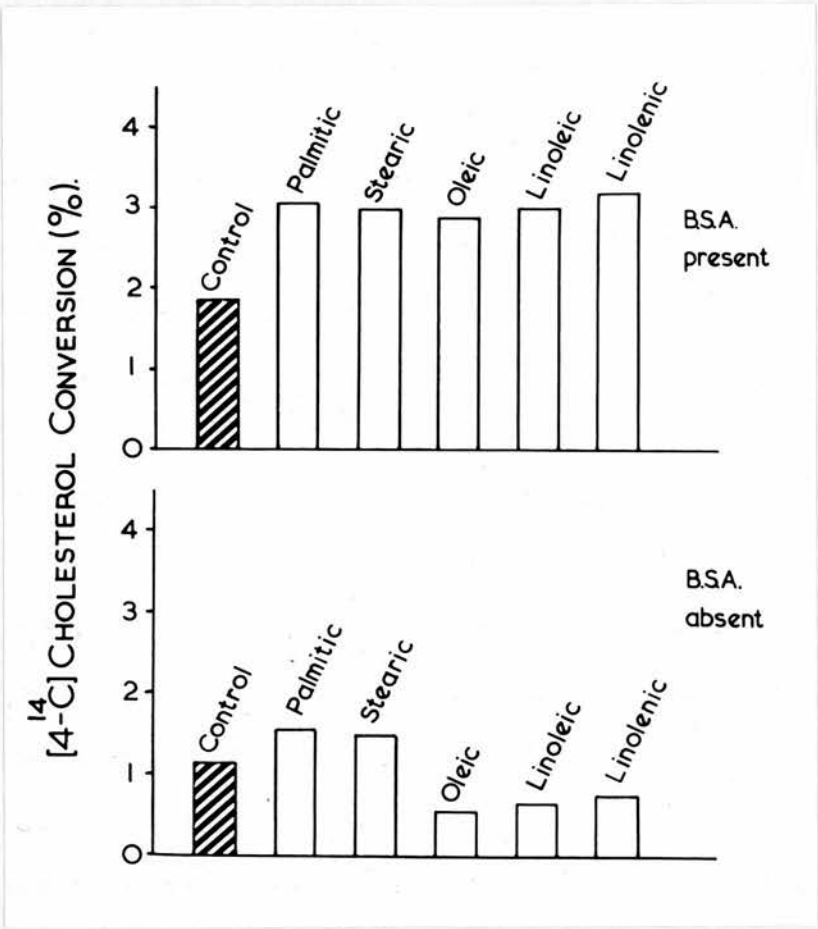


FIG. 6.3: The effect of fatty acids as electron donors for the cholesterol side-chain cleavage reaction. Incubations were carried out in medium "B", with and without BSA, as shown. Small amounts (500 μM) of succinate were also present, to allow fatty acid activation.

completely ineffective in supporting cholesterol side-chain cleavage in intact porcine luteal preparations when used with no other co-factors present. This finding was not unexpected as it is well known that fatty acids are potent uncoupling agents, and powerful inducers of mitochondrial swelling (Van den Bergh, 1967). In a discussion of mitochondrial FFA utilization, Lehninger (1965) has suggested that the addition of "catalytic" amounts of any easily permeable TCA intermediate, along with the fatty acid substrate, allows complete oxidation of the latter by providing both a "priming" source of ATP for fatty acid activation and also oxaloacetate as an acceptor for the acetyl CoA generated as a result of β-oxidation. Accordingly, the effect of including small amounts (500 μM) of succinate in incubations where fatty acids were being evaluated as electron donors for cholesterol side-chain cleavage was investigated. The results of such an experiment are shown in Fig. 6.3. It can be seen that providing 1% BSA was included in the incubation media, several fatty acids would support additional cholesterol side-chain cleavage activity when compared to succinate controls in which all constituents except the fatty acid were present. In the absence of BSA however, only the saturated acids, palmitic and stearic, supported measurably more cholesterol side-chain cleavage activity

than the appropriate controls. It can be seen that the presence of BSA, which is known to protect mitochondria from fatty acid-induced swelling or uncoupling (Bjorntorp et al., 1964), allowed more cholesterol side-chain cleavage activity to take place than when it was not included, and furthermore, in the absence of BSA, the unsaturated acids (oleic, linoleic and linolenic) actually inhibited cholesterol side-chain cleavage activity when compared to the succinate control. Unsaturated fatty acids are known to be more potent uncoupling agents than the corresponding saturated fatty acids (Borst et al., 1962), and cholesterol side-chain cleavage supported by succinate is energy dependent in this tissue (see section 5.6, Chapter 5). The results of these experiments indicated that the uncoupling characteristics of the fatty acid substrates were probably interfering with the cholesterol side-chain cleavage reaction.

6.3 A possible role for acyl-carnitines as electron donors for cholesterol side-chain cleavage activity.

The work of Fritz (1955, 1959, 1961) and Fritz and Yue (1963) established that carnitine (β-hydroxy, γ-trimethylammonium butyrate) increased rates of long-chain fatty acid oxidation by a variety of tissues. Bremer (1962, 1968) suggested that carnitine's role was in carrying acyl-groups to the inner mitochondrial

TABLE 6.1

THE EFFECT OF CARNITINE, CO-ENZYME A AND ATP ON THE SIDE-CHAIN CLEAVAGE OF CHOLESTEROL IN THE PRESENCE OF OLEIC ACID

SUBSTRATE and COFACTORS ADDED	percent conversion of [4-^{14}C] cholesterol to the following products: [4-^{14}C] PREGNENOLONE	[4-^{14}C] PROGESTERONE	TOTAL CONVERSION
No additions	0.4	0.3	0.7
Oleic Acid	0.6	0.8	1.4
Oleic acid + carnitine	0.7	0.6	1.3
Oleic acid + Co A	0.7	0.7	1.4
Oleic acid + ATP	8.4	5.6	14.0
Oleic acid + carnitine + Co A	0.8	0.6	1.4
Oleic acid + carnitine + ATP	11.0	5.9	16.9
Oleic acid + Co A + ATP	17.4	6.4	23.8
Oleic acid + Co A + carnitine + ATP	18.8	9.4	28.2
Carnitine	0.7	0.5	1.2
Carnitine + Co A	1.1	0.2	1.3
Carnitine + ATP	9.0	6.0	15.0
Carnitine + ATP + Co A	13.6	8.0	21.6
Co A	1.0	0.5	1.5
Co A + ATP	10.0	7.2	17.2
ATP	7.5	5.1	12.6

membrane, after the ATP-dependent fatty acid activation and acyl-CoA formation had taken place in an extra-mitochondrial compartment, or perhaps in the outer mitochondrial membrane (Norum et al., 1966, Farstad et al., 1967). It thus seemed relevant to investigate the possibility that acyl-carnitine formation might be involved in FFA utilization by porcine luteal mitochondrial preparations.

Incubations of mitochondria, in medium "B" containing 1% BSA were carried out as outlined in Table 6.1. The fatty acid substrate used was oleic acid, at a low final concentration (75 µM) to minimize uncoupling effects; the final concentrations of the other cofactors, CoA, carnitine and ATP, when included were 50 µM, 5mM and 3mM respectively, as suggested by Fritz and Yue (1963). Cholesterol side-chain cleavage activity in these conditions was determined in the usual way (Chapter 2), incubations being terminated after an hour.

The results (Table 6.1) demonstrated that oleic acid could support high levels of cholesterol side-chain cleavage, 28% of the radioactive substrate being cleaved in an hour, when CoA, carnitine and ATP were included. The progressive increase in activity through the series oleic acid + ATP (14% conversion), oleic acid, ATP + carnitine (16.9% conversion), oleic acid ATP, carnitine + CoA (28.2% conversion) might indicate that

a similar series of reactions to those proposed by Fritz and Yue (1963) for heart muscle preparations, was occurring in the porcine luteal mitochondrial preparations: i.e.

$$\text{Oleic acid} + \text{ATP} + \text{CoA} \xrightarrow{Mg^{++}} \text{oleyl CoA} + \text{AMP} + \text{PP}$$

$$\text{Oleyl CoA} + \text{carnitine} \rightarrow \text{oleylcarnitine} + \text{CoA}.$$

The oleyl carnitine was then presumably utilized for intramitochondrial β-oxidation, and could act as an electron donor for cholesterol side-chain cleavage.

It was apparent that even in the absence of added oleic acid, appreciable rates of cholesterol side-chain cleavage were supported when ATP, CoA and carnitine were present. This suggests that under these conditions endogenous substrates were being utilized, and the progressive increase in activity supported by ATP (12.6%), ATP + carnitine (15.0%) and ATP, carnitine + CoA (21.6%) might be a consequence of the lipid nature of these substrates.

6.4 The effect of respiratory inhibitors on cholesterol side-chain cleavage supported by palmityl-carnitine.

Palmityl-carnitine was prepared as suggested by Fritz and Yue (1963) by a method based on the palymityl choline synthesis procedure of Gomori (1948): an equimolar mixture of palymityl chloride (Eastman Chemicals, Ltd., Liverpool) and carnitine hydrochloride

(Sigma, London) was heated to about 130°C under constant stirring. Evolution of HCl ceased after 1 hour, and the mixture solidified into a thick paste. This was extracted with petroleum spirit (bp.60°-80°), and the residue was subsequently extracted by refluxing with acetone. The acetone filtrate was kept at -20° overnight while crystallization occurred. The granular white crystals were harvested by centrifugation, and purified by two further recrystallizations from hot acetone. The yield was very low, being about 2% of the theoretical, and similar to that reported by Fritz and Yue (1963). A sample of palmityl carnitine was also obtained as a gift from Dr. E. Simpson (University of Edinburgh). In subsequent studies the same results were obtained with palmityl-carnitine from either source.

The efficiency of palmityl carnitine as an electron donor for cholesterol side-chain cleavage was investigated using preparations of intact porcine luteal mitochondria. The effect of the respiratory inhibitors rotenone, amytal and antimycin on this system was also evaluated in a study analogous to that described for various TCA intermediates, in Chapter 5. The results of such experiments are given in Fig. 6.4. Palmitylcarnitine (50 μM) supported 6.3% conversion of [4-^{14}C] cholesterol to radioactive steroid products.

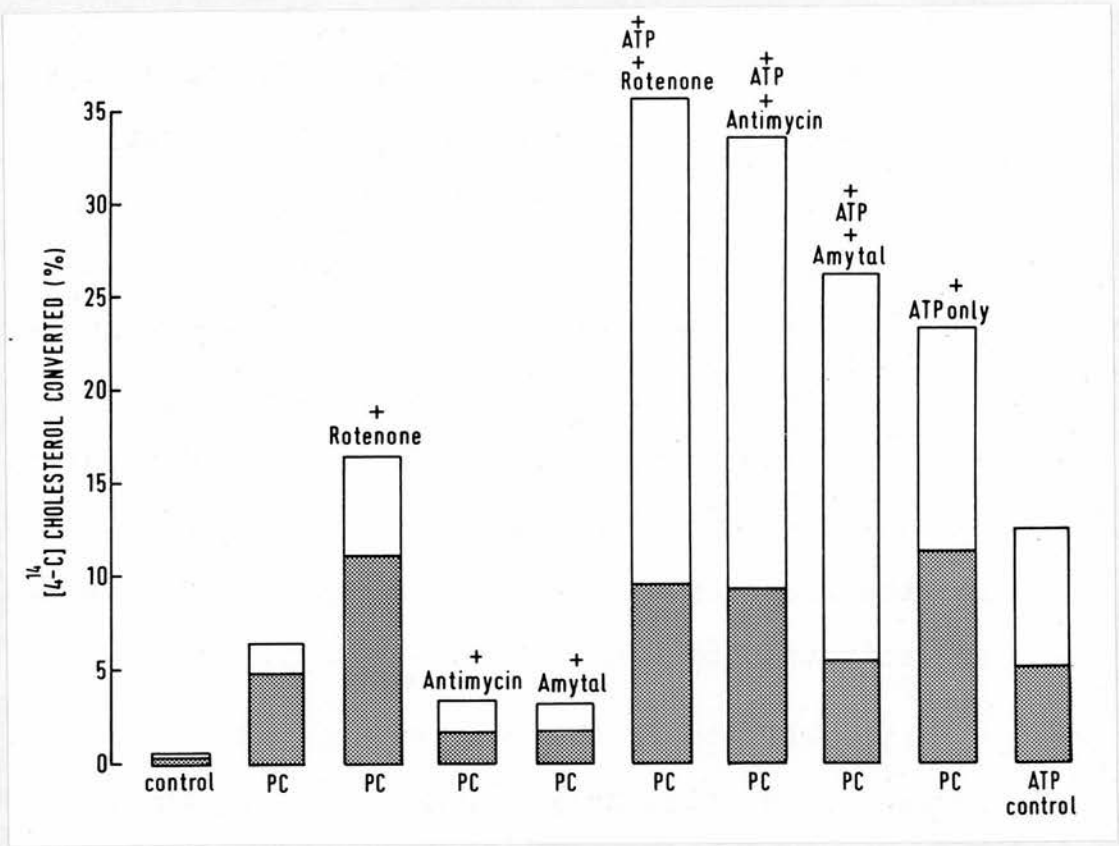


FIG. 6.4: The effect of respiratory inhibitors on cholesterol side-chain cleavage supported by 50 μM palmitylcarnitine. Rotenone, amytal, antimycin and ATP when present were at final concentrations of 10 μM, 1.8 mM, 330ng/mg of mitochondrial protein and 3 mM respectively.

Although the addition of rotenone (10 μM) to this system markedly stimulated cholesterol side-chain cleavage activity, the presence of either amytal (1.8 mM) or antimycin (330 ng per mg. of mitochondrial protein) resulted in about 50% inhibition of the reaction. ATP (3 mM) stimulated side-chain cleavage in the presence of palmitylcarnitine by about 400%. It also abolished the inhibitory effects of both amytal and antimycin on the system, resulting in the case of ATP + rotenone, or ATP + antimycin in very high levels of cholesterol side-chain cleavage activity. It can be seen that in these circumstances 50μM palmitylcarnitine was at least as efficient an electron donor for the reaction as TCA intermediates used at a final concentration of 5 mM (compare Figs 5.1 and 6.4).

The effects of the respiratory inhibitors on this system are particularly interesting: in the absence of ATP, rotenone was stimulatory, while both amytal and antimycin inhibited; in the presence of ATP all three "inhibitors" were stimulatory, although amytal somewhat less so, compared with controls in which only palmitylcarnitine and ATP were present. The mitochondrial β-oxidation of fatty acids generates, in other tissues (Lynen, 1955.) both NADH and reduced flavoprotein. The experiments described in Chapter 5 indicated that NADH could be utilized by a non-energy dependent pathway as an electron donor for cholesterol

side-chain cleavage in these mitochondrial preparations, while electron transfer from the flavoprotein level (i.e. during succinate oxidation) to the cytochrome P450 reductase system was energy-dependent.

The results shown in Fig. 6.4 demonstrate that cholesterol side-chain cleavage activity is energy requiring when palmitylcarnitine is the electron donor for the reaction; this is perhaps a consequence of an electron transfer from flavoprotein-linked acyl-CoA dehydrogenases to the cytochrome P450 reductase, similar to that occurring from succinate (see Chapter 5, section 5.6). This concept is discussed in the following Chapter.

SUMMARY

1. [4-^{14}C] cholesteryl oleate was shown to be metabolized to [4-^{14}C] steroids, as well as [4-^{14}C] cholesterol, by preparations of porcine luteal mitochondria. A time course of this process indicated that the likely order of events was hydrolysis of the ester linkage, followed by side-chain cleavage of the free cholesterol so formed.

2. Long chain fatty acids were shown to be capable of supporting cholesterol side-chain cleavage activity, when incubated in the presence of BSA, and succinate; however, much greater rates of reaction were demonstrable when ATP carnitine and CoA were also present in in-

cubations in which oleic acid was acting as electron donor.

3. The effects of amytal, rotenone and ATP on cholesterol side-chain cleavage activity supported by palmityl carnitine were complex: antimycin and amytal were inhibitory, while rotenone stimulated; ATP relieved the inhibition seen in the presence of the former two inhibitors, and in fact lead to a large stimulation of cholesterol side-chain cleavage activity.

CHAPTER 7

CHAPTER 7

GENERAL DISCUSSION

7.1 Introduction.

The mammalian corpus luteum is an ephemeral endocrine gland which, in the intact animal, develops into a morphological entity, functions for a number of days which is characteristic for each mammalian species, then ceases to function sometime prior to the next ovulation.

The present study has been concerned with the biochemistry of the porcine corpus luteum. The principal function of the corpus luteum is the biosynthesis of steroid hormones, in particular, progesterone, which then play an important role in the reproductive physiology of the animal: progesterone is responsible for stimulating the growth and development of the uterine endometrium, in preparation for subsequent implantation and continuation of pregnancy. It is relevant that an investigation of ovarian luteal biochemistry should be particularly concerned with those areas of metabolism peculiar to a steroidogenic tissue. The rate-limiting step in the ovarian steroidogenic pathway is thought to be the side-chain cleavage of cholesterol and it is in this area that the gonadotrophic hormone responsible for stimulating luteal function, luteinizing hormone (LH),

is thought to act (Hall and Koritz 1965). LH stimulation has long been known to bring about certain metabolic events in the corpus luteum, such as depletion of cytoplasmic cholesterol-esters (Claesson et al., 1947a,b) and ascorbic acid (Claesson et al., 1949), and also stimulation of glucose uptake (Channing and Villee, 1966) and utilization (Sowerby, 1968); however, neither the interrelations of these events, nor their precise relevance in the stimulation of luteal function and steroidogenesis is yet understood. The approach of this present study has been primarily to investigate those areas of luteal intermediary metabolism that could conceivably be involved in controlling steroidogenesis: in particular some potential sources of substrates such as cholesterol, and electrons for the cholesterol side-chain cleavage reaction, have been examined. Mitochondrial electron transport pathways in ovarian luteal tissue have been studied in an attempt to elucidate the routes by which reducing equivalents generated during the tissue's oxidative metabolism are directed to the cytochrome P450 multi-enzyme system associated with the side-chain cleavage of cholesterol

7.2 Source of tissue.

It was decided to utilize ovarian corpora lutea taken from mature domestic pigs at slaughter. The choice of such a tissue offered several advantages when compared with that derived from other sources: (1) the

porcine ovary is large enough to permit the easy dissection of corpora lutea free from the residual follicular and interstitial ovarian components, (2) since the domestic pig is polyoestrous and produces large litters (about 8-12 offspring per litter), relatively large amounts of luteal tissue (4 to 6 gms) are obtainable from a single animal, and tissue is available throughout the year, (3) the animals were not subject to any prior hormonal therapy, so avoiding criticisms levelled at "unphysiological" luteinized tissues such as that of the rat ovary superovulated by the technique of Parlow (1958) (e.g. see Savard et al., 1965); and (4) the proximity and co-operation of a local abattoir made collection of such tissue relatively easy.

7.3 Preliminary studies and observations.

Cholesterol side-chain cleavage activity was measured by a radiometric assay based on that established by Boyd and co-workers (Simpson and Boyd, 1966; Sulimovici and Boyd, 1968a; Mason and Boyd, 1971). The assay involved the incubation of [4-^{14}C] cholesterol of high specific activity (58μCi/mg) with various tissue preparations, and enzyme activity was expressed as the percentage conversion of the [4-^{14}C] cholesterol to [4-^{14}C] steroid products. Such an assay avoided disadvantages inherent in earlier methods: methods based on the estimation of the C_6 fragment, isocaproic acid

(Ichii et al., 1963) give no information about the fate of the relatively more important steroid moiety; measurement of steroid products by means of the Pettenkofer reaction (Koritz, 1962) is only satisfactory in tissues where pregnenolone is the sole product accumulating after cholesterol side-chain cleavage has occurred, which often is not the case in ovarian preparations (Sulimovici and Boyd, 1968a; Jackanicz and Armstrong, 1968; Flint and Armstrong, 1971a,b).

Using the radio-assay (described in Chapter 2), the products of the cholesterol side-chain cleavage reaction when assayed in intact ovarian luteal mitochondrial preparations were seen to comprise both pregnenolone and progesterone (see Figs. 2.1 and 3.1). These metabolites were identified by their chromotographic behaviour in TLC and GC systems (see Figs. 2.1 and 3.1), and further characterized by their mass spectra (see Figs. 3.2, 3.3 and 3.4). The finding that progesterone was the predominant steroid product resulting from incubation of cholesterol with mitochondrial fractions is in agreement with several other observations on ovarian tissues (Sulimovici and Boyd, 1968a, Jackanioz and Armstrong, 1968; Flint and Armstrong, 1971a,b).

The initial studies on the cholesterol side-chain cleavage system of the porcine luteal preparations

(see Chapter 3) indicated that in many respects it was similar to those investigated in other steroidogenic tissues (Sulimovici and Boyd, 1969): the reaction was mitochondrial, utilized NADPH, was associated with cytochrome P450, and had a fairly broad pH profile with a maximum near pH 7.4.

7.4 Supply of substrates for cholesterol side-chain cleavage.

Previous studies on ovarian cholesterol side-chain cleavage (Sulimovici and Boyd, 1968a,b, 1969) established that the reaction in luteinized tissue, like that in other tissues, had the characteristics of a mixed function oxidase (Mason, 1957, 1965.) A partial purification of the enzyme system resolved it into three separable components which were necessary for full activity: these comprised a flavoprotein, an iron-sulphur protein, and cytochrome P450 (Sulimovici and Boyd, 1968b). This isolated multi-enzyme system had an absolute requirement for NADPH - a characteristic shared by most other mammalian mixed function oxidases. The reaction could be summarized thus:

$$2\text{Cholesterol} + 5\text{NADPH} + 5O_2 \rightarrow 2\text{pregnenolone} + 6H_2O + 2\ \text{isocaproic aldehyde.} + 5NADP^+ \qquad (1)$$

(see also Chapter 1, section 1.4).

During the course of the present study it was decided to investigate some of the potential sources of the reactants shown in reaction (1) above; it was conceivable that control of the rate of cholesterol side-chain cleavage (and hence perhaps, the overall steroidogenic activity) could be exerted (presumably via LH, *in vivo*) by some initial effect on the supply of these necessary reactants.

(i) cholesterol

It has long been held that the profuse lipid droplets present in ovarian luteal cytoplasm are stored precursors for steroidogenesis (see Chapter 1, section 1.2.iv). The droplets are known to be rich in cholesterol, present mainly in an esterified form with long chain (and often polyunsaturated) fatty acids. The data presented in Fig. 6.2 indicated that intact porcine luteal mitochondrial preparations contained an active cholesterol esterase (sterol ester hydrolase) activity and also demonstrated that in such *in vitro* situations, cholesteryl esters (in this case, cholesteryl oleate) could provide a source of cholesterol for cholesterol side-chain cleavage.

(ii) oxygen

Of the three reactants concerned, no attempt was made to investigate the supply of oxygen, for practical reasons; it might be noted however that one of the most

rapid effects of LH in the ovary, *in vivo*, is to promote blood flow through the gland, causing noticeable hyperaemia (Maier and Staehlin, 1968). This phenomenon is also seen in the analogous adrenal situation, and it has been suggested (Grant, 1968, 1970) that the trophic effect of ACTH on that gland is mediated, at least in part, via sphincter-like musculature of adrenal arterioles, whose release directs oxygenated blood to sites of steroidogenesis within the adrenal cortex (see Chapter 1, section 1.3 2.iv)

(iii) NADPH.

Since the original hypothesis of Haynes and Berthet (1957), several theories concerning trophic hormone control of steroidogenesis have proposed that the availability of NADPH determines the net rate of this process (see section 1.3.2); although more recently some of the experimental evidence for this view, especially that pertaining to effects of exogenous NADPH on tissue slice preparations, has been dismissed as artefactual (Savard *et al.*, 1965), the problem of the supply of intramitochondrial reducing power for steroidogenesis must remain relevant. Accordingly, much of the present study was devoted to establishing possible electron donors for the cholesterol side-chain cleavage reaction in the porcine corpus luteum, and to elucidating the mitochondrial electron transport pathways involved.

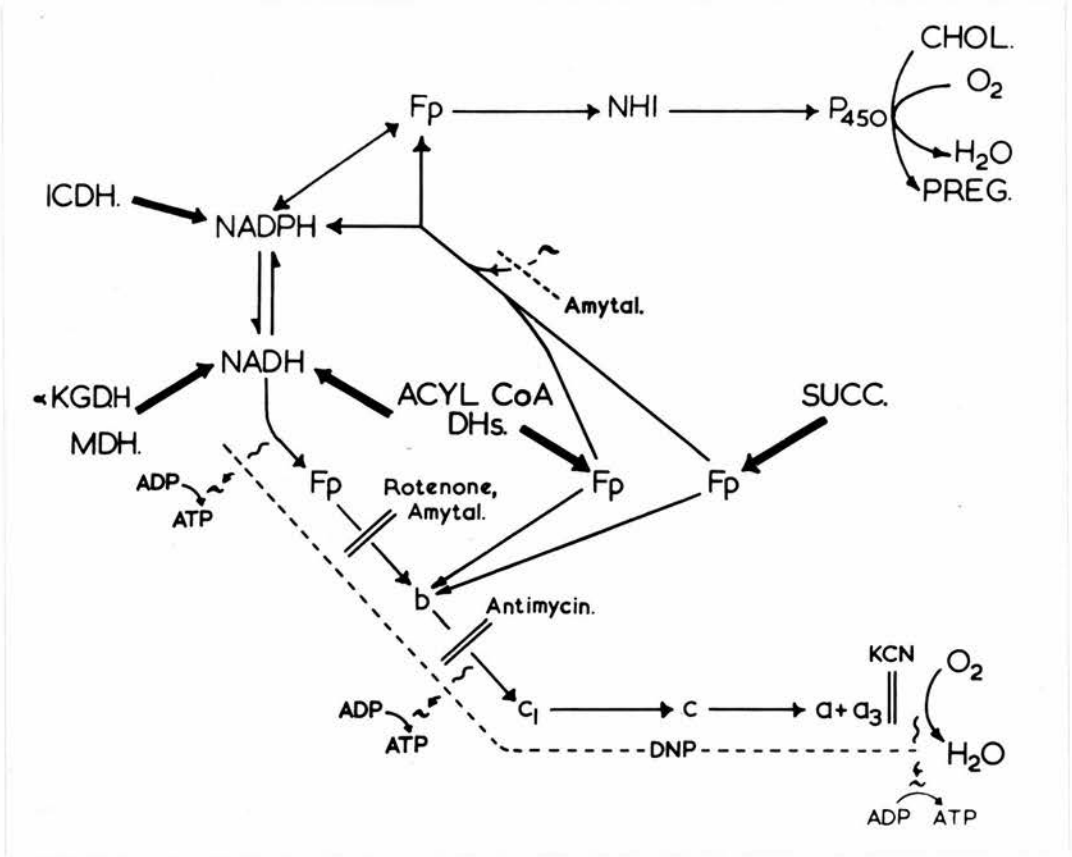


FIG. 7.1: A schematic representation of the postulated interaction between the cytochrome oxidase chain, and the P450 steroidogenic chain in porcine luteal mitochondria. The cytochromes are designated by their letter nomenclature. Sites of energy conservation and utilization are shown, and ~ represents a high energy intermediate in ATP formation. Sites of inhibition of the respiratory chain by antimycin, rotenone, amytal and KCN are shown at ||. Solid arrows (→) indicate direction of electron flow. NHI = non-haem iron protein, chol = cholesterol, preg = pregnenolone. Isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, and malate dehydrogenase are designated ICDH, αKGDH and MDH respectively.

When preparations of intact ovarian luteal mitochondria were used as an enzyme source, several compounds were found to be capable of replacing the requirement for exogenous NADPH as an obligatory cofactor for cholesterol side-chain cleavage activity; often considerably more side-chain cleavage occurred in these circumstances when compared with control incubations in which NADPH was present (e.g. see Fig. 5.1).

The data concerning such alternative electron donors may for convenience be considered under three separate categories:

A. via $NADP^+$ - dependent dehydrogenases.

B. via NAD^+ - dependent dehydrogenases.

C. via flavoprotein - dependent dehydrogenases.

A hypothetical scheme based on the results of these studies, which shows some possible routes of electron flow in porcine ovarian luteal mitochondrial preparations, is given in Fig. 7.1.

A. via $NADP^+$ - dependent dehydrogenases:

The most active intramitochondrial $NADP^+$ - dependent dehydrogenase examined in porcine luteal tissue was that of isocitrate dehydrogenase (Table 5.1); it appeared capable of generating enough reducing power to support maximal rates of cholesterol side-chain cleavage when isocitrate was present. When cholesterol side-chain cleavage was assayed in the

presence of isocitrate and arsenite (to inhibit α-ketoglutarate dehydrogenase) high levels of activity were measurable (see Fig. 5.2). The further addition of inhibitors of NADH oxidase, such as rotenone, antimycin or cyanide, resulted in stimulation of cholesterol side-chain cleavage activity of about 50% when compared with inhibitor-free controls. A stimulation of a similar magnitude was observed in the presence of these respiratory inhibitors when NADPH, rather than isocitrate, was the electron donor added (Fig. 5.4); the cause, in both cases was thought to be due to prevention of NADPH:NADH transhydrogenation, so conserving reducing power from NADPH for the NADPH - cytochrome P450 reductase and cholesterol side-chain cleavage (see Fig. 7.1).

Low levels of $NADP^+$ dependent malate dehydrogenation were detectable in some preparations of intact porcine luteal mitochondria (see Table 5.1.) Although this was at first attributed to "malic enzyme" activity, its actual source was not rigorously investigated; the enzyme responsible did appear however to be located in a mitochondrial compartment separate from that containing the cholesterol side-chain cleavage multi-enzyme. In another study on porcine luteal tissue it has recently been shown (Stevenson and Taylor, 1971) that this situation is an artefact (see Chapter 5, section 5.1). It thus seems unlikely that intra-mitochondrial "malic enzyme" activity plays a dominant

role in the supply of NADPH for steroidogenesis in the porcine corpus luteum in contrast to the situation in the bovine (and porcine) adrenal cortex (Simpson and Estabrook, 1969; Brdiczka and Pette, 1971).

B. via NAD^+ - dependent dehydrogenases:

Four substrates whose oxidation generates NADH were investigated in relation to their capability of supporting cholesterol side-chain cleavage in intact porcine luteal mitochondrial preparations: they comprised L - malate, α-ketoglutarate, oleic acid and palmityl-carnitine. All of these compounds supported appreciable levels of cholesterol side-chain cleavage activity. (see Figs. 5.1, 5.3, 6.4, and Table 6.1).

The use of an inhibitor of succinate dehydrogenase (malonate) established that α-ketoglutarate dehydrogenation, an NAD^+ specific reaction, could supply reducing power for cholesterol side-chain cleavage activity (Fig. 5.3). This suggested the possibility that NADH: NADPH transhydrogenation could occur in these mitochondrial preparations. Furthermore, since α-ketoglutarate was still effective in the presence of both antimycin and arsenate (Fig. 5.3) it seemed probable that the transhydrogenation was not energy requiring.

Additional evidence implicating a reversible pyridine nucleotide transhydrogenase mechanism like that shown in Fig. 7.1 was obtained from experiments

in which the effects of exogenous NADH and NADPH on cholesterol side-chain cleavage activity were investigated (see section 5.5). Exogenous NADPH supported the cholesterol side-chain cleavage reaction in porcine luteal mitochondrial preparations, although only to a limited extent compared with TCA substrates (see Fig. 5.1), probably because of relative mitochondrial impermeability to pyridine nucleotides. However, when NADPH was incubated with inhibitors of NADH oxidase such as rotenone or antimycin, the percentage conversion of cholesterol was markedly increased (Fig. 5.4). This suggested that in the former experiment only part of the NADPH taken up by the mitochondria was oxidized by the steroidogenic electron transfer chain, while the rest was converted to NADH and oxidized via the respiratory chain; in the latter experiment all the NADPH was available for steroidogenesis (see Fig. 7.1)

It appears that in adrenal cortex mitochondria the transfer of hydrogen from NADH to NADPH is energy dependent (Oldham et al., 1968), and that steroid hydroxylation supported by NAD^+ - linked substrates is inhibited by agents preventing the formation of ATP or high energy intermediates (Brownie and Grant, 1954; Purvis et al., 1968). Using porcine ovarian luteal mitochondria preparations however, no essential requirement for energy derived from phosphorylation to

drive a similar energy linked transhydrogenation could be demonstrated. Exogenous NADH supported cholesterol side-chain cleavage much more effectively in the presence of antimycin, rotenone or cyanide, which inhibit the formation of high energy intermediates, than in their absence (Fig. 5.4.) These results are predictable if a non energy requiring pyridine nucleotide transhydrogenase were connecting the two cytochrome chains present in these luteal mitochondria (see Fig. 7.1). They might also be explained if the cholesterol side-chain cleavage mixed function oxidase of porcine corpora lutea were not, in fact, NADPH specific, but could also use NADH directly. This latter possibility has not yet been explored. The uncoupling agent DNP inhibited cholesterol side-chain cleavage supported by NADH (Fig. 5.4., and 5.5) probably by stimulating its removal via the respiratory electron pathway. When rotenone or antimycin were added to the DNP - inhibited reactions, the inhibition was relieved (Fig. 5.5) although no high energy phosphorylation could be occurring. Another agent which will stimulate NADH oxidation maximally in preparations of coupled mitochondria, namely ADP, inhibited side-chain cleavage supported by NADH (as well as NADPH) (Fig. 5.6). This inhibition was also relieved by rotenone or antimycin.

These results are compatible with the hypothesis embodied in Fig. 7.1 where the pyridine nucleotide transhydrogenase is similar to that described by Kaplan and co-workers (1952, 1953) which, when isolated from mammalian sources (Kaplan et al., 1953) was freely reversible, with an equilibrium constant close to unity. Under the conditions employed in many of the experiments - for example, when 100 μM DNP and 10 μM rotenone were present with NADH (Fig. 5.5) - any action of an energy-requiring pyridine nucleotide transhydrogenase seems unlikely, although its possible role in more physiological conditions or in vivo in the porcine corpus luteum certainly cannot be dismissed. The presence of an energy-linked pyridine nucleotide transhydrogenase, as well as a relatively more active non-energy requiring enzyme, has recently been demonstrated by spectrophotometric assay, in sub-mitochondrial particles of porcine corpus luteum tissue (Stevenson and Taylor, unpublished results).

Oleic acid and palmityl-carnitine - substrates whose catabolism via β-oxidation might be expected to generate NADH as well as reduced flavoprotein - were also efficient as electron donors for cholesterol side-chain cleavage (see Table 6.1 and Fig. 6.4). The experimental data relating to the utilization of these fatty acid substrates will be considered below, following that concerning succinate oxidation.

C. via flavoprotein-dependent dehydrogenases:

The use of succinate as a source of reducing power for mitochondrial cytochrome P450, and cholesterol side-chain cleavage was described in section 5.6. The data obtained suggested that electron transfer from the flavoprotein level to the cytochrome P450 reductase system was energy dependent: the reduction by succinate of cytochrome P450, and its support for cholesterol side-chain cleavage activity, was prevented by the addition of respiratory inhibitors or DNP (see Tables 5.5 and 5.6 and Fig. 5.7); this inhibition could be relieved by the further addition of exogenous ATP (Table 5.6). These characteristics are similar to those described for analogous situations in adreno-cortical tissue (Koritz, 1966) where it is suggested that succinate reduction of NAD^+ takes place by an endergonic reversed electron flow (Chance and Hollunger, 1961). An attempt was made to establish if such a route were operative in porcine luteal mitochondrial preparations. Two inhibitors, rotenone and amytal, which are deduced to act at the same site in the NADH dehydrogenase - cytochrome b reductase portion of the mitochondrial respiratory chain, were employed (Table 5.6); their respective effects on succinate-supported cholesterol side-chain cleavage activity proved to be very different.

Rotenone is a more specific inhibitor of electron

transport than amytal (Ernster et al., 1963) and has been shown to become tightly bound to mitochondrial particles (Burgos and Redfearn, 1965); it is now well established that rotenone inhibits electron transport at a site on the oxygen side of the NADH dehydrogenase (NADH: (acceptor) oxidoreductase, E.C. 1.6.99.3) flavoprotein (Horgan et al., 1968) (see Fig. 7.1). Any unspecific binding of rotenone to other mitochondrial sites can be prevented by the inclusion of bovine serum albumin (BSA) in the incubation medium (Horgan et al., 1968). In the present study, rotenone was used at a concentration (10 μM) adequate to saturate the binding site described above,(Slater, 1967), and BSA was always present in the incubation media. Even though these conditions were observed, no inhibition of succinate-supported cholesterol side-chain cleavage activity could be demonstrated in the presence of rotenone (see Table 5.6); on the contrary, stimulation of the reaction was invariably observed in such situations. Recently a similar finding has been reported regarding the ineffectiveness of rotenone as an inhibitor of electron transport in bovine corpus luteum homogenates (Haksar and Romanoff, 1971).

Amytal (5-ethyl-5-isoamylbarbituric acid) is one of a group of barbiturate drugs which are all considered to inhibit mitochondrial oxidation of NADH by interaction at a similar site to that discussed

above for rotenone (Horgan et al., 1968) (see Fig. 7.1). When amytal was used in place of rotenone in porcine luteal mitochondrial incubations, marked differences in the effects of these two inhibitors became apparent: whereas rotenone slightly enhanced cholesterol side-chain cleavage supported by succinate, or succinate with antimycin plus ATP, amytal brought about inhibition of this activity (see Table 5.6). Even so, the inhibition achieved never exceeded 50% compared with amytal-free controls, although the compound was present at a final concentration of 1.8 mM which is sufficient to abolish electron flux in the NADH - cytochrome b reductase portion of the mitochondrial respiratory chain of other tissues almost completely (Slater, 1967).

The data discussed above suggest that

(i) the respiratory electron transport chain of the porcine luteal mitochondrion may differ markedly in its sensitivity to these two inhibitors when compared with the situation pertaining in mitochondria derived from most other sources or

(ii) electron flow from succinate to the mixed function oxidase associated with cholesterol side-chain cleavage does not involve reversed electron transport in the cytochrome b - NAD^+ region of the mitochondrial respiratory chain.

The inhibition that did occur in the presence of amytal could be accounted for by properties of this inhibitor not shown by rotenone: i.e. unlike amytal, rotenone does not effect the Pi-ATP exchange reaction (Low, 1959), the ATP-ase reactions in the presence or absence of DNP (Siekevitz et al., 1958) and does not lower the P:O ratio with succinate as substrate (Ernster et al., 1963). It had been demonstrated in porcine luteal mitochondrial preparations (see above) that the electron transport between succinate and cytochrome P450 was energy requiring. It is therefore conceivable that the partial inhibition by amytal of this process was a result of interference with some energy transfer mechanism (see Fig. 7.1)

Further experimental evidence which lends support to hypothesis (ii) (above) was given in section 5.6.3. Using a preparation of sub-mitochondrial particles, it was shown (Fig. 5.8) that $NADP^+$ was reduced by succinate at a much greater rate than NAD^+. This finding, like those discussed above, also suggests that NADH is not a direct intermediate on the electron transfer pathway between succinate and the site of cholesterol side-chain cleavage. The hypothesis put forward to account for these results is given in Fig. 7.1: it is suggested that an energy dependent transfer of electrons might

occur between the flavoprotein associated with succinate dehydrogenase and either $NADP^+$ or the flavoprotein of the cytochrome P450 reductase, in the porcine luteal mitochondrion. If, as has been suggested (Cooper, 1971), the latter flavoprotein has a low redox potential (about -0.3 v), such a relationship would be theoretically feasible.

In terms of the physiology of the corpus luteum, the most significant source of electrons for steroidogenesis is fatty acids. The cytoplasm of the corpus luteum is rich in long chain acyl esters of cholesterol, and on LH stimulation of the gland, hydrolysis of these esters occurs. The data presented in Chapter 6 (see Fig. 6.2 and Table 6.1) indicated that both the cholesterol and the fatty acid moieties of these esters could have a role as stored precursors for luteal steroidogenesis: the cholesterol as the prime substrate and the fatty acids, as a source of electrons, for cholesterol side-chain cleavage.

The β-oxidation of fatty acids is associated with the reduction of both NAD^+ and flavoprotein. Some indications of the routes involved in electron transfer from these reduced cofactors to the cytochrome P450 reductase in porcine luteal mitochondria can be gained from a consideration of data shown in Fig. 6.4. These results concerned the effect of respiratory inhibitors on cholesterol side-chain cleavage supported

by palmityl-carnitine. It was found that rotenone had a marked stimulating effect on cholesterol side-chain cleavage activity in such systems. Rotenone is known to prevent the oxidation of NADH while allowing ATP formation from reduced flavoprotein (see above). This respiratory inhibitor might therefore be expected to stimulate fatty acid-supported cholesterol side-chain cleavage by allowing ATP formation from reduced flavoprotein while maintaining the electron pressure from NADH to the cytochrome P450 chain via pyridine nucleotide transhydrogenation. The transfer of H^+ from NADH to cholesterol side-chain cleavage does not have an absolute requirement for energy, in porcine luteal mitochondria (see (A), above) whereas support of this activity by succinate has (see (C), above); it is therefore suggested that the inhibitory effects of amytal or antimycin on fatty acid-supported cholesterol side-chain cleavage are a consequence of the involvement of an energy requiring transhydrogenation from the acyl CoA dehydrogenase flavoprotein, similar to that proposed above for succinate. Antimycin and amytal, which inhibited in such situations (see Fig. 6.4), probably did so by interfering with ATP formation, the former compound inhibiting the oxidation of both NADH and reduced flavoprotein while the latter interfered with energy transfer reactions.

7.5 Some explanations of the respiratory characteristics of porcine luteal mitochondrial preparations.

The hypothesis diagrammatized in Fig. 7.1 can also be used to account for the low ADP:O and respiratory control ratios found in mitochondrial preparations of porcine corpus luteum (see Chapter 4 section 4.8).

One of the basic assumptions inherent in calculations of ADP:O and respiratory control ratios is the absence of any significant oxygen uptake in the presence of ADP attributable to a non-energy generating electron transport pathway (Chance and Williams, 1955a, 1956). If, in the luteal preparations, high energy phosphorylated intermediates were being used to drive electrons from succinate to the cytochrome P450 reductase associated with cholesterol side-chain cleavage, the resulting additional oxygen uptake would lead to a lowering of the calculated ADP:O ratio. Since endogenous cholesterol is always available for hydroxylation in these preparations (see section 3.4.i, and also Armstrong, 1968), it would undergo side-chain cleavage as soon as electrons reached the cytochrome P450 associated with the reaction. Further experimental evidence (section 5.6) demonstrated that electron flow from succinate to cytochrome P450 was in fact energy requiring. However, the difficulty in accepting

this explanation is that the oxygen uptake associated with cholesterol side-chain cleavage must be relatively small (see section 5.1) compared with that at the cytochrome oxidase site. Furthermore, electron transfer from NAD^+ -linked substrates to the cholesterol side-chain cleavage reaction was apparently not energy-requiring (see sections 5.4 and 5.5), yet these substrates also gave rise to low ADP:O ratios. The low redox potential created by the presence of inhibitors or high concentrations of substrates and coenzymes might artefactually open unusual electron transfer pathways; it is conceivable that the non-energy linked pyridine nucleotide transhydrogenase (Fig. 7.1) is an example of this. If the "physiological" route from NADH to the NADPH - cytochrome P450 reductase is by an energy requiring pathway akin to that proposed for succinate and acyl CoA dehydrogenases, this would explain the low ADP:O ratios obtained with NAD^+ -linked substrates.

A more likely explanation is that a product of cholesterol side-chain cleavage activity, namely progesterone, was effecting oxygen uptake. This steroid is known to be a potent uncoupling agent in other mitochondrial preparations (Wade and Jones, 1956). The synthesis of progesterone would also explain the higher rates of oxygen uptake seen after ADP utilization compared with those before its addition

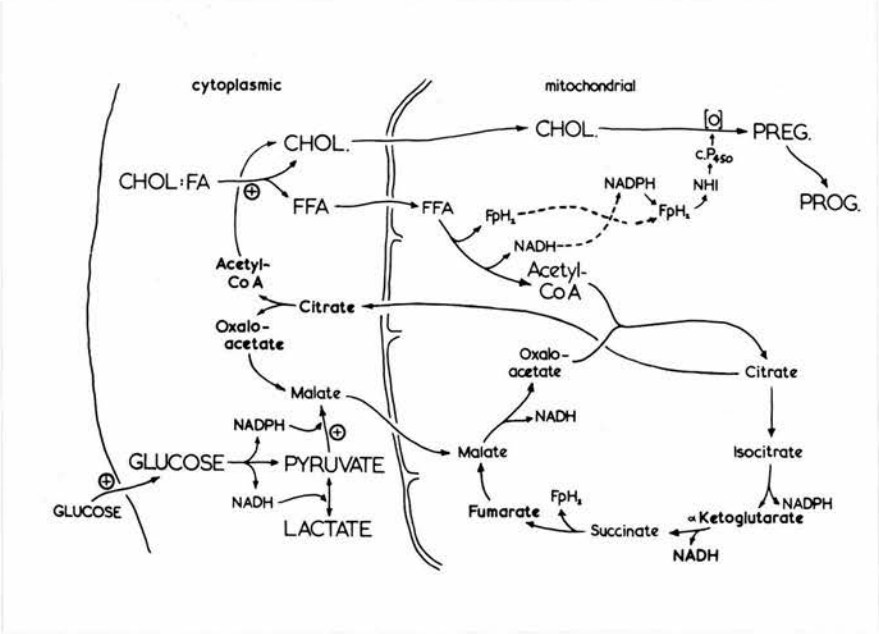


FIG 7.2 Some metabolic pathways relating to intermediary metabolism and steroidogenesis in the porcine corpus luteum: a hypothetical scheme based on work presented in this thesis, and data available in the literature. Reactions which are known to be stimulated after LH administration are indicated ⊕

(i.e. 14.1 nmoles/min/mg compared with 8.8 nmoles/min/mg - see Fig. 4.7) ; this would also account for the poor respiratory control of the luteal mitochondrial preparations.

Finally, it is possible that one of the sites of energy conservation is lacking, or inoperative, in porcine luteal mitochondrial preparations.

7.6 Relationships between intermediary metabolism and steroidogenesis.

The results presented in this thesis can be used as a basis for speculation about ovarian metabolism, when they are considered in conjunction with information already available concerning this tissue. Perhaps the best starting point for such an exercise is with some known effects of LH. Luteotropic stimulation results in an increased uptake and utilization of glucose (Channing and Villee, 1966; Sowerby, 1968); in addition cytoplasmic malic enzyme levels are enhanced in these situations (Stevenson and Forman, 1971). Hydrolysis of cytoplasmic cholesterol esters is also a well known effect of LH stimulation (see Chapter 1, section 1.3). Possible interrelationships of these events are shown in Fig. 7.2.

Increased cholesterol esterase activity has obvious relevance to the stimulation of steroidogenesis: it would result in increased cholesterol availability

for this process, and the fatty acid released might also play an important role as an electron donor for such activity. Results which indicate that such a "double-precursor" role of cholesterol esters is possible in the corpus luteum have been presented in Chapter 6. In addition, there are other indications that the oxidation of fatty acids is characteristic of such tissue: Sowerby (unpublished results) found that the RQ of luteal tissue when oxidizing endogenous substrate was 0.65, and that addition of exogenous substrates such as glucose did not result in a stimulation of oxygen uptake by these preparations; these results suggest that the preferred respiratory substrate is lipid. Studies by Flint and Denton (1970a) have also indicated that most of the tissue's oxygen uptake is a result of fatty acid oxidation.

In order to completely oxidise fatty acids a source of carbohydrate is necessary. It is possible that the stimulation of glucose uptake and utilization brought about by LH is related to this requirement. Under such circumstances intracellular pyruvate levels are elevated (Flint and Denton, 1969). It has been demonstrated that high pyruvate concentrations inhibit the cytoplasmic lactic dehydrogenase, and stimulate malic enzyme activity (Stevenson, unpublished results). The role of the cytoplasmic malic

enzyme of luteal tissue (Table 5.1) may be to convert the pyruvate resulting from glycolysis to malate, which may then penetrate the mitochondria (see Fig. 7.2). This would be compatible with the finding that [6-^{14}C] glucose is oxidized via the TCA cycle in luteinized tissue (Stevenson and Thomas, 1972). The function of the intramitochondrial malate may be to allow complete utilization of fatty acid: the subsequent conversion of malate to oxalo-acetate would enable the product of fatty acid oxidation, acetyl CoA, to be removed by condensation and formation of citrate; the equilibrium of the malate dehydrogenase reaction is known to be shifted in favour of oxalo-acetate formation in the presence of acetyl CoA when assayed in luteal tissue preparations (Stevenson, unpublished results). The citrate could enter the TCA cycle and thus provide several additional sources of electrons and energy for cholesterol side-chain cleavage (see Chapter 5). Citrate may also be involved in cytoplasmic lipid syntheses, in particular the biosynthesis of cholesterol. If citrate lyase were involved in this process, the oxalo-acetate produced might be converted to malate by the cytoplasmic malate dehydrogenase present in this tissue (Table 5.1)

Such a metabolic scheme is rather speculative, and represents only a small part of the overall biochemistry of the corpus luteum. It is however amenable to further investigation: in particular, a tracer study

using cholesteryl-esters radioactively labelled in both the cholesteryl and fatty acid moieties could provide more definitive information regarding the utilization of these lipids by luteal tissue.

Finally, one might speculate as to how the acute stimulation of steroidogenesis is achieved at the metabolic level. Several such hypotheses are available in the literature (see Chapter 1, section 1.3), and many are related to a stimulation of cholesterol side-chain cleavage activity. In the present study, it was noticeable that this reaction could be stimulated when conditions were arranged to promote electron transfer to the NADPH-cytochrome P450 reductase, at the expense of the mitochondrial respiratory electron transport chain. e.g. when NADH oxidase inhibitors were present with respiratory substrates (see Figs 5.2, 5.3, 5.4, 5.5, 5.6, and 6.4), or when ATP was added to incubations where succinate, or fatty acid derivatives were acting as electron donors for cholesterol side-chain cleavage activity (see Table 5.6 and Fig. 6.4). Even when no further increase in the reaction could be achieved by increasing the concentration of the electron donor, the addition of respiratory inhibitors (in the case of $NAD(P)^+$ linked dehydrogenases) or ATP when flavoprotein dependent dehydrogenases were supplying electrons, usually elicited an increase in the conversion of [4-^{14}C] cholesterol to [4-^{14}C] steroid metabolites. It is

suggested, therefore, that one of the means by which LH stimulates steroidogenesis might be to divert electrons from the mitochondrial respiratory cytochrome chain to the site of cholesterol side-chain cleavage, perhaps by enhancing one of the transhydrogenase reactions which are postulated in Fig. 7.1.

APPENDIX

The following chemicals were purchased from the Sigma Chemical Co.:- AMP, ADP, ATP, NAD^+, NADH, $NADP^+$, NADPH, DNP, PMS, BSA (FFA-free), EDTA, CoA, carnitine; palmitic-, stearic-, oleic-, linoleic- and linolenic acids; citrate, isocitrate, α-ketoglutarate, fumarate, malate, oxalo-acetate, pyruvate, β-hydroxybutyrate, aspartate, glutamate, G-6-P; amytal, antimycin, rotenone; catalase, cytochrome c; standard cholesterol, pregnenolone and progesterone.

[4-^{14}C] cholesterol and [4-^{14}C] cholesterol oleate were obtained from the Radiochemical Centre, Amersham.

BSA was obtained from the Armour Pharmaceutical Co. (Eastbourne)

G6P dehydrogenase was purchased from the Boehringer Corporation Ltd. (London).

Palmityl chloride was obtained from Eastman Chemicals (Liverpool).

Bis-trimethylsilyl acetamide was purchased from the Pierce Chemical Co. (Queensferry, Flintshire).

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CHOLESTEROL ESTERS AND STEROIDOGENESIS IN PIG CORPORA LUTEA

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1. Introduction

The corpus luteum of a number of species, including pig, contains quantities of cholesterol esters [1, 2, 3] which vary in concentration during the oestrus cycle [4]. Since an inverse relationship obtains between the amount of cholesterol esters present, and the progestational secretory activity [1] it was proposed that the sterol esters were stored precursors for ovarian steroidogenesis [4, 5, 6]. That luteinizing hormone, which stimulates steroidogenesis, induces depletion of ovarian cholesterol esters [7, 8] is well established. The present study demonstrates that (i) cholesterol from cholesterol-fatty acids (FA) ester can be converted to pregnenolone and progesterone in luteal mitochondria and (ii) that the FA moiety of the sterol ester provides a potential source of electrons for the steroid hydroxylation reactions necessary for this conversion. The cholesterol side-chain cleavage (SCC) reaction is energy dependent when FA is the sole source of electrons.

2. Materials and methods

4-^{14}C-cholesterol (58 μCi/mg) and 4-^{14}C-cholesteryl-oleate (46 μCi/mg) were obtained from the Radiochemical Centre, Amersham: both were purified before use as described previously [9]. Palmitic, stearic, oleic, linoleic and linolenic acids, carnitine co-enzyme A (CoA), ATP, bovine serum albumin (BSA) (fatty acid free), D,L-isocitrate and succinate were obtained from Sigma (London). Palmityl-carnitine was prepared by the method of Fritz and Yue [10].

Experimental details of the preparation of mitochondria from porcine ovarian corpora lutea, and the SCC assay were described previously [9]. Whole mitochondria (approximately 5 mg protein) were incubated in 1 ml of medium consisting of 200 mM sucrose, 25 mM Tris-HCl (pH 7.4), 10 mM potassium phosphate (pH 7.4) 20 mM KCl, 5 mM $MgCl_2$ and 0.2 mM tetra-sodium EDTA with substrates and inhibitors as required. The reaction, which was started by adding 1 μg 4-^{14}C-cholesterol (100,000 cpm), proceeded at 37° for 1 hr in an atmosphere of air, and was stopped with 1 ml methanol containing 4 μg cholesterol, 2 μg pregnenolone and 2 μg progesterone. An identical assay procedure was used when 4-^{14}C-cholesteryl-oleate was substrate, 100,000 cpm 4-^{14}C-cholesteryl-oleate replacing the 4-^{14}C-cholesterol, and cholesteryl-oleate (4 μg) as well as the other steroids being added as carriers at termination of the reaction. After extraction of the remaining substrates and products of reaction with hot acetone:methanol:ethyl acetate (2:1:1 by vol) followed by partitioning from water into chloroform, they were separated by TLC on silica gel G as before [9]. The TLC system [di-isopropyl ether:petroleum spirit (60°–80°):acetic acid (70:30:1 by vol)] proved adequate for separating 4-^{14}C-cholesteryl-oleate, 4-^{14}C-cholesterol, 4-^{14}C-pregnenolone and 4-^{14}C-progesterone: the ester ran well ahead of free cholesterol and close to the solvent front.

3. Results and discussion

3.1. *Cholesteryl-oleate as source of cholesterol for steroidogenesis*

Since Armstrong et al. [11] showed cholesteryl-oleate to be the most abundant ester in luteal tissue, this substance was chosen as a representative ester for

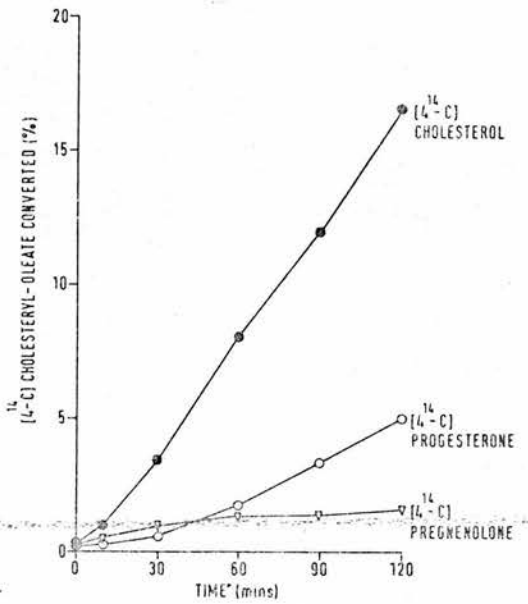

Fig. 1. Time course of 4-^{14}C-cholesteryl-oleate utilization by intact mitochondria from porcine corpora lutea. 4-^{14}-cholesteryl-oleate (100,000 cpm) was incubated with 3 mg of mitochondrial protein in an isotonic buffered sucrose containing 1% BSA, as described in the methods. The electron donor added was D,L-isocitrate (10 mM). The identified products are indicated on the figure.

our *in vitro* experiments. Fig. 1 illustrates the time course of utilization of 4-^{14}C-cholesteryl-oleate by a preparation of porcine luteal mitochondria in terms of the production of 4-^{14}C-metabolites. It can be seen that 4-^{14}C-cholesterol was the first major product of the incubation, while there was a gradual accumulation of the more polar steroids 4-^{14}C-pregnenolone and 4-^{14}C-progesterone. It is clear that cholesteryl-oleate can be hydrolysed by the intact mitochondrial fraction, and the free cholesterol used as substrate for SCC. No compound corresponding to pregnenolone-oleate was isolated: this finding together with the data shown in fig. 1 suggests that hydrolysis of the ester linkage precedes the SCC reaction. Coutts and Stanfield [12, 13] demonstrated cholesteryl esterase (sterol ester hydrolase, EC 3.1.1.13) activity in rat and bovine corpora lutea: this enzyme activity was found predominantly, in the 5000 *g* pellet.

3.2. *Utilization of free fatty acid (FFA) as electron donor for SCC*

Evidence that FFA was the preferred oxidizable substrate in the luteal ovary was obtained by Cooper and Stevenson [14] who showed that the RQ for endogenous substrate of rat luteinized ovary was 0.71 and no other added carbon source could alter this figure. Flint and Denton [15] also showed that oxygen uptake by slices of luteinized rat ovary was unaffected by the addition of glucose to the medium, and later [16] suggested that the main endogenous substrate for respiration in this tissue was FFA liberated from sterol esters.

Our experiments showed that with no added cofactors FFA was completely ineffective in supporting SCC in intact porcine luteal mitochondria. In fact the unsaturated acids, oleic, linoleic and linolenic, which are known to be potent uncouplers of oxidative phosphorylation [17] actually inhibited SCC supported by 500 μM succinate: the sypply of electrons from succinate for steroid hydroxylation is energy dependent [9]. This uncoupling effect of the unsaturated FFA's was overcome by the addition of 1% BSA, in which case there was a slight stimulation of SCC activity by all the previously mentioned FFA as well as palmitic and steraric acid.

The oxidation of FFA by heart mitochondria is enhanced when incubation conditions allow the synthesis of acyl-carnitine intermediates [10]. Our results indicate that the utilization of FFA as electron donor for ovarian luteal steroidogenesis is markedly stimulated under similar conditions. The effect on SCC activity of adding 50 μM oleate together with the FFA activating and transporting agents CoA (50 μM), ATP (3 mM) and carnitine (5 mM) is shown in table 1. The features to note in this table are (i) that ATP was essential for the utilization of oleate as an electron source, (ii) oleate supports SCC more effectively if exogenous CoA as well as ATP is added, and (iii) SCC is most active when all three cofactors are present. The results in the lower section of the table suggest that there is an endogenous supply of FA in the mitochondrial fraction.

Acyl-carnitines can be oxidized directly by mitochondria without prior need for activation [18]. Using

Table 1

The effect of fatty acid activating and transporting agents on cholesterol side-chain cleavage activity in the presence of oleic acid. The method of assay has been described in the methods, activity being expressed as percentage conversion of 4-^{14}C-cholesterol to 4-^{14}C-steroid metabolites. The fatty acid substrate, and cofactors when present were at the following final concentrations: oleic acid, 75 μM; ATP, 3 mM; carnitine, 5 mM; CoA, 50 μM. 3 mg of mitochondrial protein were present in each incubation.

Substrate and cofactors added	Conversion of 4-^{14}C-cholesterol to the following products.		
	4-^{14}C-pregnenolone (%)	4-^{14}C-progesterone (%)	Total conversion (%)
No additions	0.4	0.3	0.7
Oleic acid	0.6	0.8	1.4
Oleic acid + carnitine	0.7	0.6	1.3
Oleic acid + CoA	0.7	0.7	1.4
Oleic acid + ATP	8.4	5.6	14.0
Oleic acid + carnitine + CoA	0.8	0.6	1.4
Oleic acid + carnitine + ATP	11.0	5.9	16.9
Oleic acid + CoA + ATP	17.4	6.4	23.8
Oleic acid + CoA + carnitine + ATP	18.8	9.4	28.2
Carnitine	0.7	0.5	1.2
Carnitine + CoA	1.1	0.2	1.3
Carnitine + ATP	9.0	6.0	15.0
Carnitine + ATP + CoA	13.6	8.0	21.6
CoA	1.0	0.5	1.5
CoA + ATP	10.0	7.2	17.2
ATP	7.5	5.1	12.6

preparations of intact porcine luteal mitochondria, we investigated the relationship between palmityl-carnitine utilization and SCC in the presence of some respiratory inhibitors (fig. 2). Palmityl-carnitine itself supported 6.3% conversion of 4-^{14}C-cholesterol to radioactive products in these incubations. Rotenone (10 μM) markedly stimulated this support, but both amytal (1.8 mM) and antimycin (330 ng/mg mitochondrial protein) inhibited the conversion by approximately 50%. ATP (3 mM) greatly stimulated the SCC reaction in the presence of palmityl-carnitine, and abolished the inhibitory effects of both amytal and antimycin. It appears, therefore, that SCC, when supported by FFA is energy dependent. The β-oxidation of FFA is associated with the reduction of NAD^+, and flavoprotein. Rotenone is known to prevent the oxidation of NADH, while allowing ATP formation from reduced flavoprotein (FPH_2) [19, 20]. Rotenone then, will stimulate FFA supported SCC by allowing ATP formation from FPH_2 while maintaining the electron pressure from NADH to the cytochrome P450 chain via transhydrogenation. Since the transhydrogenase involved in transfer of H^+ from NADH to SCC is not energy dependent in pig luteal mitochondria but support of SCC by succinate is, [9] it is suggested that, in the absence of inhibitors, electrons from FA reach cytochrome P450, at least in part, via the flavoprotein dependent acyl-CoA dehydrogenase. Antimycin and amytal probably inhibit by interfering with ATP formation; the former inhibiting the oxidation of both NADH and FPH_2 while the latter interfere with energy transfer reactions [21, 22].

4. Conclusion

It has been shown that both the cholesterol and FA moieties of cholesteryl esters can play an important role in SCC. The utilization of electrons from FFA for cholesterol hydroxylation reactions appears to be energy dependent.

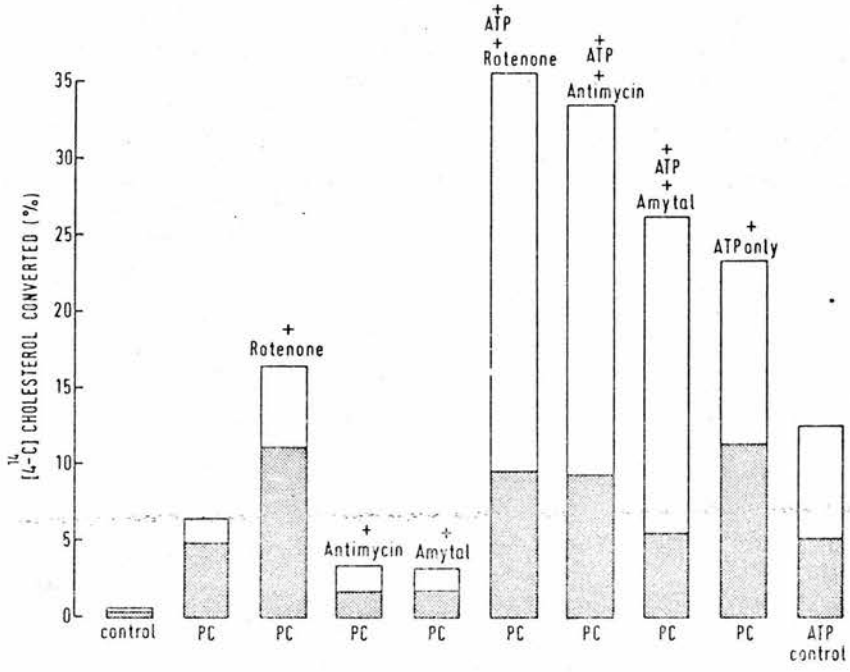

Fig. 2. The effect of respiratory inhibitors on cholesterol side-chain cleavage supported by 50 μM palmityl-carnitine (PC). The assay was described in the text. Rotenone, amytal, antimycin and ATP, present where indicated on the figure, were at final concentrations of 10 μM, 1.8 mM, 330 ng/mg of mitochondrial protein, and 3 mM respectively. The total area in each column represents total 4-^{14}C-steroid metabolites; the hatched areas are 4-^{14}C-pregnenolone, the clear areas 4-^{14}C-progesterone. No PC is present in the controls; the first contains mitochondria and 4-^{14}C-cholesterol, the second has ~~10 μM~~ 3mM ATP added.

Acknowledgements

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Oxidative phosphorylation and cholesterol catabolism in ovarian tissue.

By J. Robinson, Julia M. Cooper and Patricia M. Stevenson. *Medical Research Council Clinical Endocrinology Unit, 2 Forrest Road, Edinburgh, EH1 2QW*

The assay of the cholesterol side chain cleavage reaction in ovarian mitochondria, as used by Sulimovici & Boyd (1967), requires an NADPH generating system to supply reducing power for the concomitant hydroxylations. During the course of adapting this side chain cleavage assay for use with mitochondria prepared from pig corpora lutea, it was found that the generating system could be replaced by succinate and malate. Furthermore, it was discovered that when the mitochondria were prepared in the presence of bovine plasma albumin the total conversion of [4-^{14}C] cholesterol to pregnenolone and progesterone was stimulated when either succinate or malate was the source of electrons, but was inhibited when the NADPH generating system was used.

When the mitochondria from pig corpora lutea were prepared in an albumin-containing medium, they fulfilled criteria of integrity as defined by Chance, Bonner & Storey (1968). The ADP:O ratios for succinate and malate in these coupled mitochondria as determined by the method of Chance & Williams (1955) were consistently lower than in coupled liver mitochondria from both rat and pig: for malate the ADP:O ratio for ovarian mitochondria was 1·67 while that for succinate was 0·85.

These low ADP:O ratios, along with the evidence that succinate and malate can support cholesterol side chain cleavage activity suggests that in the corpora lutea these dicarboxylic acids are not only supplying electrons for oxidative phosphorylation through the cytochrome oxidase system, but are also being oxidized via the terminal oxidase cytochrome P-450: this cytochrome is known to be necessary for the cholesterol hydroxylation reactions. This hypothesis is analogous with that of Cammer & Estabrook (1967) who investigated the relationship between the ADP:O ratios of succinate and malate, and the deoxycorticosterone hydroxylation reaction in mitochondria from bovine adrenal cortex.

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The supply of electrons for ovarian cholesterol side-chain cleavage. By J. Robinson and Patricia M. Stevenson. *Medical Research Council Clinical Endocrinology Unit, 2 Forrest Road, Edinburgh, EH1 2QW*

The cholesterol side-chain cleavage multi-enzyme, isolated from adrenocortical and ovarian mitochondria, requires NADPH as its electron donor (Sulimovici & Boyd, 1969). Our investigation of side-chain cleavage in intact mitochondria from porcine corpora lutea has shown that all the Krebs cycle intermediates can replace NADPH; the majority were more effective than exogenous coenzyme.

Studies on an analogous system in adrenal cortex have indicated two major routes by which electron flow from Krebs cycle compounds might be diverted to support steroid hydroxylations: the first by direct intramitochondrial NADPH generation, via isocitrate dehydrogenase (ICDH) (E.C. 1.1.1.42) (Sauer & Mulrow, 1969), or via 'malic enzyme' (E.C. 1.1.1.40) (Simpson & Estabrook, 1969); and the second via an energy-linked transhydrogenation of NADH to NADPH (Sauer, 1970).

We have shown that mitochondria from porcine corpora lutea contain active $NADP^+$-linked ICDH. Correspondingly, isocitrate support for cholesterol side-chain cleavage (in the presence of arsenite to inhibit α-ketoglutarate dehydrogenation) is not abolished by respiratory inhibitors such as rotenone, antimycin A or cyanide; these compounds stimulated the reaction by 40% compared with isocitrate plus arsenite alone.

'Malic enzyme' activity in porcine corpora lutea is located (as in the rat ovary) predominantly in the cytoplasmic subcellular fraction. It seems unlikely that the mitochondrial 'malic enzyme' can generate enough NADPH for cholesterol side-chain cleavage (Stevenson & Forman, 1971). Although side-chain cleavage requires NADPH, we found with our intact mitochondria that NADH could also support the reaction. Furthermore in the presence of rotenone (10 μM), or antimycin A, NADH was more than five times as effective as in their absence; these inhibitors stimulated NADPH-supported side-chain cleavage to a much lesser extent ($< 50\%$). Similarly, the NADPH-supported reaction was only marginally stimulated by KCN (1 mM), whereas this compound doubled the NADH-supported reaction. NADH (5 mM) was 90% as efficient in supporting side-chain cleavage as NADPH (5 mM), when rotenone or antimycin A was present with each coenzyme.

Both DNP and ADP, compounds which maximally stimulate respiratory electron flow in coupled mitochondria, inhibited side-chain cleavage; the inhibition by DNP was relieved by rotenone, that of ADP by rotenone or antimycin A.

Our preliminary conclusions are that, as well as NADPH generation by ICDH, an NADH–NADPH transhydrogenation can occur in luteal mitochondria, at rates sufficient to support side-chain cleavage. Under the conditions employed, it seems unlikely that an energy-linked transhydrogenase (Danielson & Ernster, 1963) can be operating. The results suggest that a non-energy requiring reaction, akin to that described by Kaplan, Colowick & Neufield (1953), is occurring, the equilibrium of

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which could determine the relative diversion of electron flow from NADH and oxidative phosphorylation to NADPH and steroidogenesis in the luteal mitochondrion.

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Method of Determining Oxygen Concentrations in Biological Media, Suitable for Calibration of the Oxygen Electrode

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The oxygen electrode is now widely used in biochemistry and physiology to measure oxygen consumption rates. In order to obtain absolute values for oxygen uptake it is necessary to calibrate the instrument for each medium used in order to find the oxygen concentration corresponding to the 100% setting on the recorder. It is not always appreciated (*a*) that the oxygen electrode measures the activity, not the concentration of oxygen in solution and (*b*) that the presence of both electrolytes and nonelectrolytes influences the solubility and the activity coefficient of oxygen in aqueous solutions (1, 2). For these reasons equilibration of solutions with gas mixtures of known oxygen tension can give accurate calibration only if directly determined values already exist for the solubility of oxygen in the particular medium under consideration.

These solubility data are available for water, NaCl solutions, and some classical Ringer solutions (3, 4) but are not readily available for many commonly used buffer systems. Direct measurements of oxygen solubility are therefore necessary for these solutions.

Of the existing methods for such determinations, the classical method of Winkler (5) is extremely tedious for routine use. The calibration method most often applied to oxygen electrode studies is that of Estabrook and Mackler (6), in which oxygen is consumed in the stoichiometric oxidation of a specified amount of NADH, catalyzed by lysed mitochondria; the change in electrode current is thus equivalent to a known uptake of oxygen from solution. In this method the preparation of the mitochondria has the disadvantage of being unduly lengthy (more than 4 hours) for routine use and also requires equipment which may not be readily available (i.e., a high-speed refrigerated centrifuge

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and homogenizer). An additional restriction is that the medium under investigation must support mitochondrial oxidations.

This paper describes a direct method of calibration in which the mitochondrial preparation of the above method is replaced by the compound *N*-methylphenazonium methosulfate (PMS). The reactions involved are:

$$PMS + NADH + H^+ \leftrightharpoons PMSH_2 + NAD^+ \quad \text{(i)}$$

$$PMSH_2 + O_2 \rightarrow PMS + H_2O_2 \quad \text{(ii)}$$

Spontaneous decomposition of some hydrogen peroxide formed in reaction (ii) releases variable amounts of oxygen back into solution, making calculations based on the stoichiometry of reactions (i) and (ii) impossible. Therefore all the hydrogen peroxide formed in reaction (ii) was broken down by including catalase in the reaction mixture.

$$H_2O_2 \xrightarrow{\text{catalase}} H_2O + \tfrac{1}{2}O_2 \quad \text{(iii)}$$

The net reaction then becomes:

$$NADH + H^+ + \tfrac{1}{2}O_2 \rightarrow NAD^+ + H_2O \quad \text{(iv)}$$

EXPERIMENTAL

Materials

β-NADH, type III, disodium salt, was obtained from Sigma Chemical Co. (St. Louis, Mo.). A solution containing approximately 10 μmole NADH/ml in 0.1 *M* phosphate buffer, pH 7.4, was made up daily. The exact concentration was determined spectrophotometrically, throughout the experimental period.

Cytochrome c, type III, from horse heart was obtained from Sigma Chemical Co. A 90 μ*M* solution was made up when required.

N-Methylphenazonium methosulfate (phenazine methosulfate, PMS) was obtained from Sigma Chemical Co. A 1% (w/v) aqueous stock solution was made up and, provided it was rigorously protected from light, could be used for several months. This stock was diluted daily with distilled water to give a solution containing 200 μg/ml.

Catalase. Stock No. C10, 2,000 IU/mg obtained from Sigma Chemical Co. was made up daily at a concentration of 4 mg/ml in 0.1 *M* phosphate buffer, pH 7.4, or in the experimental medium being used.

0.1 M phosphate buffer, pH 7.4, was made by dissolving 2.82 gm K_2HPO_4 and 0.518 gm KH_2PO_4 in distilled water to a total volume of 200 ml. All other chemicals were of analytical-reagent grade.

Apparatus

The YSI model 53 biological oxygen monitor (Yellow Springs Instrument Co.) was used and consisted essentially of a Clark electrode covered with an FEP Teflon membrane and housed in a Lucite plunger. This probe was capable of insertion into a glass reaction chamber, making a closed system but for a narrow groove in the plunger, through which materials could be introduced into the chamber. The contents of the chamber were agitated continuously by magnetic stirring and maintained at a precise temperature (±0.01°) by means of a surrounding water bath and thermostat (Haake). The anode current from the probe was monitored using a Servoscribe recorder.

Method

The general procedure recommended in the brochure accompanying the equipment was followed. The experimental medium (3.0 ml), containing 20 μg PMS and 800 units (400 μg) catalase was added to the

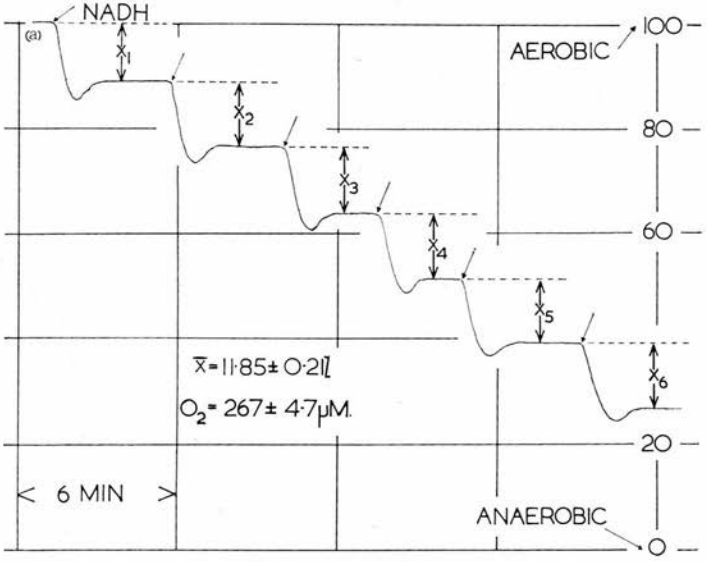

FIG. 1(a). Effect of high PMS concentrations on oxygen electrode trace recording and on calculated oxygen concentration. The reaction mixture contained 30 μg PMS and 400 μg catalase in 3 ml 0.1 *M* phosphate buffer, pH 7.4, at 25°C. Additions of 0.19 μmole amounts of NADH are indicated by arrows. Oxygen concentration was calculated from percentage deflections x_{1-6} obtained by measuring from each arrow to the new stable level. Mean percentage deflection $\bar{x}$ (±SD) and calculated oxygen concentration (±SD) are shown; the concentration obtained from other methods (see text) is 244 ± 3.9 μ*M*.

water-jacketed chamber and stirred for 20 min to allow temperature equilibration and saturation with air.

The probe was then inserted, care being taken to exclude all air bubbles. After stirring for a further 2 min to allow probe stabilization, the recorder was zeroed and then set at 100% for the air saturated solution. NADH (0.1–0.3 μmole) was added to the stirred solution in a volume of 10 or 20 μl from an Agla syringe fitted with a 3½″ long needle.

The current was allowed to reach a steady value and then further stepwise additions of NADH were made until the oxygen tension was zero. Rapid stirring and accurate temperature control were essential and were maintained throughout. It was found advisable to protect the reaction mixture from direct sunlight to minimize photodecomposition of PMS: a simple light shield round the water-jacketed chamber was found to be adequate. Under laboratory conditions in Edinburgh such precautions were not normally necessary. Direct sunlight for 30 min did not alter the accuracy of calibration but increased the reaction time.

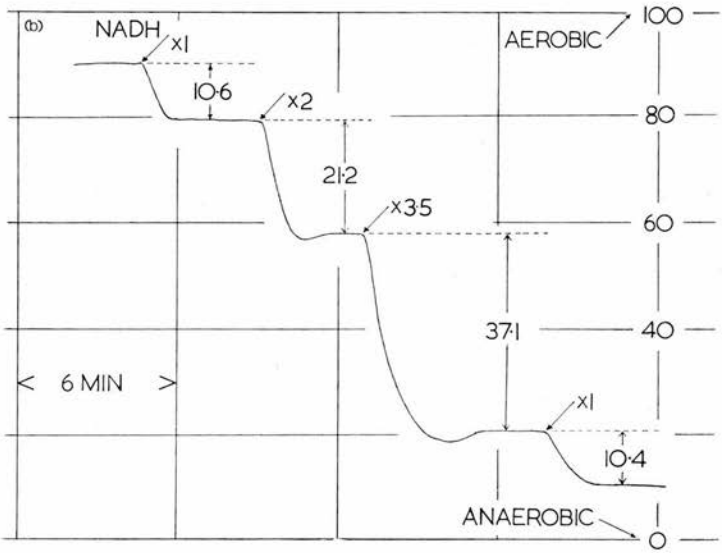

FIG. 1(b). Effect of addition of increasing amounts of NADH on oxygen electrode trace recording and on percentage deflection. The reaction mixture contained 20 μg PMS and 400 μg catalase in 3 ml 0.1 M phosphate buffer, pH 7.4, at 25°C. Additions of NADH are indicated by arrows; the numbers ×1, ×2 or ×3.5 signify additions of 0.155, 0.310, or 0.5425 μmole NADH, respectively. Percentage deflection (x) for each addition is shown; the calculated oxygen concentrations are 243, 243, 243, and 248 μM, respectively.

The oxygen concentration (c) of the air-saturated solution was then calculated from the expression

$$c = \frac{100}{2x} \cdot \frac{n}{v} \mu\text{mole/ml}$$

where n μmoles of NADH added to a volume v ml of experimental medium causes a recorder deflection of $x\%$.

The oxygen consumed by the electrode itself over an average experimental period of 15 min was less than 0.5% of the total oxygen present in the 3 ml reaction mixture. Diffusion of oxygen from the atmosphere into the solution was negligible, and the amount of oxygen introduced in the 10 μl or 20 μl additions of NADH was small enough to be ignored. However, with stepwise additions of volumes greater than 20 μl, the introduction of oxygen must be allowed for in calculating results.

The relative concentrations of NADH, PMS, and catalase were varied to find optimum conditions for calibration.

Catalase

Using 20 μg PMS per 3 ml reaction mixture the formation of hydrogen peroxide was rapid and reaction (iii) tended to be rate limiting unless high catalase concentrations were used. Accordingly, for a rapid calibration method, relatively high concentrations of catalase (400 μg/3 ml) were used; concentrations greater than this lead to a noisy trace recording of the electrode current, due to poisoning of the electrode membrane. With catalase concentrations lower than 40 μg/3 ml a temporary accumulation of hydrogen peroxide occurred, detectable by the "overshoot" on the trace recording of the electrode current. When this occurred the oxygen concentration calculated after equilibrium was reached was identical to that obtained in the presence of greater amounts of catalase, but the reaction time was markedly increased. Hence 400 μg catalase per 3 ml reaction mixture was used routinely.

NAD and PMS

In the presence of 400 μg catalase per 3 ml medium, H_2O_2 temporarily accumulated when PMS concentrations exceeded 20 μg/3 ml, due to the increased rate of reaction (i) and perhaps some inhibition of catalase (7). The oxygen concentration calculated from the trace recording shown in Figure 1(a) was found to be 9.6% higher than values obtained with lower PMS concentrations, or values obtained using the mitochondrial method of Estabrook and Mackler (6).

Addition of large amounts of NADH (0.6 μmole) to the reaction mixture described in the section on methods also caused H_2O_2 accumula-

tion (Fig. 1(b)), but unlike results obtained with large amounts of PMS it did not lead to inaccurate results. It was concluded that the spurious values for oxygen concentration in the presence of large amounts of PMS were due not to the build-up of H_2O_2, but to the high PMS/NADH ratio. An explanation for this is considered in the discussion.

A rapid and reproducible method of calibration was obtained using a 3 ml reaction volume containing 400 μg catalase and 20 μg PMS to which stepwise additions of 0.1–0.3 μmole NADH were made. A calibration can be completed in 15 min.

Validity of Method

The method was checked by comparison with the method of Estabrook and Mackler (6) over a range of oxygen concentrations produced by saturating the medium with air at different temperatures. A range of different oxygen concentrations was also produced by increasing the NaCl content of the medium. In the latter case the oxygen tensions

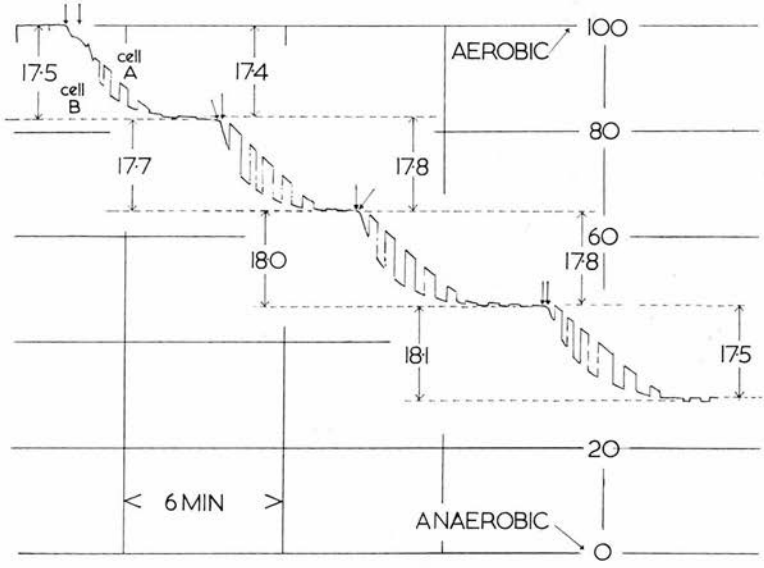

FIG. 2. Comparison of two oxygen electrode trace recordings using (A) calibration method of Estabrook and Mackler (6) and (B) PMS method. Cell A contained 4.5×10^{-9} mole cytochrome *c* and 4 mg mitochondrial protein in 3 ml 0.1 *M* phosphate buffer, pH 7.4, at 25°C. Cell B contained 20 μg PMS and 400 μg catalase in 3 ml 0.1 *M* phosphate buffer, pH 7.4, at 25°. Addition of 0.255 μmole NADH to each cell is indicated by arrows, and percentage deflection (*x*) for each cell is shown. Two oxygen electrodes were used and anode currents were recorded from cell A and B alternately.

found experimentally using the PMS method were compared with values cited for similar solutions in the *International Critical Tables* (4).

(*a*) *Comparison with mitochondrial method.* Traces obtained from an alternating recording of two electrodes immersed in separate chambers containing the same medium (0.1 *M* phosphate, pH 7.4) at 25° are shown in Figure 2. One cell (A) contained 4.5×10^{-9} mole cytochrome *c* and 4 mg mitochondrial protein (prepared according to Estabrook and Mackler (6)), while the other cell (B) contained PMS and catalase. NADH (0.255 μmole) was added to each chamber and the oxygen content of the medium calculated from the trace recordings of the electrode currents. Values of oxygen concentrations obtained from similar experiments over a range of temperatures are shown in Figure 3. Both methods gave the same results over the entire range tested. Values for air-saturated pure water taken from the *International Critical Tables* (4) are also shown in Figure 3. As expected the oxygen content of pure water is slightly higher than that of 0.1 *M* phosphate.

In the Estabrook method of calibration the rate of reaction is virtually independent of oxygen concentration, due to the very high affinity of cytochrome oxidase for oxygen. PMS has a relatively low affinity for

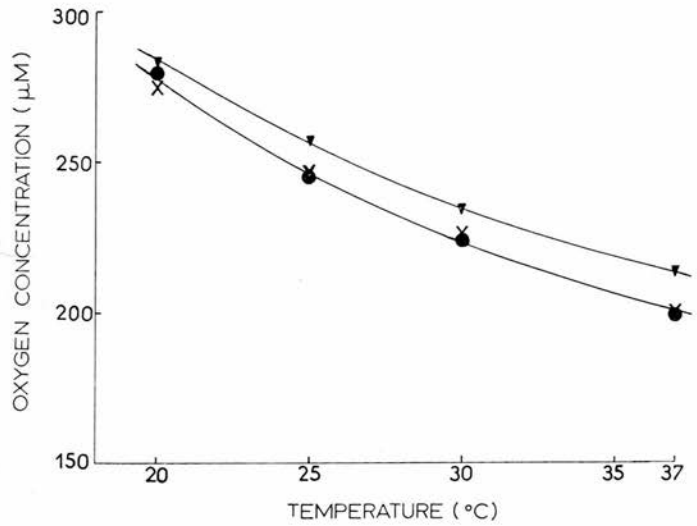

FIG. 3. Oxygen concentrations for media at different temperatures obtained from tables and experimentally: (▲) data for pure water obtained from *International Critical Tables* (4), (●) oxygen concentrations for 0.1 *M* phosphate buffer obtained using calibration method of Estabrook and Mackler (6), (×) oxygen concentrations for 0.1 *M* phosphate buffer, pH 7.4, obtained using PMS method described in this paper.

oxygen, resulting in a slower over-all reaction rate as the oxygen tension drops; at oxygen concentrations less than 48 μM the time for complete oxidation of the added NADH is doubled (see Fig.1(b)). However, since the final percentage deflection (x) is unaltered, the method, although slower, is still accurate at low oxygen tensions.

(*b*) *Oxygen content of 0.01 M phosphate, pH 7.4,* containing 0.5, 1.0, 2.0, 3.0, or 5.43 M NaCl was determined by the PMS method described above. Using these solutions of very different oxygen content clearly confirmed that the electrode measures activities, not concentrations; the electrode current with the probe immersed in air-saturated 3.0 M NaCl was less than 5% lower than that from 0.5 M NaCl, whereas the oxygen content is more than 60% lower. For the purpose of determining the actual oxygen content, the recorder was reset for each air-saturated NaCl solution to give 100% deflection. The superimposed trace recordings of the electrode current after addition of NADH to each of these solutions are shown in Figure 4. The decreased amount of

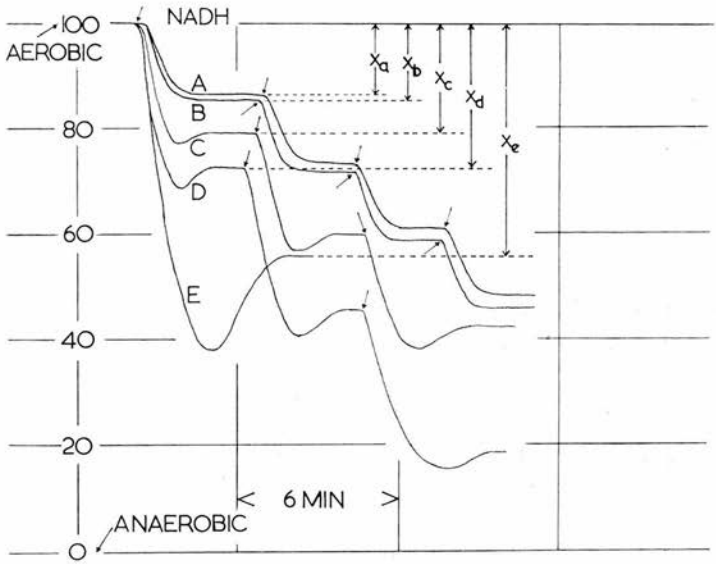

FIG. 4. Effect of varying NaCl concentrations on oxygen electrode trace recording. The reaction mixtures were 20 μg PMS and 400 μg catalase in 3.0 ml 0.01 M phosphate buffer containing different amounts of NaCl equilibrated with air at 25°C. NaCl concentrations were (A) 0.5 M; (B) 1.0 M; (C) 2.0 M; (D) 3.0 M, and (E) 5.43 M (saturated). The recorder was reset for each air-saturated NaCl solution to give 100% deflection and trace recordings were superimposed. Additions of about 0.14 μmole NADH are indicated by arrows, the exact amount in each case being determined by extinction at 340 mμ. x_a to x_e indicate initial percentage deflections for each solution.

oxygen in solution caused by the increasing concentration of NaCl is demonstrated by the progressively larger trace deflections on addition of the same amount of NADH (see Fig. 4). The actual oxygen concentrations calculated from these results are shown in Figure 5 together with data taken from the *International Critical Tables* (4). The values confirm that the PMS method described in this paper is giving true values for oxygen concentrations of solutions.

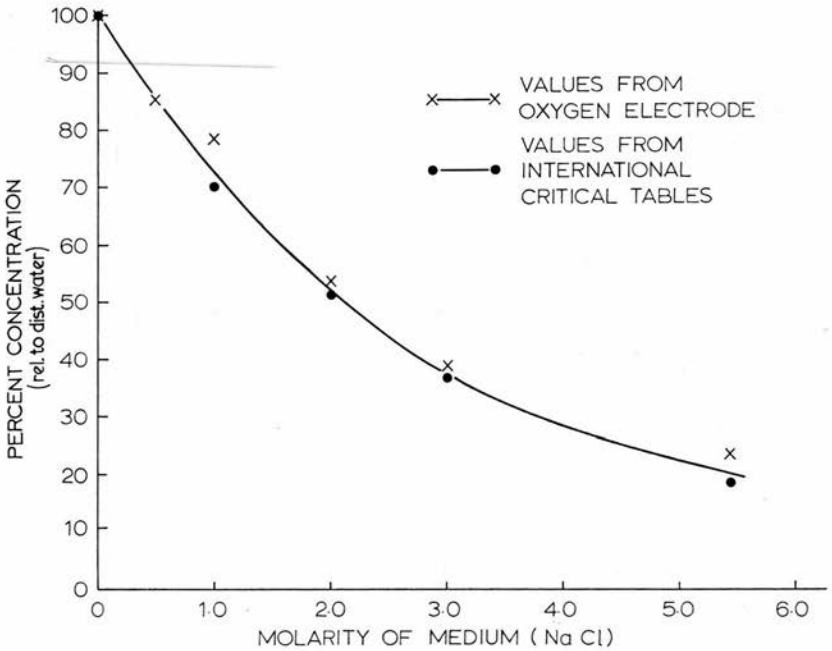

FIG. 5. Oxygen concentrations relative to distilled water for NaCl solutions of varying molarity (oxygen concentration of distilled water is taken as 100%): (●) data taken from *International Critical Tables* (4) for NaCl in pure water, at 25°C, (×) data obtained using PMS calibration method as described in Figure 4 for NaCl in 0.01 *M* phosphate buffer, pH 7.4, at 25°.

DISCUSSION

Errors of as much as 25% can be introduced into experimental results if it is assumed that polarography measures oxygen concentration rather than activity (8). Application of a simple method of calibration should eliminate these inaccuracies.

The method described in this paper has the same advantages as the method utilizing the mitochondrial oxidation of NADH (6); it allows a direct determination of the oxygen concentration of the medium under

the same physical conditions as its experimental application, and at the same time can be used to test the response of the electrode over a full range of oxygen concentrations. The advantages of the PMS over the mitochondrial method are, first, that the necessity for a lengthy preparation of mitochondria that are permeable to NADH has been eliminated and, second, that the PMS method is less restricting on the composition of solutions tested than a method involving mitochondrial oxidations.

The only situation in which spurious oxygen concentrations were obtained was when low NADH/PMS ratios were used. A similar phenomenon in which a fraction of $PMSH_2$ apparently remained unoxidized has been reported by Sowerby and Ottaway (9) and Ottaway (10). Ottaway (10) postulated the formation of a NAD-$PMSH_2$ complex with NADH/PMS ratios less than unity in order to explain his spectrophotometric data. Formation of such an unreactive type of $PMSH_2$ would explain the data obtained in this laboratory (see results).

Provided conditions under which low NADH/PMS ratios pertain are avoided, true values for the oxygen content of media can be determined in 15 min prior to any experiment employing the oxygen electrode.

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