STEROL METABOLISM IN

EXTRA-HEPATIC TISSUES

A thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

in the

UNIVERSITY OF OXFORD

by

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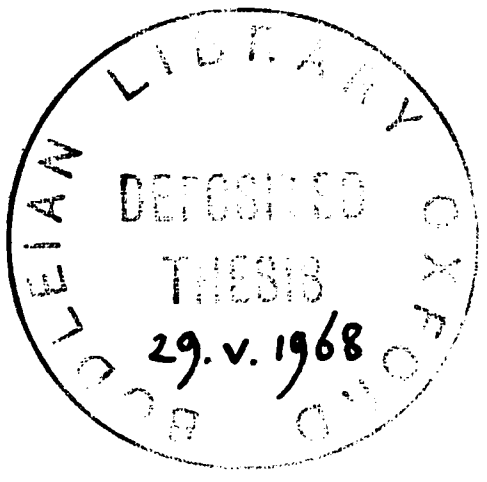
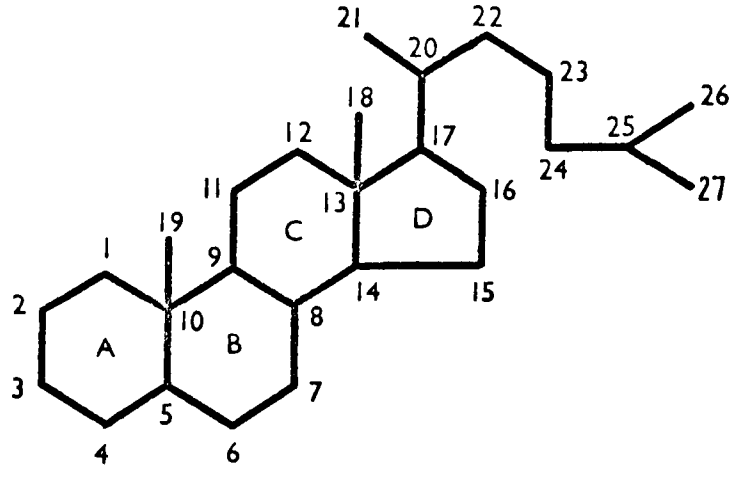
October, 1967.





ABBREVIATIONS

ACTH :- adrenocorticotrophic hormone.

ATP :- adenosine-5'-triphosphate.

cholesterol oxidase :- (E.C. 1.1.3.6) the enzyme system which cleaves the side-chain of cholesterol between C-20 and C-22 producing pregnenolone and isocaproic acid.

cyclic-3',5'-AMP :- adenosine-3',5'-cyclic-monophosphate.

EDTA :- ethylenediaminetetraacetate.

G6P :- glucose-6-phosphate.

G6PDH :- glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49).

isocaproic acid :- 4-methyl-pentanoic acid.

NAD :- nicotinamide-adenine dinucleotide.

NADH :- reduced nicotinamide-adenine dinucleotide.

NADP :- nicotinamide-adenine dinucleotide phosphate.

NADPH :- reduced nicotinamide-adenine dinucleotide phosphate.

pregnenolone :- pregn-5-ene-3β-ol-20-one.

tris :- 2-amino,2-hydroxy-methyl propane-1,3-diol.

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ABSTRACT

REFERENCES

"Some metabolic properties of cholesteryl-3β-sulphate and cholest-4-ene-3-one" by P.R. Raggatt, P.D.G. Dean and M.W. Whitehouse (Biochem. J., 1965, 96, 26P).

"Substrate and inhibitor specificity of the cholesterol oxidase in bovine adrenal cortex" by P.R. Raggatt and M.W. Whitehouse (Biochem. J., 1966, 101, 819).

ACKNOWLEDGEMENTS

I would like to thank Professor Sir Hans Krebs, in whose Department this work was carried out, for his support and cooperation.

I am very grateful to Dr. M.W. Whitehouse for his supervision, support and encouragement in the first two years of this work. My thanks and appreciation are also due to Dr. D.S. Robinson for his supervision in the final year of this work and for his helpful criticism of this thesis.

I wish to thank Professor J.C. Moir and the staff of the Nuffield Maternity Home and of the Maternity Ward, Churchill Hospital, for their willing cooperation in the collection of placentae and the Oxford Cooperative Wholesale Society and the staff of its slaughterhouse for their help in the collection of adrenal glands.

I am also grateful to Mr. R.L.J. Gardener who occasionally helped with experiments, to Dr. P.D.G. Dean and Dr. V. Petrow who donated steroids and to the members of this and other Departments for their help and advice from time to time.

This work was carried out during the tenure of a Medical Research Council Scholarship for Training in Research and I am grateful for this financial support.

Department of Biochemistr
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October, 1967.

INTRODUCTION

The Scope of this Thesis

The steroid hormones produced in animal tissues are all ultimately derived from cholesterol. Their synthesis occurs in the adrenal cortex, the ovary, the testis and the placenta and each of these organs, while chiefly producing its own characteristic hormones, also produces a range of other hormones in smaller quantities. The rates of biosynthesis of individual hormones, as judged by measures of secretion rates, vary considerably; besides the variations from organ to organ and from one individual to another, there are a number of regular cycles of variation of steroid hormone production as in pregnancy and during the menstrual cycle. In addition there are large changes in secretion rates in various pathological conditions (Dorfman & Ungar, 1965).

The great variety of steroid hormones, each with its own important and specific physiological effect, and the variations in secretion rates of particular hormones that are known to occur, require the existence in the body of sensitive systems for the metabolic control of the rates of formation of the different hormones. Clearly several such control mechanisms must exist, both for the regulation of the overall rate of steroid hormone biosynthesis and for the regulation of the rates of biosynthesis of individual hormones.

A "map" of the pathways of steroid hormone biosynthesis and metabolism which shows the complexity of the interrelationships between

the steroids also shows that there is a single major route along which steroid molecules must pass in the process of conversion into hormones. This "bottleneck" is formed by the oxidative sequence of reactions from cholesterol to pregnenolone and is illustrated in Figure 1. Such a "bottleneck" clearly provides a point at which control of the overall rate of steroid hormone formation in a particular organ could be exerted. The hormones of the anterior pituitary are, for example, now believed to act at this site to regulate steroid hormone formation. The present work is concerned with this sequence of reactions as it occurs in bovine adrenal cortex and in human term placenta.

Feed-back inhibition of the rate of conversion of cholesterol into steroid hormones by pregnenolone has been described by Koritz and Hall (1964). The nature of this inhibition has been investigated in this study in some detail and in addition the metabolism by adrenal cortex preparations of cholesteryl-3β-sulphate and cholesteryl fatty acyl esters, which have been suggested as possible precursors of steroid hormones, has been investigated. The thesis also includes studies on the conversion of cholesterol to pregnenolone in human term placenta. This latter work was an extension of some studies by Miss P. Bardsley and myself in which preliminary evidence had been obtained that the enzyme system concerned was unlike that in other endocrine organs in that it was not inhibited by pregnenolone and that it was a cytoplasmic enzyme. Using improved techniques both these postulates were disproved.

Part of this work has appeared in the Biochemical Journal (Raggatt,

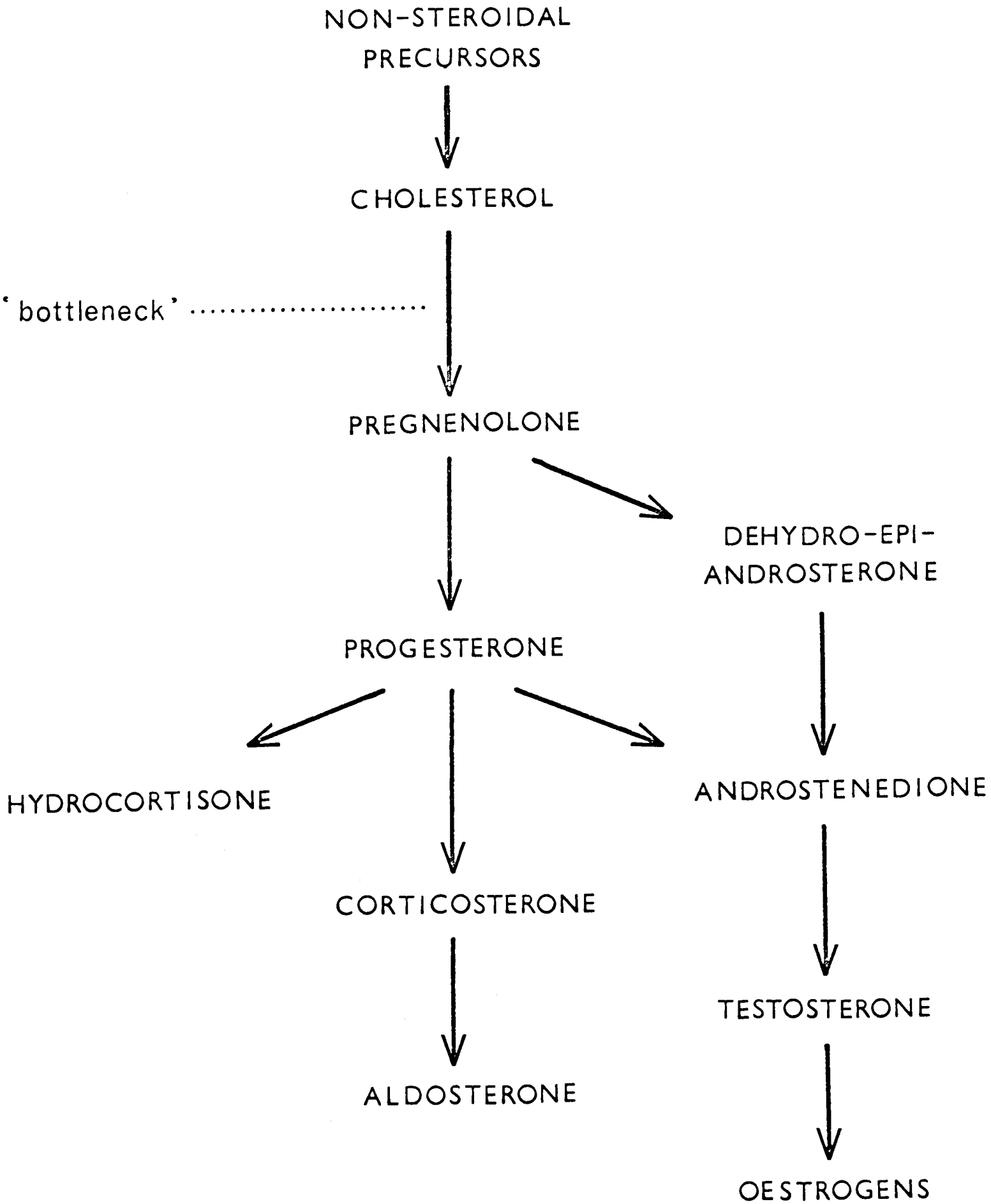


Figure 1

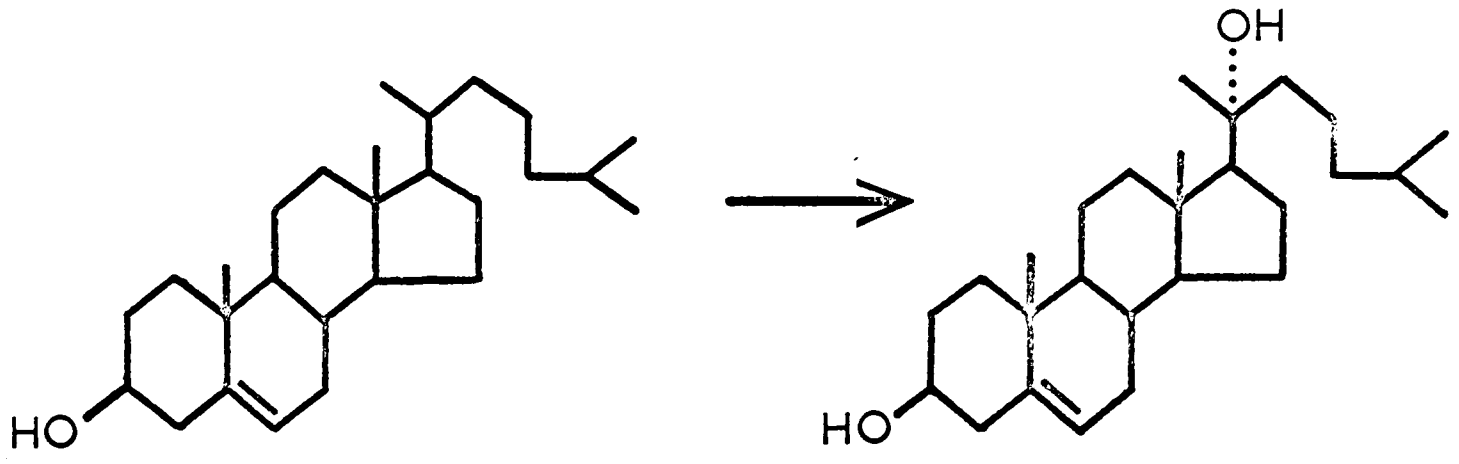
Principal pathways of biosynthesis of steroid hormones.

Dean & Whitehouse, 1965; Raggatt & Whitehouse, 1966) and copies of these papers are to be found at the end of the thesis.

Pathways of Cholesterol Oxidation to Steroid Hormones in Endocrine Organs

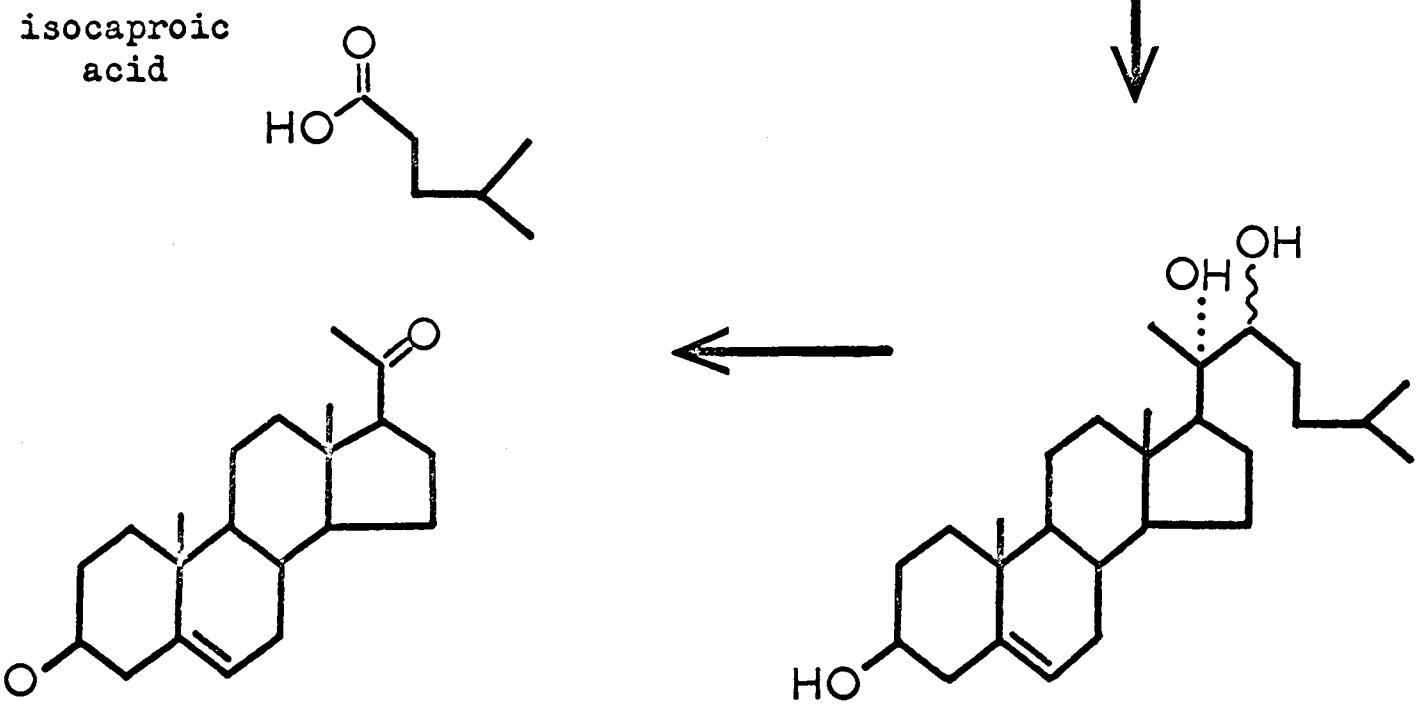
Cholesterol has long been considered to be a precursor of steroid hormones. The early work in the field is reviewed by Hechter and Pincus (1954). However, little progress was made in understanding the mechanisms involved until 1954 when Lynn, Staple and Gurin reported that the side-chain of cholesterol could be cleaved enzymically by extracts of adrenal cortex. It was shown that the product of this cleavage was pregnenolone and that this in turn was converted into physiologically active hormones. Since then there have been a large number of studies concerned with the reactions leading from cholesterol to pregnenolone and thence to steroid hormones and the individual steps in the main reaction sequence have been well established. The work has been the subject of a number of reviews (Hayano, 1962; Dorfman & Ungar, 1965) and therefore will be reviewed here in outline only.

As shown in Figure 2, cholesterol is first hydroxylated at C-20 and then at C-22 (Solomon, Levitan & Lieberman, 1956; Shimizu, Gut & Dorfman, 1962; Constantopoulos, Satoh & Tchen, 1962). The resulting 20α,22ξ-dihydroxy-cholesterol is then cleaved between C-20 and C-22. The steroid product is pregnenolone (Staple, Lynn & Gurin, 1956) while the C_6-product is isocaproic acid or isocaproic aldehyde, there being



cholesterol

20α-hydroxy-cholesterol



pregnenolone

20α,22ξ-dihydroxy-cholesterol

Figure 2

Principal pathway of biosynthesis of pregnenolone

some controversy on this point (Constantopoulos & Tchen, 1961; Lantos, McNiven, Ichii, Bedoukian & Dorfman, 1964; Constantopoulos, Carpenter, Satoh & Tchen, 1966). There is considerable evidence that this pathway of cholesterol oxidation is also present in testis, ovary (corpus luteum) and placenta (Hall & Koritz, 1964; Morrison, Meigs & Ryan, 1965; Menon, Drosdowsky, Dorfman & Forchielli, 1965).

Although the above sequence of reactions is generally considered to form the main pathway of cholesterol oxidation to steroid hormones in endocrine organs in vivo, there is evidence that other pathways may also exist. For example Dorfman and others (Dorfman, 1959; Burstein & Dorfman, 1962; Gual, Lemus, Kline, Gut & Dorfman, 1962; Krum, Morris & Bennett, 1964) have produced evidence which shows both that pregnenolone and cholesterol are obligatory precursors of steroid hormones in vivo and that they are not! Some of these alternative pathways are now described.

One possibility which has been considered is that 22-hydroxylation of cholesterol may precede 20-hydroxylation (Constantopoulos & Tchen, 1961; Chaudhuri, Harada, Shimizu, Gut & Dorfman, 1962). However, this is not widely accepted as providing a quantitatively significant route.

There is also some evidence that, instead of hydroxylation at C-20 and C-22 and cleavage between C-20 and C-22 to form a C_{21}-steroid and a C_6-compound, hydroxylation may occur at C-17 and C-20 to form a C_{19}-steroid and a C_8-fragment (Shimizu, 1964; Shimizu, 1965a; Shimizu, Shimao & Tanaka, 1965). This would also seem to be a very minor pathway

quantitatively and has not been confirmed independently.

There has been one report (Shimizu & Gut, 1965) that desmosterol (3β-hydroxy-cholesta-5,24-diene) can give rise to 4-methyl-pent-3-enoic acid (and presumably pregnenolone); i.e. C-20 - C-22 cleavage of the side chain of desmosterol, a precursor of cholesterol. This has not been confirmed.

Cholest-4-ene-3-one or cholest-5-ene-3-one and their derivatives could clearly be precursors of steroid hormones if oxidation of steroid ring A preceded instead of followed side-chain cleavage. There is a report that 20α-hydroxy-cholest-4-ene-3-one can be converted at a very slow rate to progesterone by an extract of calf adrenal (Shimizu, 1965b) and cholest-4-ene-3-one is more readily oxidised (to bile acids) than cholesterol in the liver (Stevenson & Staple, 1962; Raggatt et al. 1965). However such compounds do not appear to be precursors of steroid hormones in endocrine organs (Kowal, Forchielli & Dorfman, 1964; Krusemper, Forchielli & Ringold, 1964; Raggatt & Whitehouse, 1966).

Cholesterol esters have also been considered as possible precursors of steroid hormones in the adrenal cortex. About 80% of the total cholesterol of the adrenals is esterified, mainly with fatty acids, though this proportion varies considerably with the species and with the nutritional state of the animal (Goodman, 1965). After administration of ACTH or in situations of stress the output of steroid hormones from the adrenal glands increases. Simultaneously the adrenal cortex is depleted of cholesterol fatty-acyl esters but not of free cholesterol,

suggesting that these esters may be precursors of steroid hormones (Riley, 1963; Griffiths, Grant & Symington, 1963; Goodman, 1965; Davis & Garren, 1966). Enzymes esterifying cholesterol and hydrolysing cholesterol esters are found in the adrenal cortex (Kritchevsky, 1958; Shyamala, Lossow & Chaikoff, 1965).

A possible function of cholesteryl-3β-sulphate as a precursor of steroid hormone sulphates is suggested by some experiments of S. Lieberman' group. Both cholesteryl-3β-sulphate and pregnenolone-3β-sulphate were oxidised to dehydroepiandrosterone-3β-sulphate in vivo and double-labelling of these sulphates with ^{3}H and ^{35}S showed that the sulphate group was retained during oxidation of the steroid side chain (Calvin, Vande Wiele & Lieberman, 1963; Roberts, Bandi, Calvin, Drucker & Lieberman, 1964). Cholesteryl-3β-sulphate has been isolated from bovine adrenals (Drayer, Roberts, Bandi & Lieberman, 1964), from human blood and gallstones (Drayer & Lieberman, 1965) and from several other human tissues (Moser, Moser & Orr, 1966). Wieland, de Courcy, Levy, Zala & Hirschman (1965) have found 17α-hydroxy-pregnenolone-3β-sulphate in human adrenal venous blood. Pregnenolone-3β-sulphate is hydroxylated at C-17 in vitro by hyperplastic adrenal tissue without hydrolysis of the sulphate group (Calvin & Lieberman, 1964). Endocrine organs metabolise certain other steroid sulphates without hydrolysing the sulphate group (Baulieu & Carpechot, 1965; Sandberg, Gurpide & Lieberman, 1965; Pasqualini, 1965; Baillie, 1965, Baillie & Griffiths, 1965). Enzymes which sulphate steroids and which hydrolyse steroid sulphates are found

in adrenals and other tissues (Burstein & Dorfman, 1963; Wengle, 1964; Adams, 1964; Baulieu & Carpechot, 1965; Payne & Mason, 1965; Nichol & Roy, 1965; Warren & French, 1965). E-E. Baulieu has suggested that the formation of dehydroepiandrosterone-3β-sulphate from precursor sulphates without hydrolysis of the sulphate group is quantitatively a major pathway in adrenal cortex (Baulieu & Carpechot, 1965).

Although there is little supporting evidence to show that any of the alternative pathways described above are of major importance in vivo compared with the main pathway, it is possible that certain hormones, for example dehydroepiandrosterone-3β-sulphate, may be synthesized other than by the main pathway and although synthesized in minute quantities might be important physiologically.

Metabolic Control of Cholesterol Oxidation in Endocrine Organs

The enzyme system which hydroxylates and then cleaves the side-chain of cholesterol between C-20 and C-22 producing pregnenolone and isocaproic acid ('cholesterol oxidase', E.C. 1.1.3.6) is located in the mitochondria of adrenal cortex, ovary and testis. It requires NADPH and molecular oxygen (Halkerston, Eichhorn & Hechter, 1961) though there is some evidence that NADH can also be used as a cofactor (Staple et al. 1956; Toren, Menon, Forchielli & Dorfman, 1964; Satoh, Constantopoulos & Tchen, 1966), possibly because of the action of a transhydrogenase (see below). The various mechanisms for the regulation of the overall rate of steroid hormone formation that have been suggested are all

thought to operate at this cholesterol oxidase step. They are of three general types: firstly, regulation through control of the NADPH supply; secondly, regulation by pituitary hormones such as ACTH; thirdly, regulation through feed-back inhibition of cholesterol oxidase by the products of cholesterol oxidation, especially pregnenolone.

The role of NADPH.

Most of the work which has been done on the production and utilisation of NADPH in mitochondrial steroid oxidations has been with reference to steroid 11β-hydroxylase (E.C. 1.14.1.6). This enzyme (which converts deoxycorticosterone to corticosterone) is also mitochondrial and requires NADPH and molecular oxygen and therefore the findings are relevant to cholesterol oxidase also. Two recent papers by F.G. Peron's group contain admirable reviews and discussions of this topic with extensive bibliographies (Guerra, Peron & McCarthy, 1966; Peron, McCarthy & Guerra, 1966).

Since intact mitochondria are normally impermeable to pyridine nucleotides (Lehninger, 1953; Bücher & Klingenberg, 1958), steroid hydroxylation in intact, competent mitochondria depends on the supply of Krebs-cycle intermediates which can give rise to the required NADPH inside the mitochondria. However, addition of Ca^{2+} or other mitochondrial swelling agents (Hirschfield & Koritz, 1964) allows entry of extra-mitochondrial NADPH and stimulates 11β-hydroxylation and pregnenolone formation. At the same time, use of Krebs cycle intermediates is inhibited (Guerra et al. 1966). There is some evidence that added

NADH + NADP can serve as a source of NADPH for steroid hydroxylation in such damaged mitochondria via transhydrogenase action (Peron *et al*. 1966).

Regulation of the activity of the enzymes producing NADPH in the mitochondria could thus regulate the rate of steroid hormone production *in vivo*. The most effective NADPH-generating system *in vitro* seems to vary with the experimenter, but two systems have achieved particular prominence (and also have been used in the work reported below). These are the enzyme systems utilising malate and those utilising glucose-6-phosphate. The first of these has been stated to be the principal source of the NADPH required for cholesterol oxidation in adrenal cortex mitochondria (Kimura, 1962).

The chief enzymes in the cell utilising malate are malic enzyme (E.C. 1.1.1.40) and malate dehydrogenase (E.C. 1.1.1.37). The former is NADP-linked and it is generally thought to be mainly a cytoplasmic enzyme. Malate dehydrogenase is mainly mitochondrial but is NAD-linked (Kun, 1963). It is therefore considered that the mitochondrial NADPH is generated *in vivo* by transhydrogenation from NADH formed by malate dehydrogenase or other mitochondrial NAD-linked dehydrogenases. Transhydrogenase (E.C. 1.6.1.1) (Ernster & Lee, 1964, 1967) has been shown to be present in adrenal cortex mitochondria (Peron *et al*. 1966). Some NADPH may however be generated directly should the malic enzyme be, in part, mitochondrial.

McKerns (1962) reported that oestrogens inhibited several NADP-linked dehydrogenases including malic enzyme. In so far as this enzyme

may be concerned in NADPH generation it is possible that oestrogens could exert some regulatory control over steroid hormone production.

Glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49) is thought to be the principal source of NADPH for extra-mitochondrial steroid hydroxylations (Kimura, 1962). It is well established that mammalian forms of this enzyme (but not that of yeast or spinach) are inhibited non-competitively by steroids and these could therefore regulate the production of NADPH by this enzyme. Pregnenolone is an especially powerful inhibitor of glucose-6-phosphate dehydrogenase and dehydroepiandrosterone and oestrogens are also inhibitors (Marks & Banks, 1960; McKerns & Kaleita, 1960; de Sousa & Manso, 1963; Nielson & Warren, 1965).

Glucose-6-phosphate dehydrogenase has been shown histochemically to be concentrated in the zona fasciculata of the adrenal cortex, the zone associated with formation of the glucocorticoids; but after administration of ACTH, the enzyme was increased largely in the fasciculata-reticularis border zone (Greenberg & Glick, 1960). This may be one way in which ACTH exerts control over steroid hormone biosynthesis. (see below).

Oxidation of the NADPH that is associated with steroid hydroxylations inside the mitochondria and inside the microsomes is believed to occur by a specific electron-transport chain consisting of a flavoprotein, a non-haem iron protein and a specific cytochrome (cytochrome P_{450}) (Guerra et al. 1966; Peron et al. 1966; Simpson & Boyd, 1966; Kimura & Suzuki, 1967; Malkin & Rabinowitz, 1967). Cytochrome P_{450} is regarded

as the terminal oxygen activator which, in conjunction with the specific hydroxylase, activates the molecular oxygen used to hydroxylate the steroid. The cytochrome P_{450} electron-transport chain has been implicated in mitochondrial cholesterol oxidation to pregnenolone (Simpson & Boyd, 1966), mitochondrial 11β-hydroxylation (Cooper, Levin, Narasimhulu & Rosenthal, 1966) and microsomal 21-hydroxylation (Estabrook, Cooper & Rosenthal, 1963). There is evidence however that in 11β-hydroxylation and in cholesterol oxidation to pregnenolone the classical electron-transport chain is also involved, linked by transhydrogenase (Guerra et al. 1966; Koritz, 1966a).

The role of pituitary hormones and 3',5'-cyclic AMP.

Pituitary hormones stimulate steroid hormone production by their respective target organs and there is good evidence that this effect is mediated by 3',5'-cyclic AMP (Sutherland, Øye & Butcher, 1965). Recent evidence to support this view is described below.

Stimulation of cholesterol oxidation and steroid hormone production by ACTH and by 3',5'-cyclic AMP has been demonstrated in adrenal cortex slices (Karaboyas & Koritz, 1965; Roberts, Creange & Young, 1965). Interstitial-cell stimulating hormone (ICSH) and 3',5'-cyclic AMP stimulate testosterone production in vivo and in testis slices (Hall, 1963; Sandler & Hall, 1966). Progesterone synthesis in ovarian tissue is stimulated by luteinising hormone and by 3',5'-cyclic AMP (Savard & Casey, 1964; Dorrington & Kilpatrick, 1966a, 1966b). That the increased steroid hormone production is a consequence of 3',5'-cyclic AMP action

is suggested by the observation that luteinising hormone caused rapid accumulation of 3',5'-cyclic AMP and this preceded the increased production of progesterone (Marsh, Butcher, Savard & Sutherland, 1966).

The effects of pituitary hormones and 3',5'-cyclic AMP are not additive; while both ACTH and 3',5'-cyclic AMP separately cause effects of similar magnitude on adrenal steroid hormone biosynthesis, when the tissue is maximally stimulated by ACTH, addition of 3',5'-cyclic AMP causes no further stimulation (Koritz, 1962; Karaboyas & Koritz, 1965). Similar results have been obtained using luteinising hormone and ovarian tissue (Kilpatrick & Dorrington, 1966; Marsh & Savard, 1966).

The view that 3',5'-cyclic AMP may mediate the action of pituitary hormones on steroid biosynthesis is also supported by the finding that inhibitors of the action of ACTH also inhibit the action of 3',5'-cyclic AMP (Ferguson, 1963; Farese, 1964; Garren, Ney & Davis, 1965). These inhibitors (puromycin, cycloheximide and chloramphenicol) are also inhibitors of protein synthesis and it has therefore been suggested that protein synthesis is associated in some way with the action of ACTH. Garren et al. (1965) and Garren & Crocco (1967) have suggested that ACTH causes the synthesis of a protein which functions as a specific regulator of steroid hormone production.

Although stimulation of cholesterol oxidation by both ACTH and 3',5'-cyclic AMP in cell-free preparations of adrenal cortex has been reported in one study (McKerns, 1964), most workers have been able to observe an effect of ACTH only in preparations containing whole cells,

suggesting that the cell membrane is involved in the mechanism of action of ACTH. Sutherland et al. (1965) have suggested that adenyl cyclase, the enzyme which produces 3',5'-cyclic AMP from ATP, is located in the cell membrane and that this enzyme is an essential mediator of the action of ACTH.

Farese (1967) has recently shown that incubation of quartered rat adrenals in the presence of ACTH or 3',5'-cyclic AMP induces the synthesis of a soluble factor which stimulates cholesterol side-chain cleavage and total corticosteroid production in isolated adrenal cortex mitochondria. The inductive process, but not the subsequent stimulation of steroid hormone production from cholesterol, was blocked by puromycin. The induced factor had some protein-like properties but showed no enzymic activity. From quartered adrenals incubated similarly, but without addition of ACTH or 3',5'-cyclic AMP, a similar factor was isolated which depressed steroidogenesis by isolated mitochondria.

As an explanation of his findings, Farese has suggested that extra-cellular ACTH acts initially on the adenyl cyclase of the cell-membrane causing production of 3',5'-cyclic AMP within the cell. This then causes synthesis of Farese's factor which penetrates the mitochondria and stimulates cholesterol oxidation, perhaps by reversing the normal inhibition. The net result would be stimulation of corticosteroid biosynthesis.

Other explanations for the action of ACTH have been suggested. One attributes it to changes in the size of the pool of precursor cholesterol

(Kobayashi, Yago, Marisaki, Ichii & Matsuba, 1963; Ichii, Kobayashi & Matsuba, 1965; Billiar & Eik Nes, 1965). Another suggests that ACTH inhibits glucose-6-phosphatase (E.C. 3.1.3.9) and thus favours formation of NADPH (Hilf, Burnett & Borman, 1962).

Although the overall effect of ACTH is to increase the rate of biosynthesis of corticosteroids, it is thought that its site of action, however mediated, is at some point between cholesterol and pregnenolone (Stone & Hechter, 1954; Karaboyas & Koritz, 1965). The stimulation of steroid hormone production in other endocrine organs which is caused by other pituitary hormones is also probably exerted at a site between cholesterol and pregnenolone (Hall, 1963; Mason & Savard, 1964). Griffiths & Glick (1966) report that ACTH stimulates 11β-hydroxylase in the fasciculata-reticularis border zone of the rat adrenal but this may be an indirect effect.

Pituitary hormones could control the formation of steroid hormones by affecting the NADPH-generating enzymes. This hypothesis has been elaborated by Haynes (Haynes & Berthet, 1957; Haynes, Sutherland & Rall, 1960) who suggests that ACTH specifically increases the formation of 3',5'-cyclic AMP which is known to activate phosphorylase (E.C. 2.4.1.1) (Rall & Sutherland, 1958). This would lead to increased formation of NADPH by the pentose phosphate pathway and thus would stimulate oxidation of cholesterol to pregnenolone. A similar theory could account for the observation of Brown, McLean & Greenbaum (1966), that hypophysectomy lowers the activity of malic enzyme, NADP-linked isocitric dehydrogenase

(E.C. 1.1.1.42) and other enzymes in the adrenals and the testes as well as reducing the rate of biosynthesis of steroid hormones.

The evidence that ACTH acts by stimulating production of 3',5'-cyclic AMP has already been discussed. However, the concentration of NADPH and the rate of corticosteroid biosynthesis seem less closely linked than the Haynes theory would suggest. Harding & Nelson (1964) report that, after hypophysectomy, adrenal corticosteroid production fell after 30 minutes but no change in adrenal NADPH levels occurred until 72 hours after operation. In experiments with guinea pig adrenal slices, Billiar & Eik Nes (1965) found significant differences in the time courses of the stimulation of steroid hormone production by ACTH and by a NADPH-generating system. Koritz & Peron (1958) found that when rat adrenal slices were maximally stimulated by a NADPH-generating system, addition of ACTH increased steroidogenesis further. The inhibitors puromycin and chloramphenicol, which abolish the stimulatory effects of ACTH and 3',5'-cyclic AMP on adrenal slices, had no effect on the stimulation by a NADPH-generating system (Ferguson, 1963; Farese, 1964), suggesting that the effect of NADPH is independent of the effects of ACTH and 3',5'-cyclic AMP.

Another objection to the Haynes theory is that while 3',5'-cyclic AMP is reported to stimulate the hydroxylation of certain steroids beyond pregnenolone in the biosynthetic pathway (Creange & Roberts, 1965), ACTH does not (Karaboyas & Koritz, 1965). If the effect of ACTH is solely to increase the production of NADPH, it is hard to see why it

should not stimulate all hydroxylations equally. This is not to say, however, that ACTH has no effect on NADPH production or that the rate of NADPH production has no effect on the rate of biosynthesis of steroids.

The role of feed-back inhibition by pregnenolone.

Part of this work is concerned with the inhibition of cholesterol oxidase by the products of the enzyme's action, especially pregnenolone. This inhibition could represent a kind of negative feed-back control of the activity of the enzyme by which the rate of utilisation of pregnenolone for subsequent steroidogenesis exerted control over the rate of its production.

Pregnenolone has been shown to inhibit cholesterol oxidase in the adrenal cortex, in the placenta and in the corpus luteum (Ichii, Forchielli & Dorfman, 1963; Koritz & Hall, 1964; Raggatt & Whitehouse, 1966). Pregnenolone acts by inhibiting cholesterol 20α-hydroxylase, the enzyme catalysing the first step in steroid hormone biosynthesis from cholesterol. This has been shown by its lack of effect on the oxidation of 20α-hydroxy-cholesterol to steroid hormones (Koritz & Hall, 1964; Ichii, Omata & Kobayashi, 1967).

3β-Hydroxy-steroid dehydrogenase (E.C. 1.1.1.51) and 3-keto-steroid Δ^5-Δ^4-isomerase (E.C. 5.3.3.1), the enzymes required to make progesterone from pregnenolone, are microsomal enzymes (Cheatum, Douville & Warren, 1967). Steroid-21-hydroxylase (E.C. 1.14.1.8), one of the enzymes needed to convert progesterone into corticosteroids, is also microsomal (Ryan & Engel, 1957). Steroid-17α-hydroxylase (E.C. 1.14.1.7), one of

the enzymes needed to convert pregnenolone into androgens, is a cytoplasmic enzyme (Young, Bryson & Sweat, 1965). Thus, all the main pathways of pregnenolone metabolism in the adrenal cortex have some steps which take place outside the mitochondrion and pregnenolone must leave the mitochondrion after its formation from cholesterol. On the basis of these findings, Koritz (1966b) has recently suggested that control over the rate of exit of pregnenolone from the mitochondrion (however exerted) may be coupled with the negative feed-back inhibition of cholesterol oxidase to provide a mechanism for the regulation of cholesterol oxidation.

EXPERIMENTAL

Materials

Bovine adrenals were kindly supplied by the Oxford Co-operative Wholesale Society Slaughterhouse. They were removed from cows (1 - 3 years old) within a few minutes of death and transported to the laboratory on ice. Human term placentae (by normal or Caesarean delivery) were kindly made available by the Nuffield Maternity Home, Oxford, and the Maternity Department of the Churchill Hospital, Oxford. They were collected within a few minutes of delivery and transported to the laboratory in ice cold physiological sodium chloride solution (0.9%).

Hexokinase (from yeast, activity of approx. 140 μmole substrate converted/min./mg. at 25°) and glucose-6-phosphate dehydrogenase (from yeast, activity of approx. 140 μmole substrate converted/min./mg. at 25°) were obtained from Boehringer und Soehne GmbH (Mannheim).

The following substances were obtained from the Sigma Chemical Co. (London):- cholesteryl linolenate, ATP, NAD, NADP, NADH, NADPH, glucose-6-phosphate.

The following substances were obtained from British Drug Houses Ltd. (Poole, Dorset):- pregnenolone, pregnenolone acetate, progesterone, cholesterol, cholesteryl acetate, cholesteryl oleate, cholesteryl stearate, cholesteryl palmitate, androst-4-ene-3,17-dione, digitonin, naphthalene, dimethyl formamide, sodium lauryl sulphate.

The following substances were obtained from Mann Research Laboratories, Inc., (New York):- lithocholic acid, 22,23-bisnor-chol-5-enoic acid, corticosterone, dehydroepiandrosterone, dehydroepiandrosterone sulphate, oestrone, testosterone.

The following substances were obtained from Koch-Light & Co. Ltd (Colnbrook, Buckinghamshire):- isocaproic acid, palmitic acid, 17α-hydroxy-pregnenolone, 3β-acetoxy-22,23-bisnor-chol-5-enoic acid.

PPO (2,5-diphenyl oxazole) and dimethyl-POPOP (1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene) were obtained from Thorn Electronics, (Surbiton, Surrey).

25-Oxo-27-nor-cholesterol was obtained from The Schering Corp. (Bloomfield, New Jersey). Pregn-5-ene-3β-20α-diol was obtained from Schering A.G. (Berlin).

3β-Hydroxy-chol-5-enoic acid was a 'Rousel' product (M.R.C. Reference Collection).

3β-Hydroxy-androst-5-ene-17β-carboxylic acid was obtained from Zori Ltd. (Tel Aviv).

Su4885 (2-methyl-1,2-di(pyrid-3-yl)-propan-1-one) was obtained from Ciba Pharmaceutical Products (Basle).

Bovine serum albumin was obtained from The Armour Pharmaceutical Co. (Eastbourne).

Silica-H (Merck) was obtained from Camlab Ltd. (Cambridge).

A small sample of 20α-hydroxy-cholesteryl-3β-acetate was a gift from Dr. V. Petrow (British Drug Houses Ltd.).

The following compounds were prepared and donated by P.D.G. Dean, Esq., of the Department of Biochemistry, University of Oxford:-

25-Oxo-27-nor-cholesteryl-3β-acetate, (m.p. 139-140°), prepared by acetylation of 25-oxo-27-nor-cholesterol with acetic anhydride in pyridine.

25-Hydroxy-27-nor-cholesterol, (m.p. 160-165°), prepared by borohydride reduction of 25-oxo-27-nor-cholesteryl-3β-acetate.

25-Hydroxy-cholesterol (m.p. 182-183°) prepared by the method of Ryer, Gebert & Murrill (1950).

[26-^{14}C]-Cholesterol (24.0 mc/mmole) and [4-^{14}C]-cholesterol (34.0 mc/mmole) were obtained from The Radiochemical Centre (Amersham, Buckinghamshire). They were purified before use by chromatography on thin layers of silica in systems II and IV (see below) and suitably diluted with non-radioactive cholesterol (recrystallised from ethanol, m.p. 146-148°) just before use. [^{14}C]-Cholesterol was stored dissolved in hexane under nitrogen at -15° in the dark.

[4-^{14}C]-Cholesteryl linolenate (19.4 mc/mmole) was supplied by Professor A. Crastes de Paulet of the Institute of Biology of the University of Montpellier under an arrangement with Euratom. It was rechromatographed on thin layers of silica in system I (see below).

Sodium [1-^{14}C]-isocaproate (2.12 mc/mmole) was obtained from The Radiochemical Centre (Amersham, Buckinghamshire).

Other reagents were 'analytical grade' and were obtained from normal commercial sources. Solvents for thin-layer chromatography were

dried and redistilled before use. Dioxane for scintillant was purified by passing it through a column of alumina.

Syntheses

Sodium cholesteryl-3β-sulphate was made by the method of Roy (1956) using pyridine-SO_3 made by the method of Sobell, Goodman and Blau (1951). Chlorosulphonic acid was redistilled (b.p. 151 - 152°) and shaken with dry, redistilled carbon tetrachloride to produce a saturated solution. This solution was cooled to about -20° and added dropwise to a solution of pyridine (3 ml., redistilled over KOH) in dry, redistilled carbon tetrachloride (20 ml.) cooled in solid CO_2:ethanol. The chlorosulphonic acid solution was added until no more white precipitate of pyridine-SO_3 formed. Dry, redistilled benzene was added and the precipitate was collected and washed with more benzene by centrifugation. The white pyridine-SO_3 was used at once. It was found that careful purification of all materials and maintainance of a low temperature were essential to avoid a discoloured product.

Cholesterol (3g.) was dissolved in dry benzene (20 ml.) and a four-fold excess of pyridine-SO_3 suspension in benzene (5 ml.) was added. The mixture was refluxed for 1½ hr. After cooling and addition of dry light petroleum (b.p. 40 - 60°) and standing at 0°, crystals of cholesteryl pyridinium sulphate formed and were collected. Recrystallisation of this product from chloroform:light petroleum (b.p. 40 - 60°) gave colourless needles of cholesteryl pyridinium sulphate, m.p. 179 - 182° (decomp.); found 72.4%C, 9.6% H, 2.8% N, 6.0% S;

$C_{32}H_{51}NO_4S$ requires 72.6% C, 9.64% H, 2.65% N, 6.05% S. The crude cholesteryl pyridinium sulphate was dissolved in 50% aqueous ethanol and refluxed for 1 hr. with M Na_2SO_4 solution. A white precipitate formed and when cool was recovered and washed with cold water and with ether by centrifugation and recrystallised from a mixture of butan-2-one, ethanol and water (approx. 5:5:1 by vol.). The product, sodium cholesteryl sulphate, melted at 175 - 176° and had 66.4% C, 9.2% H and 6.3% S; $C_{27}H_{45}NaO_4S$ requires 66.4% C, 9.22% H and 6.56% S. It moved as a single spot (R_f 0.58) on chromatography on thin layers of silica-H using benzene:butan-2-one:ethanol:water 3:3:3:1 (by vol.).

Sodium [^{14}C]-cholesteryl sulphate was prepared similarly, from either [26-^{14}C]- or [4-^{14}C]-cholesterol (10 μc, about 0.1 mg.) and a large excess of pyridine-SO_3. After the initial condensation (1½ hr.) the pH of the solution was adjusted to 8.0 with 1N-NaOH; then pyridine and solvent were removed *in vacuo* and the residue was dissolved in ethanol: butan-2-one (2:1, v/v) and applied quantitatively to a 20 x 20 cm. chromatoplate of silica-H. After chromatography in butan-2-one, the band corresponding to cholesteryl sulphate (R_f 0 - 0.2) was removed and extracted with ethanol:butan-2-one (2:1, v/v) and rechromatographed with butan-2-one; this procedure removes all unchanged cholesterol and a yellow, non-radioactive impurity. The product was further purified by thin layer chromatography on silica-H in benzene:butan-2-one:ethanol: water 3:3:3:1 (by vol.) when it moved as a single narrow band (R_f 0.58) coincident with authentic non-radioactive cholesteryl sulphate. This

method of purification proved better than simple rechromatography in the benzene:butan-2-one:ethanol:water system, as in the latter the yellow impurity 'streaked' into the cholesteryl sulphate region. The overall yield was 20 - 25%. It was stored as a dry film under nitrogen at -15° in the dark and used as soon as possible. It was rechromatographed on the same day as it was used as a substrate. Storage in ethanol:butan-2-one solution at -15° resulted in solvolysis; e.g. one 2 μc batch (0.04 mg.) in 5 ml. ethanol;butan-2-one 1:1 (v/v) was almost completely solvolysed to cholesterol in 3 months (see also McKenna & Norymberski, 1957a).

Sodium 25-oxo-27-nor-cholesteryl-3β-sulphate was made in the same way as cholesteryl sulphate. [Yield 51%; m.p. 185 - 190° decomp; found 58.9% C, 8.59% H and 6.34% S; $C_{26}H_{42}NaO_5S$ requires 59.6% C, 8.50% H and 6.54% S. R_f 0.44 on thin layers of silica-H in the system benzene: butan-2-one:ethanol:water 3:3:3:1 (by vol.).]

Sodium pregnenolone 3β-sulphate was made in the same way as cholesteryl sulphate; however, care was needed to prevent solvolysis during crystallisation. [Yield 67%, m.p. 197° decomp. (from ethanol:butan-2-one), found 59.4% C, 7.64% H and 7.46% S; $C_{21}H_{31}NaO_5S$ requires 60.0% C, 7.46% H and 7.66% S. It moved as a single spot (R_f 0.51) on thin layer chromatography on silica-H with benzene:butan-2-one:ethanol:water 3:3:3:1 (by vol.).]

[4-^{14}C]-and [26-^{14}C]-cholesteryl-3β-acetate. Either [^{14}C]- or [26-^{14}C]-

cholesterol (10 μc, about 0.1 mg.) was dissolved in pyridine (0.5 ml.) and acetic anhydride (0.5 ml.) was added. After standing at 20° in the dark overnight, water (2ml.) was added and the mixture was warmed to 50° for 5 min. After adjusting the pH to 8.0 with 1N-NaOH the cholesteryl acetate was extracted with benzene (4 x 3 ml.); the benzene solution was washed with 1N-HCl, (1 ml.), water (1 ml.), 5% $NaHCO_3$ (1 ml.) and water (1 ml.) and then dried with $MgSO_4$. The solution was concentrated by evaporation under nitrogen at 50° and the product applied to a 20 x 20 cm. chromatoplate of silica-H. After chromatography in iso-pentyl acetate: hexane 1:9 (v/v), the band corresponding to cholesteryl acetate was removed. The product was extracted from the silica with ethyl acetate and rechromatographed in the same system. It was finally chromatographed on silica-H in benzene:ethyl acetate 9:1 (v/v). The radioactive product had identical mobility (R_f 0.63) to authentic cholesteryl acetate (m.p. 115°) and moved as a single narrow band on thin layer chromatography. The overall yield was 81%. It was stored in benzene solution at -15° in the dark.

[26-^{14}C]-cholest-4-ene-3-one was made by Oppenauer oxidation of cholesterol (Serini, Köster & Strassberger, 1945). The cholesterol (14 μc, 0.322 mg.) was dissolved in dry redistilled xylene (0.1 ml.) and aluminium isopropoxide (1 mg.) was added. After heating at 95° for 5 min., dry redistilled cyclohexanone (0.1 ml.) was added and the mixture heated at 95° for 4 hr. in the dark. A few drops of water were added and then the

solvents were removed in vacuo. The residue was extracted with diethyl ether, and applied to a 20 x 20 cm. chromatoplate of silica-H. After chromatography with ethyl acetate:hexane 3:7 (v/v), the cholestenone band was removed, extracted with ether and rechromatographed in the same system, and finally purified by thin-layer chromatography in ethyl acetate:hexane 1:5 (v/v). The radioactive product had exactly the same mobility as authentic non-radioactive cholestenone (m.p. 78°), and in both these systems it moved as a single narrow band. The overall yield was 87%. It was stored in benzene solution at -15° in the dark.

27-Nor-cholest-4-ene-3,25-dione was made from 25-oxo-27-nor-cholesterol (m.p. 127°) in the same way as cholestenone (yield 75%) and after two recrystallisations first from hexane and then from aqueous acetone had m.p. 129 - 130°; λmax. 240 - 243 mμ, $\varepsilon = 1.76 \times 10^4$ (in ethanol). [Cholest-4-ene-3-one had λ max. 240 - 243 mμ and $\varepsilon = 1.69 \times 10^4$ (in ethanol.] Selye (1942) reports the m.p. of 27-nor-cholest-4-ene-3,25-dione (supplied to him by the Schering Corp.) as 114 - 116° but gives no details of its synthesis.

Dihydrogen cholesteryl-3β-phosphate. Cholesterol (2 g.) was dissolved in dry redistilled pyridine (10 ml.) and the solution added dropwise to freshly redistilled $POCl_3$ (2 ml.) in dry acetone (10 ml.) at -40°. The reaction mixture was allowed to stand at room temperature in the dark overnight. The white precipitate which formed was collected by centrifugation and washed with acetone; this product contained pyridine

and on treatment with water gave chloride and hydrogen ions. It was heated with water (50 ml.) at 95° for 2 hr. and the white, flocculent solid which formed was collected and washed with water by centrifugation. After recrystallisation from ethanol-benzene mixtures, it softened at 160° and melted rapidly with decomposition at 194 - 196°. It contained 69.4% C, 9.8% H and 6.8% P; $C_{27}H_{47}O_4P$ requires 69.5% C, 10.0% H and 6.5% P. Among other products of this method was a white solid of m.p. 63 - 65° (from hexane-ethanol) and R_f 0.82 on silica-H in ethylacetate: hexane 3:7 (v/v), probably di-cholesteryl ether [m.p. 74° (Plimmer and Burch, 1929), 65° (Venner, 1960)].

The chemical synthesis of cholesteryl-3β-phosphate by the action of phosphoryl chloride ($POCl_3$) in the presence of pyridine has been described by three groups (von Euler et al., 1927, 1929; Plimmer and Burch, 1929; Venner, 1960) and all these methods were tried with very limited success. The course of the reaction is far from simple as is indicated by the variety and nature of the products identified by various workers:- cholesteryl dihydrogen phosphate (and its pyridine adduct), di-cholesteryl hydrogen phosphate (and its pyridine adduct), di-cholesteryl ether, cholesteryl-3β-chloride, cholesteryl dihydrogen phosphate dimer and cholesteryl phosphoryl chloride. It seems likely that the initial reaction results in formation of a phosphate ester bond but that subsequent rearrangement occurs including cleavage of the O-C bond of the phosphate ester linkage. This could give rise to cholesteryl chloride and dicholesteryl ether, or could be followed by

dehydrogenation giving rise to a variety of ring-unsaturated steroids. This is supported by the observations of McKenna and Norymberski (1957a, 1957b) who have studied the solvolysis of steroid sulphates and Blumenthal and Herbert (1945) who have demonstrated analogous mechanisms in the acid hydrolysis of other phosphate esters.

Venner's method described crystallisation of the initial white product from glacial acetic acid. This gave rise almost wholly to cholesteryl-3β-chloride and this product was used in later experiments. The cholesteryl-3β-chloride had m.p. 94 - 96° (from hexane), R_f 0.75 on silica-H in ethyl acetate:hexane 3:7 (v/v), 79.8% C, 11.2% H, 8.9% Cl; $C_{27}H_{45}Cl$ requires 80.0% C, 11.2% H and 8.8% Cl. A small amount of the product tentatively identified as dicholesteryl ether (m.p. 63 - 65° from hexane-ethanol) was also formed but little or no cholesteryl phosphate.

The method of Turnbull and Wilson (1954) using diphenyl phosphoryl chloride gave a good yield of cholesteryl diphenyl phosphate, [m.p. 115 - 116° (from ethanol), R_f 0.6 on silica-H in ethyl acetate:hexane 3:7 (v/v), found 75.0% C, 9.1% H, 4.4% P; $C_{39}H_{55}O_4P$ requires 75.7% C, 10.3% H, 5.0% P]. However, attempts to hydrolyse this according to Turnbull and Wilson gave little or no cholesteryl phosphate. Likewise the method of Nussbaum and Tiberi (1965) gave no isolable cholesteryl phosphate. This method condenses sterols with S-ethyl pyridinium thiophosphate in the presence of dicyclohexyl-carbo-di-imide.

The isolation of cholesteryl phosphate is hindered by its low

solubility in most solvents and its tendency to form gels in the presence of water. Attempts to prepare and purify [^{14}C]-cholesteryl phosphate failed.

Pregnenolone-3β-palmitate was made by the method of Pinter, Hamilton and Muldray (1964). Palmitoyl chloride was made from palmitic acid (0.5 g.) and oxalyl chloride (1.0 ml.) and allowed to react with pregnenolone (0.3 g.) in di-isopropyl ether. [Yield 60%, m.p. 94 - 95° (from benzene-ethanol), found 79.1% C, 11.0% H; $C_{37}H_{61}O_3$ requires 80.1% C, 11.1% H]. It moved as a single spot (R_f 0·59) on thin layer chromatography on silica-H with iso-pentyl acetate:hexane 1:9 (v/v).

20α-hydroxycholesterol and 20α-hydroxycholesteryl-3β-acetate.

Pregnenolone 3β-acetate was condensed with 4-methyl-pentyl magnesium bromide according to Petrow and Stuart-Webb (1956). The 4-methyl-pentyl bromide was made by reduction of isocaproic acid with lithium aluminium hydride followed by bromination with PBr_3. The 20α-hydroxy-cholesteryl-3β-acetate produced had m.p. 156 - 157° (from methanol), [found 78.6% C, 10.9% H; $C_{29}H_{48}O_3$ requires 78.4% C and 10.8% H] and moved as a single spot (R_f 0.61) on thin layer chromatography on silica-H with ethyl acetate:hexane 3:7 (v/v), identical with a sample of 20α-hydroxycholesteryl-3β-acetate supplied by Dr. V. Petrow. The yield was 20 - 30%. The acetate was hydrolysed by 0.2N-methanolic NaOH under nitrogen in the dark overnight at room temperature (Bush, 1961). The 20α-hydroxycholesterol produced had m.p. 136 - 137°; found 80.5% C, 11.6% H; $C_{27}H_{46}O_2$ requires 80.6% C, 11.4% H. It moved as a single spot

(R_f 0.26) on thin layer chromatography on silica-H with ethyl acetate: hexane 3:7 (v/v).

An improved version of this method was used subsequently in which pregnenolone-3β-tetrahydropyranyl ether instead of pregnenolone-3β-acetate was condensed with 4-methyl-pentyl magnesium bromide. This method has the advantage that none of the Grignard reagent is used up by the acetate ester group.

Pregnenolone-3β-tetrahydropyranyl ether was made by the method of Ott, Murray and Pederson (1952). Pregnenolone (1 g.) and anhydrous redistilled dihydropyran (4 ml.) were dissolved in anhydrous ether (45 ml.) and one drop of concentrated hydrochloric acid was added. The mixture was shaken and then dry chloroform (25 ml.) was added. The mixture was shaken mechanically for 1 hour and then left at room temperature in the dark for 18 hours. Pellets of NaOH were added and the mixture was shaken and then filtered. After evaporation of the solvents in vacuo the product was crystallised from hexane when it had m.p. 134 - 135°, and moved as a single spot (R_f 0.75) on thin layers of silica-H in the system ethyl acetate:n-hexane 3:7 (v/v) and had 78.0% C and 9.9% H; $C_{26}H_{40}O_3$ requires 78.1% C, 10.1% H. The condensation with the Grignard reagent was carried out in ether in the usual way. After the condensation the mixture was shaken vigorously for 15 minutes with 2N-H_2SO_4; this hydrolyses the tetrahydropyranyl ether to the alcohol (20α-hydroxy-cholesterol) which was recovered and crystallised as above. It had identical properties to that made from pregnenolone acetate. The yield

was 78%, a considerable improvement on the original method and there were also fewer by-products detectable by thin layer chromatography.

Melting points were determined in thin walled capillaries in an air bath and are uncorrected.

Elemental analyses were performed by Drs. Weiller and Strauss & Co., Oxford.

Enzyme preparations.

All operations were performed at 0 - 5°. Whole adrenals were removed from cows (1 - 3 years old) at the slaughterhouse within 10 min. of death, chilled and transported to the laboratory on ice. The adrenals, when dissected free of fat and connective tissue, weighed 8 - 12 g. each. The cortex was dissected from the medulla and the cortical tissue scraped off the capsule; 4.5 - 6.0 g. wet weight of cortex was obtained from each adrenal. The cortical tissue was suspended in 2.5 ml. of "Supplemented Sucrose" (Halkerston et al. 1961) per g. of tissue and homogenised in a stainless-steel homogeniser. ["Supplemented Sucrose" contains sucrose (0.44 M), EDTA (1 mM), sodium fumarate (5 mM), nicotinamide (5 mM) and potassium phosphate buffer (10 mM) at pH 7.4.] The homogenate was centrifuged at 1200 g for 30 min., the sediment discarded and the resulting supernatant centrifuged at 16,000 g for 20 min. The "mitochondrial pellet" so obtained was washed with

Supplemented Sucrose and recentrifuged at 16,000 g for 20 min. It was resuspended in Supplemented Sucrose for incubation. To prepare the "microsomal fraction", the 16,000 g supernatant was centrifuged at 105,000 g for 30 min. The fatty layer was removed from the 105,000 g supernatant before decanting the supernatant from the centrifuge tubes and the "microsomal pellet" was resuspended in Supplemented Sucrose. The various pellets were resuspended using a loose-fitting Teflon-in-glass homogeniser.

Placentae were collected from the hospital within 20 minutes of delivery (normal or Caesarean) and transported to the laboratory in ice cold physiological saline (0.9% w/v NaCl). The external membranes were removed from the placenta and discarded and clots of blood were removed and the surface rinsed with fresh saline. The soft tissue was cut into pieces weighing 1 - 2 g., avoiding large blood vessels and the connective tissue. These pieces were washed three times in ice-cold saline to remove as much blood as possible and then chopped with scissors and minced in a stainless steel hand mincer. The mince was diluted with $2\frac{1}{2}$ volumes of Supplemented Sucrose and homogenised and differentially centrifuged by the procedure used for adrenal cortex. In some experiments a Virtis high speed homogeniser was used.

In some experiments mitochondrial suspensions were sonicated in an M.S.E. (100 watt, 20 Kc/sec.) Ultrasonic Disintegrator for 3 min. The suspension was cooled in ice and sonication was carried out in three one-minute stages with stirring between stages to prevent the temperature

rising above 5°. The sonicated mitochondrial preparation was then centrifuged at 105,000 g for 30 min. at 0°. The sediment was discarded and the supernatant is referred to as the 'sonicated supernatant'.

Acetone-ether dried powders were prepared in some experiments after homogenisation and centrifugation had been carried out as described above. The mitochondrial fraction was resuspended in 0.1 M sodium phosphate buffer, pH 7.4, and the suspension was poured into 10 vol. of acetone:ether 5:1 (v/v) at -10° and agitated with a Waring Blender. All subsequent operations were carried out at +1°. After standing for 10 min., the powder was filtered and washed with fresh acetone and anhydrous ether and dried *in vacuo* over $CaCl_2$ and anhydrous glycerol for 1 - 2 days. It was then pulverised, redried in the same way and then stored over dry silica gel at -15°. Before use, it was homogenised with Supplemented Sucrose, allowed to stand at 0° for 30 min., rehomogenised and centrifuged at 600 g for 15 min. The sediment was discarded.

Acetone-ether dried powders of the sonicated supernatant were prepared similarly. These dissolved completely and were not centrifuged.

Incubation Procedure.

Steroid solutions were measured out dissolved in a suitable volatile solvent. The solvent was removed by evaporation at about 50° under a stream of nitrogen and the substances were dissolved in N,N-dimethyl formamide. The dimethyl formamide solution (0.1 ml.) was

measured into 25 ml. incubation flasks and the enzyme preparation (3 ml.) was then added to this, followed by 2 ml. of a cofactor mixture at pH 7.4, giving the following final concentrations:- $MgCl_2$, 1.5 mM; sodium phosphate buffer, 10 mM; disodium ATP, 1.25 mM; NAD, 1.25 mM; sodium hydrogen malate, 3 mM. The final incubation volume was 5.1 ml. This dilution produced final concentrations of the substances in the suspending medium as follows:- sucrose, 0.26M; EDTA, 0.59 mM; sodium fumarate, 3 mM; nicotinamide, 3 mM; potassium phosphate buffer, 5.9 mM. In some experiments, centre wells contained 0.2 ml. of 1N-NaOH.

In some experiments with fresh mitochondrial preparations and in all experiments with the sonicated supernatant and acetone dried preparations, a different cofactor mixture was used. This consisted of 0.01 ml. (0.01 mg., about 1.4 units i.e. converts 1.4 µmole of substrate in 1 min. at 25°) of glucose-6-phosphate dehydrogenase suspension which was added after the enzyme preparation and was followed by 2 ml. of a mixture of glucose-6-phosphate, NADP, $MgCl_2$ and sodium phosphate buffer, pH 7.4. The final concentrations were:- glucose-6-phosphate, 2.5 mM; NADP, 0.5 mM; $MgCl_2$, 1.5 mM; phosphate, 5.9 mM.

Incubations were for 10 - 180 min. at 37° in air in a shaking water bath in the dark. Controls contained enzyme denatured either by heat (30 min. in a boiling water bath) or by acid (0.2 ml. of 18N H_2SO_4) and converted $< 0.03\%$ of the [26-^{14}C]-cholesterol to [^{14}C]-isocaproic acid in 20 min.

Extraction of Products.

Extraction of [^{14}C]-isocaproic acid from incubation mixtures.

Incubations were terminated by the addition of 0.2 ml. of 18N-H_2SO_4 and cooling to -15°. After thawing, the incubation mixtures were transferred to separate centrifuge tubes and each incubation flask was rinsed with 1 ml. of 5N-NaOH containing 20 mg. of non-radioactive isocaproic acid as carrier; this was added to the appropriate tube. After mixing, the tube contents were acidified with 18N-H_2SO_4 (0.2 ml.), mixed well and centrifuged at 4,800 g for 10 min. at 10°. The clear supernatants were decanted, the sediments washed with water (1 ml.) and the washings added to the supernatants. The residual cholesterol was then removed from the supernatants by making alkaline with 16N-KOH (0.3 ml.) and extracting with methanol:ether (1:3, v/v, 5 ml.) and with ether (5 ml.). The aqueous layer was acidified with 18N-H_2SO_4 (0.2 ml.) and extracted with ether (3 x 3 ml.) to remove isocaproic acid. These ether extracts were made up to 9.0 ml. with ether and 3.0 ml. portions removed for liquid scintillation counting.

This method was developed and tested using [1-^{14}C]-isocaproic acid and [4-^{14}C]-cholesterol; it proved capable of quantitative (>90%) recovery of isocaproate, uncontaminated with cholesterol.

Extraction and analysis of [^{14}C]-steroids.

After incubation with [4-^{14}C]-steroids, the products were extracted by shaking with successive portions of suitable solvents (e.g. n-butanol for steroid sulphates, dichloromethane for free steroids) and the

combined extract was washed with water and reduced in volume under a jet of nitrogen. The recovery of radioactivity was 80 - 90%. Carrier steroids were added and the extract applied to 20 x 20 cm. thin layers of silica-H and chromatographed. After chromatography and recovery of the steroids from the silica, (described below), portions were assayed for radioactivity.

Thin-layer Chromatography.

Silica-H plates were made by spreading a slurry of 24 g. of Merck Silicagel-H in 75 ml. of water using a Desaga Spreader (Desaga, Heidelberg). This was sufficient for either four 20 x 20 cm. glass plates or sixteen 5 x 20 cm. glass plates. The slurry was allowed to set (30 min.) at room temperature and the plates were then dried at 120° for 1 hr. They were stored in a desiccator.

A 0.5 cm. band of silica at each side of the plate was removed (to avoid edge effects) and then solutions of steroids were applied to the plates using glass capillaries, either as discrete spots (for qualitative work) or as a streak (for quantitative work). A mark was made in the silica 15 cm. from the origin to limit the movement of the solvent front. The plate was then chromatographed with a suitable solvent mixture in a tank lined with filter-paper soaked in the solvent.

Many different solvent systems were used; details of four systems which were used extensively, together with R_f-values for a number of steroids in these systems are given in Table 1. R_f-values were only

Table 1

R_f-values for steroids on thin-layer chromatography.

Adsorbent: silica-H dried at 120° for 2 hrs.

Solvent Systems: System I :- isopentyl acetate:n-hexane 1:9 (v/v)

System II :- ethyl acetate:n-hexane 3:7 (v/v)

System III :- benzene:butan-2-one:ethanol:water 3:3:3:1 (by vol.)

System IV :- butan-2-one:n-hexane 1:1 (v/v)

Steroid	Solvent System			
	I	II	III	IV
androst-4-ene-3,17-dione	-	-	-	0.50
cholesterol	0.01	0.36	0.84	0.64
cholesteryl-3β-acetate	0.63	0.76	-	-
cholesteryl-3β-chloride	-	0.75	-	-
cholesteryl-3β diphenyl phosphate	-	0.60	0.90	-
cholesteryl-3β-linolenate	0.68	-	-	-
cholesteryl-3β-oleate	0.71	-	-	-
cholesteryl-3β-palmitate	0.75	-	-	-
cholesteryl-3β dihydrogen phosphate	-	-	0.03	-
cholesteryl-3β-stearate	0.75	-	-	-
cholesteryl-3β sodium sulphate	-	-	0.58	-
corticosterone	-	-	-	0.20
dehydroepiandrosterone	-	-	-	0.48
dehydroepiandrosterone-3β sodium sulphate	-	-	0.39	-
20α-hydroxy-cholesterol	0.05	0.26	-	0.60
20α-hydroxy-cholesteryl-3β-acetate	0.27	0.61	-	-
oestrone	-	-	-	0.33
25-oxo-27-nor-cholesteryl-3β sodium sulphate	-	-	0.44	-
pregnenolone	-	0.18	-	0.51
pregnenolone-3β-acetate	-	0.50	-	-
pregnenolone-3β-palmitate	0.59	-	-	-
pregnenolone-3β sodium sulphate	-	-	0.51	-
progesterone	-	0.23	-	0.57
testosterone	-	-	-	0.40

constant on plates dried and stored as described above and using dry, redistilled solvents in tanks lined with filter-paper soaked in the solvent mixture. On damp plates, i.e. not stored in a desiccator, the R_f-values were larger and more variable than on dry plates.

Chromatography was allowed to proceed until the solvent front reached the mark in the thin-layer when the plate was removed from the tank and allowed to dry. In some experiments the chromatography was repeated after allowing the solvent from the first chromatography to evaporate; this procedure gives a greater separation than using a different solvent mixture in certain cases.

Marker steroids were visualised by spraying with 20% (w/v) phosphomolybdic acid in ethanol and heating for 3 - 5 min. at 120°. This method proved capable of detecting concentrations of cholesterol on the plate of 0.2 $\mu g/cm^2$; i.e. spots containing about 0.1 µg.

In quantitative experiments, appropriate bands of silica were removed from the plate using the apparatus shown in figure 3. This apparatus was made by Mr. H. Vincent. Using a moderate suction provided by a water pump, the powder was sucked off the plate and trapped on the sintered glass disc. A small amount of solvent was sucked through the apparatus to ensure that all the silica was swept onto the sinter disc and then the suction was turned off and solvent allowed to percolate through the silica under gravity into the tube below. Experiments with [^{14}C]-cholesterol showed that by this technique steroids could be removed from the plate and recovered from the silica quantitatively (>98%)

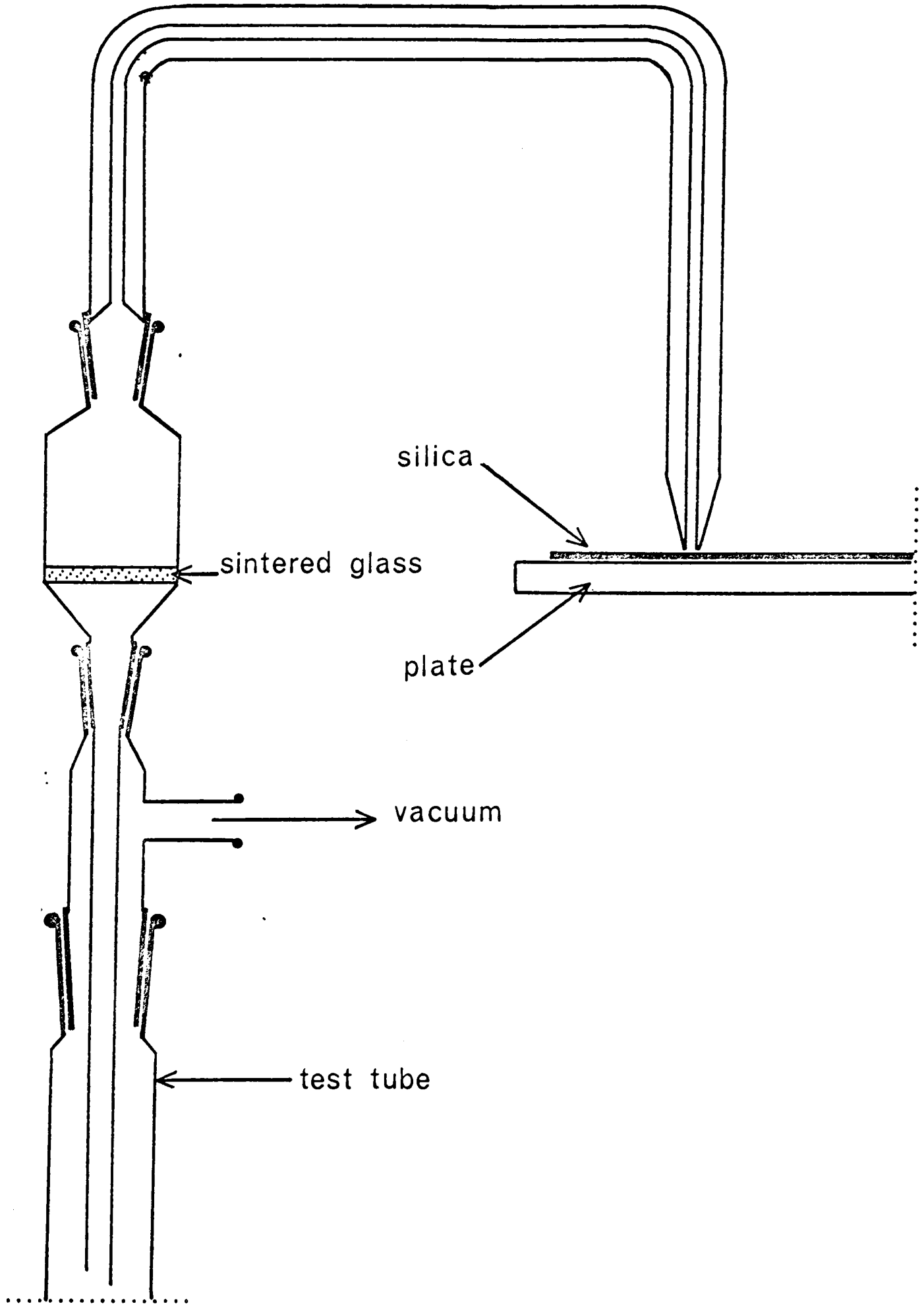


Figure 3

Apparatus used for quantitatively removing zones of thin-layer chromatograms from the supporting glass plates

within experimental error.

Liquid Scintillation Counting.

3.0 ml. portions of [^{14}C]-isocaproic acid in ether were added to 4.0 ml. of a solution of 7.5 g. of 2,5-diphenyl-oxazole (PPO), 0.15 g. of dimethyl-2,2-phenylene bis-[5-phenyl-oxazole] (dimethyl-POPOP) and 160 g. of naphthalene in 1 l. of dry toluene:dioxane, 1:1 (v/v).

Other solutions were added to sufficient of a solution of 5 g. of PPO, 0.1 g. of dimethyl-POPOP, and 80 g. naphthalene in 1 l. of dry toluene:dioxane:methanol, 5:5:3 (by vol.) to give a total volume of 7 ml.

Solutions of $^{14}CO_2$ in 1N-NaOH (0.2 ml.) were added to 7 ml. of a solution of 5 g. of PPO, 0.1 g. of dimethyl-POPOP, 80 g. of naphthalene and 2 g. of "Cab-O-Sil" (Packard Instrument Co., finely divided silica) in 1 l. of dry toluene:dioxane:methanol, 5:5:3 (by vol.) and shaken to produce a transparent gel.

The solutions were counted in an "IDL Tritomat" liquid scintillation counter (Isotope Developments Ltd., Reading) for times sufficient to give at least 10^4 observed counts. Efficiency was about 65%.

Determination of amounts of endogenous steroids in tissue preparations.

In some early experiments the method of Cook (1958) was used to extract and determine cholesterol and its esters. Lipids are extracted

with acetone:ethanol (1:1, v/v) and free sterol precipitated with digitonin. The cholesterol esters are thus separated from free cholesterol, and after hydrolysis with potassoim hydroxide may be in turn precipitated as sterol digitonides. The amounts of cholesterol in the precipitates are determined by the Lieberman-Burchard method using acetic anhydride:concentrated sulphuric acid (1:20, v/v) which gives a colour at 630 mμ. This method is somewhat cumbersome and, as the Lieberman-Burchard reaction gives best results in the range 0.1 - 0.5 mg. cholesterol, requires fairly large amounts of tissue preparations.

The method of Mann and Zak (1961) was also used. This uses the colour at 560 mμ which is produced by the action of concentrated sulphuric acid on cholesterol in the presence of ferric chloride. This is a much more sensitive method (10 μg. - upwards) but the extraction method recommended by Mann and Zak also proved troublesome. The extraction and hydrolysis procedure was not only time-consuming and laborious but gave results of poor reproducibility, especially with tissue preparations containing very small amounts of cholesterol. In addition there was a quite large background colour, the cause of which appeared to be small but variable amounts of sucrose which passed through the extraction procedure and gave a dark colouration with concentrated sulphuric acid. A new procedure was therefore devised.

Suitable amounts (10 - 500 μl.) of tissue preparations were dried on to semicircles (diameter 7 cm.) of Whatman No. 52 filter paper using a stream of warm air. When completely dry the semicircles were folded

using clean forceps and inserted into test-tubes (13 x 1.3 cm.). Ether:methanol (5 ml., 1:1, v/v) was added and the tubes were heated at 40° in the dark by standing in a water bath. Only the lower parts of the tubes were immersed so that the cooler upper parts acted as partial condensers. After 1 hr., dichloromethane:methyl acetate (5 ml., 1:1, v/v) was added, and heating continued at 40°. After a further hour the filter papers were raised with forceps to the tops of the test-tubes, above the surface of the liquid, and the temperature was raised to 50°. The solvent vapour continually condensed on the filter papers which were thus subjected to a kind of Soxhlet extraction. After a further hour, dichloromethane (5 ml.) was added slowly over the filter papers and when they had fully drained they were removed and discarded. The extracts were evaporated to dryness at 50° under a stream of nitrogen, redissolved in small quantities of dichloromethane, applied quantitatively to 5 x 20 cm. thin layers of silica-H and chromatographed in ethyl acetate:n-hexane, 3:7 (v/v). Bands corresponding to pregnenolone (R_f 0.18), free cholesterol (R_f 0.36) and cholesterol esters (R_f 0.75 - 1.0) were removed and extracted quantitatively from the silica using dichloromethane, ether and methyl acetate respectively.

This method proved capable of routine recovery of >90% of the three steroid groups and was tested using [^{14}C] labelled steroids. It has the advantages that it is reliable, quantitative, requires only intermittent attention and, apart from the chromatography, requires little labour. It also allows extraction and separation of three groups

of steroids, free from sucrose and other contaminants, from one sample of tissue preparation.

Cholesterol and cholesterol esters were determined by the Mann-Zak method (1961). The extracts were evaporated to dryness under nitrogen and redissolved in 2 ml. of glacial acetic acid. To this was added 1 ml. of a reagent made by diluting 1 ml. of a solution of ferric chloride in glacial acetic acid (2.8 g./25 ml.) to 100 ml. using concentrated sulphuric acid. After 25 min. at room temperature the optical density was measured at 560 mμ using separate standards of cholesterol and cholesteryl stearate. The lower limits (optical density 0.05) were about 7 μg. for cholesterol and about 16 μg. for cholesteryl stearate.

Pregnenolone was determined by the method of Oertel and Eik-Nes (1959). The extracts were evaporated under nitrogen and then redissolved in 3 ml. of a reagent consisting of ethanol:concentrated sulphuric acid, 1:2 (v/v). The ethanol was redistilled over silver oxide before use. After 30 min. at room temperature the optical density was measured at 405 mμ using pregnenolone as standard. The lower limit (optical density 0.05) was about 2 μg.

Protein content of tissue preparations.

This was measured by the biuret method (Gornall, Bardawill and David, 1949).

RESULTS

1) Definition of the conditions for cholesterol oxidation by adrenal cortex in vitro.

The cofactor requirements for cholesterol oxidation by adrenal cortex *in vitro*.

Mitochondrial preparations.

Halkerston *et al*. (1961) have published a careful investigation of cholesterol oxidation by bovine adrenal cortex mitochondria and their methods, especially their method of preparing the mitochondria, were largely adopted in the present work. However, it seemed necessary to check that the incubation conditions were optimum as there were certain differences in technique.

The standard cofactor mixture finally used with fresh mitochondria (the malate cofactor system), consisted of malate, NAD, ATP and $MgCl_2$ at pH 7.4. Phosphate, potassium and sodium ions were also present and the medium used to prepare the mitochondria (supplemented sucrose) contained fumarate, nicotinamide and EDTA (see experimental section for details).

Using mitochondria prepared in a medium identical to supplemented sucrose except that it contained no fumarate, a number of likely substances were tried as potential cofactors for cholesterol oxidation (table 2). The malate-NAD-ATP system seemed better than any other and was therefore used subsequently. The presence of ATP in the incubations has an additional potential advantage because it is reported to prevent the swelling of mitochondria which is caused by metal ions, proteolytic

Table 2

Cofactor requirements for cholesterol oxidation *in vitro* by adrenal cortex mitochondria.

Additions	Net % conversion of cholesterol into isocaproic acid
None	1.1
NAD	2.5
NADP	2.7
NADH	1.7
NADPH	3.2
fumarate	7.3
fumarate + NAD	10.0
fumarate + NAD + ATP	9.4
fumarate + NADP	8.8
malate	7.2
malate + NAD	12.3
malate + NAD + ATP	12.1
malate + NADP	6.9
succinate	5.4
citrate	5.8
α-ketoglutarate	4.2

Incubations were for 30 min. Each incubation contained adrenal cortex mitochondria, prepared and washed in a medium containing sucrose (0.44 M), EDTA (1 mM), nicotinamide (5 mM) and potassium phosphate buffer pH 7.4 (10 mM). Incubations also contained [26-^{14}C]-cholesterol (10.1 μM) in dimethyl formamide (0.1 ml.), sodium phosphate buffer pH 7.4 and $MgCl_2$ in a total volume of 5.1 ml. to give the following final concentrations:- sucrose, 0.26 M; EDTA, 0.59 mM, nicotinamide, 3 mM; phosphate, 5.9 mM; $MgCl_2$, 1.5 mM. The concentration of all nucleotides where present was 1.25 mM and of all Krebs-cycle intermediates 3 mM. The results are averages of duplicates.

enzymes and detergents (Hirschfield & Koritz, 1964). Halkerston *et al.* (1961), using mitochondria prepared in the same way, found that fumarate was the preferred Krebs-cycle intermediate in their experiments and in view of this, fumarate was retained in the homogenisation medium.

Other tissue preparations.

Cholesterol oxidation by extracts of acetone-ether powders of mitochondria, by the 105,000 g supernatant of sonicated mitochondria and by acetone-ether powders made from this sonicated supernatant required added NADPH or an added NADPH-generating system (table 3). The NADPH-generating system used in this work was glucose-6-phosphate plus NADP plus yeast glucose-6-phosphate dehydrogenase (see experimental section for details). This proved more effective in practice than an equivalent amount of preformed NADPH added at the beginning of the experiment. This may have been partly because of the presence in the tissue preparations of enzymes which can destroy NADPH. Thus when NADPH was incubated at 25^{o} in the presence of acetone-ether powders of the sonicated supernatant dissolved in supplemented sucrose and phosphate buffer pH 7.4, the optical density at 340 mμ was found to decline with time. In an incubation identical except that it contained no adrenal enzyme, no such decline occurred. This indicates that the adrenal enzyme, in the absence of any steroid substrate, accelerated the decomposition of NADPH. In incubations with the glucose-6-phosphate dehydrogenase cofactor system, the excess of glucose-6-phosphate should maintain the NADPH level in spite of this decomposition.

Table 3

Cofactor requirements for cholesterol oxidation by an acetone-ether powder made from the 105,000 g supernatant of sonicated adrenal cortex mitochondria

Additions	Net % cholesterol oxidation
None	0.3
NAD + malate + ATP	0.7
NADH	0.6
NADPH	7.9
G6P + NADP + G6PDH	11.3

Incubations were for 30 min. Each incubation contained 3 ml. of acetone-ether powder (1 mg./ml.) dissolved in supplemented sucrose, phosphate buffer pH 7.4 (5.9 mM), $MgCl_2$ (1.5 mM) and [26-^{14}C]-cholesterol (5.9 μM) in dimethyl formamide (0.1 ml.). The concentration of all nucleotides was 1.25 mM and of malate and glucose-6-phosphate 3 mM. Where stated, incubations contained 0.01 mg. of yeast glucose-6-phosphate dehydrogenase. The results are averages of duplicates.

Oxygen

Cholesterol oxidase is an oxygenase using molecular oxygen (Hayano, 1962). However, under the conditions of these experiments, no advantage in incubating in an atmosphere of oxygen, rather than air, was detected. Therefore, all incubations were performed in an atmosphere of air, except where specifically stated.

Dimethyl formamide

Dimethyl formamide was used to solubilise cholesterol and other steroids (see also below). Experiments were therefore performed to examine whether this substance affected cholesterol oxidation. The standard procedure used 0.1 ml. of dimethyl formamide solution in 5.1 ml. total incubation volume. Solutions of [26-^{14}C]-cholesterol were made up so that different volumes of dimethyl formamide solutions would give the same final concentration of cholesterol when diluted to 5.1 ml. with the enzyme and the cofactor mixture. Incubations were performed in this way with fresh mitochondria under the standard conditions and the [^{14}C]-isocaproic acid formed was extracted and counted. No significant effect of dimethyl formamide was detected even at four times the usual concentration (table 4).

Table 4

The effect of dimethyl formamide on cholesterol oxidation by adrenal cortex mitochondria

Volume of dimethyl formamide (ml.)	Net % conversion of cholesterol into isocaproic acid	
	Experiment 1	Experiment 2
0.05	12.4	6.6
0.1	12.5	6.3
0.2	12.8	6.4
0.3	11.9	6.7
0.4	12.3	6.6

Incubations were for 30 min. Each incubation contained 3 ml. of adrenal cortex mitochondria in supplemented sucrose, malate cofactor mixture in the standard proportions and [26-^{14}C]-cholesterol (5.3 μM). The total volume was 5.1 ml. in each case.

The intracellular location of cholesterol oxidase and its extraction from mitochondria by sonication.

Halkerston et al. (1961) have shown that most of the cholesterol oxidase activity of bovine adrenal cortex is in the mitochondria. Using a similar method of separation of subcellular components, it was confirmed that the bulk of the cholesterol oxidase was associated with the mitochondria (table 5).

A technique was devised for solubilising the mitochondrial cholesterol oxidase by sonication. A similar method has been used by Simpson and Boyd (1966). The details of this technique are given in the experimental section. Sonication increased the total ability of the enzyme preparation to convert [26-^{14}C]-cholesterol into [^{14}C]-isocaproic acid, both on a volume basis and on a protein basis (table 5). This is presumably due to the increased availability of the enzyme to the added labelled substrate. On centrifuging the sonicated mitochondria at 105,000 g for 30 min. about two-thirds of the total protein, but only about one-third of the cholesterol oxidase activity, was sedimented. This supernatant is referred to as the 'sonicated supernatant'.

Acetone-ether powders were made from the sonicated supernatant and, in the presence of the glucose-6-phosphate dehydrogenase cofactor system, proved to have high cholesterol oxidase activity. The acetone-ether powders made from the sonicated supernatant usually contained about one-third of the cholesterol oxidase activity which had been present in the original sonicated supernatant. The activity of the

Table 5

Intracellular distribution and the effect of sonication on cholesterol oxidase of bovine adrenal cortex

Fraction	Added cholesterol oxidised (mμmole/hr.)	
	per ml. of 1200 g supernatant	per mg. protein
Mitochondria	0.76	0.11
Microsomes	0.057	0.038
105,000 g supernatant	0.041	0.0027
Sonicated mitochondria	2.62	0.37
105,000 g sediment after sonication	1.04	0.24
105,000 g supernatant after sonication	1.47	0.57

Subcellular fractions were prepared in supplemented sucrose as described in the experimental section. The volume of cell-free supernatant (1200 g supernatant) in this experiment was 96 ml. Each of the fractions was diluted to 96 ml. and 3 ml. of the appropriate fraction was used per incubation flask. The cofactor system in this experiment consisted of the components of both the malate and the glucose-6-phosphate dehydrogenase systems. Incubation was for 30 min. The table shows net conversion of the added [26-^{14}C]-cholesterol (2.7 μM) to [^{14}C]-isocaproic acid and the results are the averages of duplicates. Protein was determined by the biuret method using bovine serum albumin as standard.

sonicated supernatant preparation was calculated from the rate of production of [^{14}C]-isocaproic acid from [26-^{14}C]-cholesterol of known specific activity and the measured total cholesterol content of the preparation. The calculation assumes that all of the endogenous cholesterol in the sonicated supernatant was metabolisable and that the added labelled cholesterol was completely miscible with it. Neither of these assumptions is likely to be wholly true and so the estimates of cholesterol oxidase activity must be regarded as approximate. Sonicated supernatant made from 1 g. of fresh adrenal cortex was capable of converting 60 - 110 mμmoles of cholesterol into isocaproic acid in 1 hr. Acetone-ether dried sonicated supernatant made from 1 g. of fresh adrenal cortex was capable of converting 20- 40 mμmoles of cholesterol per hour and weighed 6 - 8 mg.

Generation of NADPH

Hydroxylation of the cholesterol side-chain at C-20 and C-22, followed by oxidative cleavage resulting in the formation of isocaproic acid and pregnenolone, requires 4 moles of NADPH per mole of cholesterol. Therefore experiments were performed to measure rates of NADPH-formation under incubation conditions similar to those used routinely. Only glucose-6-phosphate and malate are considered as generators of NADPH in this section as these were the only systems used in the cholesterol oxidation experiments.

Measurements of optical density were made at 340 mμ using a

Beckman spectrophotometer with a Gilford automatic cuvette positioner and absorbance converter; the optical density was plotted automatically against time using a Servoscribe chart recorder. The cuvette chamber was maintained at 37° and all solutions were at 37° when added to cuvettes. All components of incubations except enzymes were at the same concentrations as in the cholesterol oxidation experiments. In all cases appropriate blanks were incubated simultaneously. The time course of NADPH generation was followed for about 10 min. and initial rates were calculated from the linear portions of the graphs. The time for which the graph was linear varied with the amount of enzyme present. In all cases the enzyme was prepared in supplemented sucrose and incubated in the presence of phosphate buffer pH 7.4 (5.9 mM) and $MgCl_2$ (1.5 mM).

Malate as a source of NADPH.

With fresh mitochondrial preparations it was not possible to derive accurate values for rates of reduced pyridine nucleotide formation because the initial optical density was large (about 3) and small changes in density could not be measured accurately. However, it was clear that with NADP (0.5 mM) and malate (3 mM), the initial rate of NADPH generation was high and of the order of 0.5 μmole/min. This rate seemed to be unaffected by pregnenolone (91 μM). When NAD or NAD plus ATP were substituted for NADP, generation of reduced pyridine nucleotides occurred at a much slower rate (about 0.03 μmole in 10 min.). It appears therefore, that under the usual incubation conditions and in experiments

with intact mitochondria, most of any NADH or NADPH which is formed remains within the mitochondrion and therefore is not measured under the conditions of these experiments. With added NADP and malate, the reduced NADPH formed may have been the result of the activity of a proportion of the total cell malic enzyme which is present in the mitochondria (see Introduction), but in this case, the NADPH appears to have been released into the medium.

Using the 105,000 g supernatant made from sonicated mitochondria the initial optical density was much lower and more accurate measurements were possible. The initial rate of NADPH generation from malate (3 mM) and NADP (0.5 mM) was 0.64 μmole/min. but no reduced pyridine nucleotide appeared to be formed from NAD. Similarly using a reconstituted acetone-ether powder of the sonicated supernatant (3 mg./ml., 1.8ml. per 3.1 ml. cuvette), the initial rate of NADPH generation from malate (3 mM) and NADP (0.5 mM) was 0.51 μmole/min., whereas from malate and NAD (0.5 mM) it was only 0.001 μmole/min. These findings support the conclusion that the adrenal cortex mitochondria contained some NADP-linked malic enzyme (E.C. 1.1.1.40). However any NAD-linked malate dehydrogenase (E.C. 1.1.1.37) that may be present in the mitochondria does not appear to be active in the sonicated supernatant preparations. Either the malate dehydrogenase is not extracted or, because at pH 7.4 the malate dehydrogenase catalysed equilibrium lies very much on the side of malate and NAD, little free NADH is formed under the incubation conditions.

$$K = \frac{[\text{oxaloacetate}][\text{NADH}][H^+]}{[\text{malate}][NAD^+]} = 1 \times 10^{-13} \quad \text{(Kun, 1963)}$$

The reconstituted acetone-ether powder of the sonicated supernatant showed no ability to use ATP (2 mM) to convert NAD (0.5 mM) into NADP. The NADP was measured by using a NADP-trap which consisted of glucose-6-phosphate (3 mM) and yeast glucose-6-phosphate dehydrogenase (0.01 mg.) which converts NADP very rapidly into NADPH which absorbs light at 340 mμ. Yeast glucose-6-phosphate dehydrogenase has no action on NAD. Control experiments showed that the NADP-trap was working and was not affected by the presence of the adrenal enzyme, but no NADP ($<$ 0.001 μmole) could be detected in 6 minutes incubation. Addition of malate (3 mM) or more $MgCl_2$ had no effect.

Glucose-6-phosphate as a source of NADPH.

Incubation of glucose-6-phosphate (3 mM) in the presence of yeast glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49) (Boehringer) at a variety of concentrations and NADP (0.5 mM), gave initial rates of NADPH generation which were about 106% of that claimed in its specification (specification 140 μmoles converted/mg./min. at 25°). In subsequent experiments the amount of enzyme was adjusted to give suitable rates of NADP reduction at 37°.

Yeast glucose-6-phosphate dehydrogenase activity proved to be unaffected by dimethyl formamide, pregnenolone or reconstituted adrenal cortex acetone-ether powders at the concentrations used in the cholesterol oxidation experiments (table 6). It was unable to reduce NAD under the experimental conditions.

The adrenal cortex preparations themselves contained some endogenous

Table 6

Experiments on yeast glucose-6-phosphate dehydrogenase.

Additions	Initial rate of NADPH formation (μmole/min.)
None	0 (no detectable change in 12 min.)
G6PDH	0.89
G6PDH, DMF	0.90
G6PDH, adrenal enzyme	0.94
adrenal enzyme	0.036
G6PDH, DMF-pregnenolone (50 μM)	0.88
G6PDH, adrenal enzyme, DMF-pregnenolone (50 μM)	0.92

Each incubation contained 1.8 ml. of supplemented sucrose or 1.8 ml. of a solution in supplemented sucrose of an acetone-ether powder made from the 105,000 g supernatant of sonicated adrenal cortex mitochondria (adrenal enzyme) (1 mg./ml.). Incubations also contained phosphate buffer pH 7.4 (5.9 mM), NADP (0.5 mM), $MgCl_2$ (1.5 mM). Where appropriate, dimethyl formamide (DMF) (0.06 ml.), containing pregnenolone where stated, was present. The final pregnenolone concentration was 50 μM. Where appropriate, yeast glucose-6-phosphate dehydrogenase (G6PDH) (0.006 mg.) was added after all other components had been added. Total incubation volume 3.1 ml.

glucose-6-phosphate dehydrogenase activity. Thus, using freshly prepared mitochondria, despite the difficulty of measuring small changes in an optical density of about 3, qualitative evidence of glucose-6-phosphate dehydrogenase activity was obtained (table 7). This activity was inhibited by pregnenolone. The supernatant from sonicated mitochondria and the acetone-ether powders made from it also contained glucose-6-phosphate dehydrogenase activity. The effect of pregnenolone and of cholesterol on the glucose-6-phosphate dehydrogenases obtained from yeast and from adrenal cortex were compared and the results are shown in table 8; pregnenolone inhibited the adrenal cortex enzyme but not the yeast enzyme, while cholesterol affected neither.

The 16,000 g supernatant (microsomes + cytoplasm), left after sedimentation of the mitochondria contained no glucose-6-phosphate dehydrogenase activity (table 7), even after the addition of extra $MgCl_2$, NADP or glucose-6-phosphate. This indicates that in adrenal cortex, glucose-6-phosphate dehydrogenase is probably largely mitochondrial.

Oxidation of cholesterol to pregnenolone requires 4 moles of NADPH per mole of cholesterol and the highest rate of oxidation of added cholesterol observed in this work was about 40 mμmoles/hr. It is important to emphasise that in the cholesterol oxidation experiments which are to be described, the supply of NADPH for steroid hydroxylation was not rate-limiting and was almost certainly in great excess. The endogenous glucose-6-phosphate dehydrogenase activity was small compared with the amount of yeast enzyme normally added and thus the presence of

Table 7

Glucose-6-phosphate dehydrogenase activity of adrenal cortex preparations

Enzyme	Additions	Initial rate of NADPH generation (μmole/min.)	Time for 1.3 μmole of NADPH (min.)
Mitochondria	0	0.4	2.9
"	0	0.6	3.0
"	0	0.5	3.1
"	Pregnenolone (50 μM)	0.3	4.5
"	Pregnenolone (50 μM)	0.2	4.7
16,000 g supernatant	0	0	No change in 12 minutes

Each incubation contained enzyme prepared in the standard way in supplemented sucrose (1.8 ml.), phosphate buffer pH 7.4 (final concentration 5.9 mM) and dimethyl formamide (0.06 ml.). Where stated the dimethyl formamide contained pregnenolone to give a final concentration of 50 μM. The concentrations of other components were:- $MgCl_2$ (1.5 mM), glucose-6-phosphate (3 mM) and NADP (0.5 mM). Total incubation volume 3.1 ml.

Table 8

Comparison of the effect of pregnenolone on glucose-6-phosphate dehydrogenase from adrenal cortex mitochondria and from yeast

Source of Enzyme	Additions	Initial rate of NADPH generation (μmole/min.)
Yeast	0	1.21
"	Pregnenolone (50 μM)	1.20
"	Pregnenolone (84 μM)	1.22
"	Cholesterol (45 μM)	1.22
Adrenal Cortex	0	0.106
"	Pregnenolone (50 μM)	0.060
"	Pregnenolone (84 μM)	0.046
"	Cholesterol (45 μM)	0.102
"	Cholesterol (75 μM)	0.090

Each incubation contained 1.8 ml. of either an acetone-ether powder of the 105,000 g supernatant of sonicated adrenal cortex mitochondria dissolved in supplemented sucrose (3 mg./ml.) or yeast glucose-6-phosphate dehydrogenase in supplemented sucrose (0.005 mg./ml.). The enzymes were always added last. The incubations also contained phosphate buffer pH 7.4 (5.9 mM) and dimethyl formamide (0.06 ml.) either alone or containing pregnenolone or cholesterol. The concentrations of other components were:- glucose-6-phosphate (3 mM), NADP (0.5 mM), $MgCl_2$ (1.5 mM). Total incubation volume 3.1 ml.

pregnenolone would not limit the supply of NADPH.

Stability of cholesterol preparations.

The method of solubilising cholesterol and other steroids in the aqueous incubation medium that was used in this work was to dissolve the steroid in N,N-dimethyl formamide and then to add the enzyme preparation to this. A dispersion of the steroid in an aqueous medium containing 2% (v/v) of dimethyl formamide was thus obtained. Such preparations made up in supplemented sucrose and the standard cofactor mixtures appeared completely transparent at low steroid concentrations ($<20\ \mu M$) but at higher steroid concentrations were opalescent.

As the steroids so treated were intended to react with enzymes over considerable periods of time it seemed important to gain some information on the behaviour of such preparations under incubation conditions. Cholesterol in dimethyl formamide (0.06 ml.) was dispersed in supplemented sucrose (1.8 ml.) and 1.2 ml. of the standard malate cofactor mixture was added. These are the same proportions as used in cholesterol oxidation experiments. The optical density at 600 mμ was then measured without delay with a Beckman spectrophotometer with a Gilford automatic cuvette positioner and absorbance converter and the results plotted against time using a Servoscribe chart-recorder. The cuvette chamber was maintained at 37^{o} and all the solutions were at 37^{o} when added to the cuvettes.

At cholesterol concentrations up to 21 μM, no change in optical density was detected during 20 min. incubation. At higher cholesterol

concentrations, the optical density increased with time, apparently tending to a maximum. The higher the cholesterol concentration the more rapidly the optical density increased and the greater the value of the maximum (figure 4). When the cofactor mixture, which contains phosphate buffer, was replaced by water, similar results were obtained but the optical density increased much less rapidly. These results suggest that, in the absence of protein, cholesterol dispersed using dimethyl formamide slowly forms solid particles which absorb light at 600 mμ and that this process is accelerated by ions in the cofactor mixture.

In the presence of bovine serum albumin or acetone-ether powder of the 105,000 g supernatant made from sonicated adrenal cortex mitochondria, the optical density increased only at a slow rate and although the preparations were still opalescent they appeared to be more stable. These results are shown in figure 5.

To investigate the possibility of an interaction between the adrenal cortex mitochondria and the cholesterol dispersions, [4-^{14}C]-cholesterol was diluted with varying amounts of unlabelled cholesterol, and dispersed in mitochondrial preparations using dimethyl formamide in the usual way. After incubating for various times at 37^{o}, the suspensions were centrifuged in separate tubes at 16,000 g for 20 min. at 0^{o} and samples of the supernatant were removed carefully and added to scintillant for counting.

The amount of labelled cholesterol precipitated with the mitochondria was found to be related approximately linearly to the concentration of

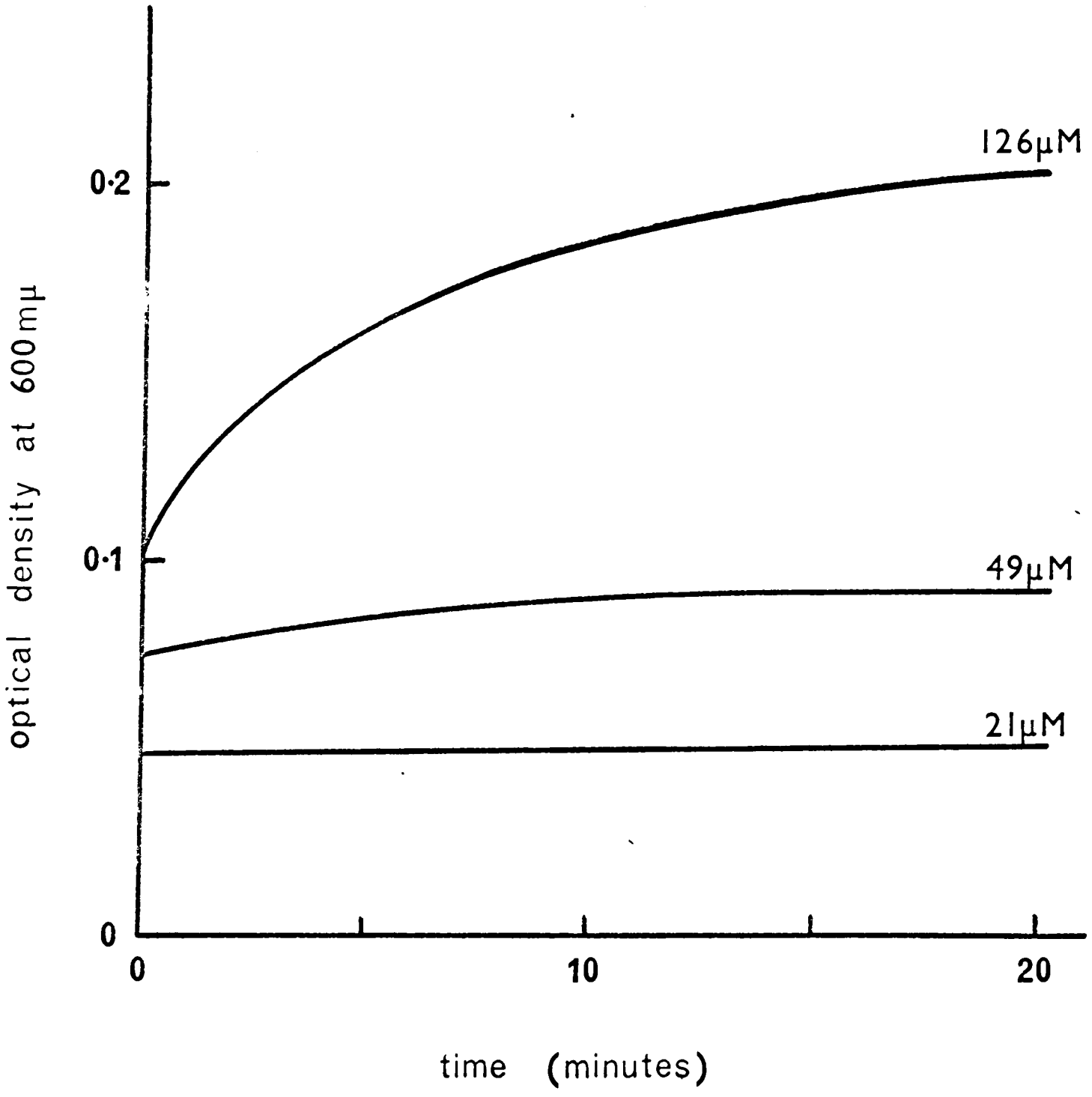


Figure 4

The effect of concentration on the behaviour of cholesterol dispersions

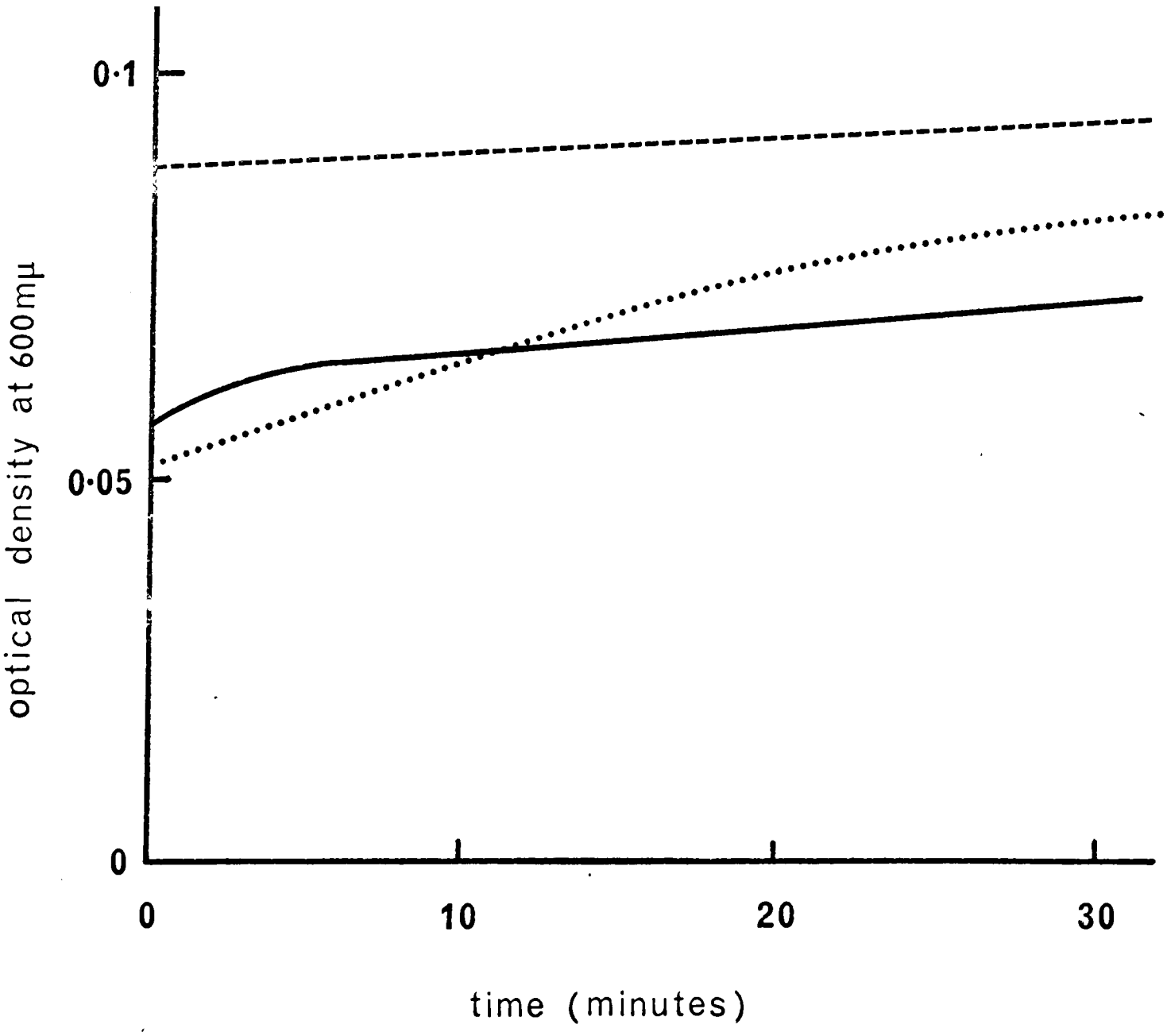


Figure 5

The effect of protein on the stablity of cholesterol dispersions (112μM)

------ in the presence of adrenal protein (1.75mg/ml.)

——— in the presence of bovine serum albumin (1.75mg/ml.)

····· in the absence of protein

added cholesterol (figure 6). In the absence of mitochondria, the amount of cholesterol disappearing from the supernatant also increased with concentration, but less rapidly and much less regularly. It was consistently observed that the amount of cholesterol precipitated together with heat-denatured mitochondria was less than that with fresh mitochondria at the same cholesterol concentration (figure 6).

The interaction between cholesterol and both fresh and denatured mitochondria appeared to be a very rapid process. No difference in the amount removed from solution by centrifugation could be detected when the mitochondria were kept at 0^{o} the whole time nor was there any difference between incubation for 2 min. or 20 min., either at 0^{o} or at 37^{o}. Omission of the cofactors had no effect.

To investigate the extent to which the removal of cholesterol from solution was a reversible process, [4-^{14}C]-cholesterol was first incubated with mitochondria as described above for 2 min. The mixture was then centrifuged at 16,000 g for 20 min. to sediment the mitochondria. The supernatant was decanted and the precipitate was dispersed in fresh non-radioactive medium and this mixture was incubated for 10 min. at 37^{o}, and then resedimented by centrifugation. The supernatant was now found to contain some radioactivity. This indicates that cholesterol taken up by the mitochondria is at least partially free to re-enter solution. However, the results obtained by this technique proved very variable and no other conclusions were drawn.

To discover whether cholesterol taken up by the mitochondria was more easily oxidised than the cholesterol remaining in solution, six

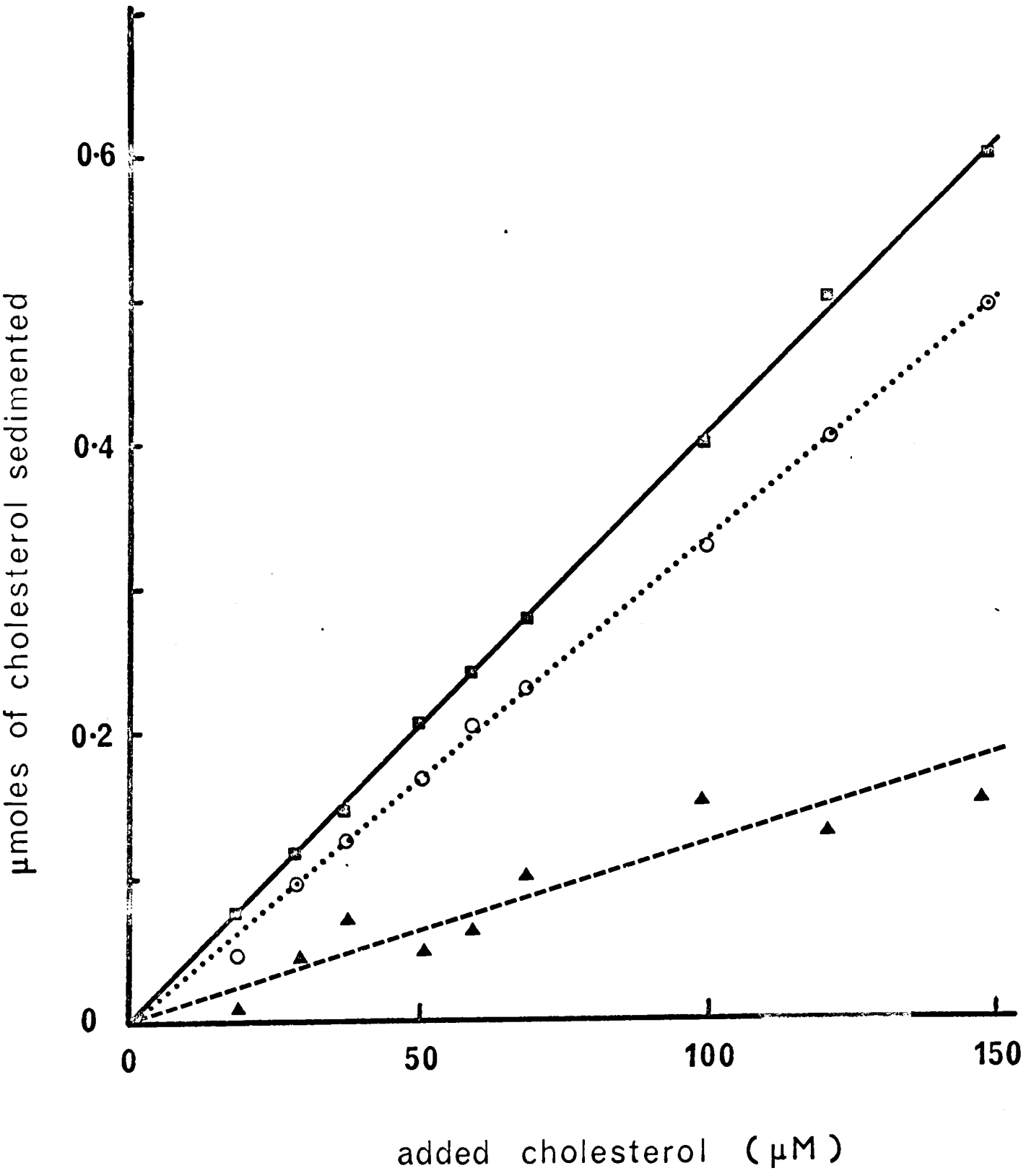


Figure 6

The effect of adrenal cortex mitochondria on the sedimentation of cholesterol at 16,000g.

■——■ in the presence of mitochondria

○····○ in the presence of denatured mitochondria

▲----▲ in the absence of protein

identical incubations, in three pairs, were prepared in the standard way, each containing [26-^{14}C]-cholesterol at 2.7 μM. All were incubated at 37° for 2 min. and then cooled to 0°. Four of the incubations were centrifuged at 16,000 g for 20 min. at 0°; after this 38% of the initial radioactivity remained in the supernatant. The supernatants of two of the incubations were decanted and replaced by fresh medium. All the pellets were then resuspended, two in the new medium and two in the old. The two remaining incubations remained at 0° for this time. All six flasks were then incubated at 37° for 30 min. and the [^{14}C]-isocaproic acid formed was extracted and counted in the usual way. The results are shown in table 9.

In the incubations in which the mitochondria had been sedimented and then resuspended in the original medium, less isocaproic acid was formed than in those which had not been centrifuged. However in the third pair of incubations in which the medium had been replaced, even less isocaproic acid was formed and a smaller precentage of the total available cholesterol was oxidised. This indicates that the cholesterol taken up by the mitochondria is not preferred as a substrate and that cholesterol in solution (not sedimentable) is metabolised.

That the uptake of cholesterol by the mitochondria, whether denatured or not, occurred as readily at 0° as at 37° and was a rapid process, and the relation between cholesterol absorbed and cholesterol concentration suggests that the removal from solution is a physical absorption process and is not dependent on the metabolic activity of

Table 9

An experiment to discover whether cholesterol taken up by mitochondria is preferred to cholesterol in solution as a substrate for cholesterol oxidase

Number	[26-^{14}C]-cholesterol		[^{14}C]-isocaproic acid formed	
	percentage sedimented with mitochondria	present during incubation (counts/sec.)	(counts/sec.)	as percentage of cholesterol present
1		2964	189.9	6.4
2		2964	186.5	6.3
3	60.9	2964	162.0	5.5
4	62.3	2964	159.9	5.4
5	61.8	1832	69.9	3.8
6	62.9	1864	64.4	3.5

All incubations contained washed adrenal cortex mitochondria in supplemented sucrose (3 ml.), malate cofactor mixture (2 ml.) and [26-^{14}C]-cholesterol (296400c/100 sec.) in 0.1 ml. of dimethyl formamide, to give a cholesterol concentration of 2.7 μM. Numbers 1 & 2 (duplicates) were allowed to stand at 0° while numbers 3 - 6 were centrifuged at 16,000 g for 20 min. at 0°. The supernatants of numbers 5 & 6 (duplicates) were decanted and replaced by fresh medium (supplemented sucrose + malate cofactor system). The pellets in numbers 3 - 6 were then resuspended and all flasks (1 - 6) were incubated at 37° for 30 min. Numbers 3 & 4 are duplicates.

the mitochondria. The absorbed cholesterol may thus be less, rather than more, readily available for metabolism by the mitochondria.

The steroid content of subcellular fractions of adrenal cortex.

The amounts of free cholesterol, cholesteryl esters and pregnenolone in freshly prepared subcellular fractions of adrenal cortex were determined by the method which is described in detail in the experimental section. Duplicate portions of each fraction were carried through the procedure together with known quantities of the different steroids to serve as internal standards. These standards were dried onto the filter-paper semi-circles and then portions of the sucrose medium in which the subcellular fractions were suspended were added and dried on in the same way.

The recovery of steroids as judged by the internal standards was about 90%; in an experiment in which a known amount of [4-^{14}C]-cholesterol was used as an internal standard and counted after the extraction process had been carried out, the recovery was 93.4%.

The results are expressed in table 10 as µg. of steroid per ml. of tissue preparation and as µg. of steroid per mg. of protein as measured by the biuret method using bovine serum albumin as standard. The equivalent concentrations given in table 10 are the concentrations of endogenous steroids which would be present in the standard incubation mixture used in this work in which 3 ml. of tissue preparation was incubated in a total volume of 5.1 ml. These of course represent total,

Table 10A

Amounts of steroids in subcellular fractions of adrenal cortex

A : CHOLESTEROL

Experiment number	Subcellular fraction	Cholesterol			Protein (mg./ml.)
		(μg./ml.)	(μg./mg. protein)	Equivalent concn. (μM)	
266	Mitochondria	118	23.2	180	5.1
271	"	298	28.4	453	10.5
277	"	210	30.0	319	7.0
266	Microsomes	490	49.5	-	9.9
271	"	632	71.8	-	8.8
277	"	330	47.1	-	7.0
266	105,000 g supernatant	64	4.6	-	13.8
271	"	117	7.5	-	15.7
277	"	85	5.8	-	14.7
266	Sonicated supernatant	41	20.5	62.4	2.0
271	"	63	20.3	95.9	3.1
277	"	34	12.1	51.7	2.8

Subcellular fractions were prepared in supplemented sucrose and the steroids extracted and determined colorimetrically as described in the experimental section. Each assay was performed in duplicate; averages are reported here and are corrected for internal standards (see text). Protein was determined by the biuret method using bovine serum albumin as standard. The Equivalent concentration given in the table is the concentration which would be obtained under standard conditions, i.e. 3 ml. of enzyme in a total volume of 5.1 ml.

Table 10B

B : CHOLESTERYL ESTERS

Experiment number	Subcellular fraction	Cholesteryl esters		% of total cholesterol esterified*	Protein (mg./ml.)
		(µg./ml.)	(µg./mg. protein)		
266	Mitochondria	50	9.8	19.9	5.1
277	"	95	13.6	20.8	7.0
266	Microsomes	90	9.1	9.7	9.9
277	"	64	9.2	10.3	7.0
266	105,000 g supernatant	47	3.4	30.0	13.8
277	"	52	3.5	26.8	14.7
266	Sonicated supernatant	24	12	25.4	2.0
277	"	16	5.7	21.8	2.8

Cholesteryl stearate was used as the standard for the cholesteryl ester determinations.

* % of total cholesterol esterified = $\frac{\text{cholesterol in esters} \times 100}{(\text{free cholesterol} + \text{cholesterol in esters})}$

Table 10C

C : PREGNENOLONE

Experiment number	Subcellular fraction	Pregnenolone			Protein (mg./ml.)
		(μg./ml.)	(μg./mg. protein)	Equivalent concn. (μM)	
266	Mitochondria	19	3.7	35	5.1
275	"	20	3.6	37	5.5
277	"	30	4.3	56	7.0
266	Microsomes	14	1.4	-	9.9
275	"	26	2.4	-	10.8
277	"	16	2.3	-	7.1
266	105,000 g supernatant	8	0.6	-	13.8
275	"	7	1.1	-	6.3
277	"	22	1.5	-	14.7
266	Sonicated supernatant	5	2.5	8	2.0
275	"	8	2.7	15	3.0
277	"	9	3.2	17	2.8

and not effective, concentrations.

The equivalent concentration of free cholesterol using the mitochondrial preparations was 200 - 450 μM and of pregnenolone 35 - 55 μM. The equivalent concentration using the sonicated supernatant was 50 - 90 μM for free cholesterol and 10 - 15 μM for pregnenolone. No cholesterol or pregnenolone was detected in the acetone-ether powders.

The products of cholesterol oxidation.

There is very good evidence that oxidation of cholesterol by preparations of adrenal cortex gives rise in turn to 20α-hydroxy-cholesterol, 20α,22ξ-dihydroxy-cholesterol and pregnenolone and isocaproic acid and that, in appropriate preparations, the pregnenolone is then oxidised to progesterone (Staple et al. 1956; Shimizu, Hayano, Gut & Dorfman, 1961; Shimizu et al. 1962; Constantopoulos et al. 1962). It thus seemed unnecessary to identify rigorously the oxidation products formed by the preparations used in the present work.

However, in order to check that the products of oxidation of cholesterol by adrenal cortex preparations under the in vitro conditions used in this work were as described in the literature, the products formed from [4-^{14}C]-cholesterol by incubation with adrenal cortex mitochondria and the malate cofactor system were extracted and subjected to thin-layer chromatography in solvent systems II and IV. Most of the radioactivity that was not unchanged cholesterol was found in the pregnenolone-progesterone region, with smaller amounts (<10%) corresponding to more polar steroids. No trace of [^{14}C]-20α-hydroxy-cholesterol was

detected in any experiment, even when non-radioactive 20α-hydroxy-cholesterol was added as a trapping agent. Essentially similar results were obtained when [4-^{14}C]-cholesterol was incubated with the 105,000 g supernatant of sonicated mitochondria or with reconstituted acetone-ether powders made from this supernatant.

In the solvent systems used for thin-layer chromatography in this work, pregnenolone is only partially separated from progesterone (table 1). However by dividing the pregnenolone-progesterone zone into two parts it was possible to estimate approximately the ratio of pregnenolone to progesterone, though it should be noted that the more mobile progesterone zone contained some pregnenolone and _vice versa_. In experiments with fresh mitochondria and the malate cofactor system (which contains NAD) about one third of the product of cholesterol oxidation was progesterone. In experiments with acetone-ether powders and the sonicated supernatant, both of which were incubated with the glucose-6-phosphate dehydrogenase cofactor system (no NAD), the proportion of progesterone was much less (<10%). The formation of progesterone is probably an indication that the 'mitochondria' are in fact contaminated with microsomes, which are reported to contain the enzymes which catalyse the formation of progesterone from pregnenolone and NAD (Cheatum _et al_. 1967).

The radioactive acidic product formed from [26-^{14}C]-cholesterol by all the adrenal cortex preparations was shown to be isocaproic acid by chromatography on Whatman No. 1 paper in the system n-butanol:ethanol:

3N aqueous ammonia 4:1:5 (v/v). The paper was equilibrated in the tank for 6 hrs. and then chromatographed in an upward direction for about 24 hrs. Aliphatic acids were visualised by spraying with 0.04% bromocresol purple in 50% aqueous ethanol at pH 9. Isocaproic acid had R_f 0.62 in this system. Radioactivity was found only in the isocaproic acid region.

Oxidation of isocaproic acid by adrenal cortex mitochondrial preparations

As the production of [5-^{14}C]-isocaproic acid from [26-^{14}C]-cholesterol was used as a measure of cholesterol oxidation in many of the experiments described here, it seemed necessary to show that no significant amount of [^{14}C]-isocaproic acid was lost to the atmosphere as $^{14}CO_2$ due to subsequent oxidation of the isocaproic acid.

[26-^{14}C]-Cholesterol (0.83 μM) was incubated with adrenal cortex mitochondria and the malate cofactor system under the standard conditions in stoppered flasks with centre-wells containing 0.2 ml. of 1N-sodium hydroxide. The flasks were filled with oxygen before sealing and incubating. After 3 hrs., 0.2 ml. of 18N-sulphuric acid was added to the incubations and the flasks were quickly restoppered and reincubated at 37° for 15 min. Only 0.1% of the [26-^{14}C]-cholesterol was converted into $^{14}CO_2$, compared with 0.01% in parallel incubations with heat-denatured enzyme. In the same incubations 20.1% of the [26-^{14}C]-cholesterol was converted into [5-^{14}C]-isocaproic acid, compared with 0.03% by the

denatured enzyme (averages of duplicates). Evidently the loss of [^{14}C]-isocaproic acid by subsequent oxidation to $^{14}CO_2$ is negligible, especially as most cholesterol oxidation experiments were incubated for much less than 3 hrs.

When [1-^{14}C]-isocaproic acid (approx. 0.1 μmole) was incubated for 1 hr. with adrenal cortex mitochondria under the standard conditions in stoppered flasks with centre-wells containing 0.2 ml. of 1N-sodium hydroxide, 1.32% was converted into $^{14}CO_2$. In parallel incubations with heat-denatured enzyme, 0.12% of the [1-^{14}C]-isocaproic acid was converted into $^{14}CO_2$.

Thus isocaproic acid is oxidised to CO_2 under the conditions used, but not very readily. It should be noted that the [5-^{14}C]-isocaproic acid formed from [26-^{14}C]-cholesterol would give rise to $^{14}CO_2$ less readily than would the [1-^{14}C]-isocaproic acid used in the above experiment. It was therefore concluded that no appreciable error was introduced by using the production of isocaproic acid as a measure of the cleavage of the cholesterol side-chain.

The fate of pregnenolone

While cholesterol oxidase is a mitochondrial enzyme system, most of the enzymes which convert pregnenolone to other steroid hormones are extramitochondrial (Ryan & Engel, 1957; Young *et al.* 1965; Cheatum *et al.* 1967). Thus *in vivo* the pregnenolone formed from cholesterol must leave the mitochondria and be transferred to the microsomes for further

metabolism, for example to progesterone. Therefore experiments were devised to investigate whether microsomes might affect the distribution of the pregnenolone produced by mitochondrial oxidation of cholesterol in vitro.

Fresh adrenal cortex was fractionated into mitochondria, microsomes and 105,000 g supernatant in the usual way and [4-^{14}C]-cholesterol was incubated for 1 hr. with mitochondria either alone or in the presence of microsomes or supernatant. At the end of the incubation the contents of the incubation flasks were transferred to centrifuge tubes and centrifuged at 16,000 g for 20 min. at 0^{o} to sediment the mitochondria. The supernatants were decanted into clean centrifuge tubes and centrifuged at 105,000 g for 30 min. to sediment the microsomes. The mitochondria, microsomes and supernatant were separately extracted with ethanol:dichloromethane (5:2, v/v) and then with pure dichloromethane. Measurement of the radioactivity in samples of the extracts showed that extraction had been quantitative. The extracts were then evaporated to dryness at 50^{o} under a jet of nitrogen, redissolved in small amounts of dichloromethane and applied quantitatively to thin layers of silica. After chromatography in system II, the band corresponding to pregnenolone was removed and extracted quantitatively and counted in the usual way. It is to be noted that this band may have contained some progesterone. The results are shown in table 11.

Only a small proportion of the total [^{14}C]-pregnenolone was in the microsomes. Some radioactivity was in the incubation medium and the

Table 11

The fate of pregnenolone

Incubation system	Distribution of [^{14}C]-pregnenolone formed from [4-^{14}C]-cholesterol (percentages)		
	Mitochondria	Microsomes	Incubation Medium
Unfractionated homogenate (1,200 g supernatant)	47	17	36
Mitochondria + sucrose	72	-	28
Mitochondria + microsomes	44	1.5	54.5
Mitochondria + supernatant	52	-	48
Mitochondria + microsomes + supernatant	40	3	57

[4-^{14}C]-Cholesterol was incubated as shown with adrenal cortex mitochondria, microsomes and 105,000 g supernatant using the standard malate cofactor system. After incubation (1 hr.), the contents of the incubation flasks were centrifuged at 16,000 g for 20 min. and then at 105,000 g for 30 min. The subcellular fractions so recovered were extracted for steroids and the pregnenolone was separated by thin layer chromatography and counted. The table shows the distribution between the various subcellular components of the [^{14}C]-pregnenolone formed.

proportion therein was increased in the presence of microsomes or 105,000 g supernatant of adrenal cortex. However a considerable proportion remained in the mitochondria in all the experiments. These results indicate that, under these conditions, the products of cholesterol oxidation are not taken up by the microsomes but either remain in the mitochondria or are released into the medium. It should be emphasised that the reassembled subcellular components in these experiments are probably in a very different condition from that in an intact cell and that the results must be treated with reserve for this reason. In this connection it is of interest that a higher porportion of the total pregnenolone radioactivity was found in the microsomes in the experiments with whole homogenates than in those with reassembled subcellular components. It is also important to emphasise that the conclusions that can be drawn from these particular experiments are limited by the fact that microsomes are known to inhibit the oxidation of cholesterol by mitochondria (see below) and that the addition of microsomes and/or supernatant to the incubation mixture involves the introduction of more unlabelled cholesterol and pregnenolone into the system.

2) The substrate specificity of cholesterol oxidase of adrenal cortex.

The substrate specificity of cholesterol oxidase of adrenal cortex

While it is generally accepted that cholesterol is the principal precursor of steroid hormones it has been frequently suggested that other substances may also give rise to steroid hormones *in vivo* (see Introduction) However the *in vivo* experiments that have been reported give no information on the relative efficiency of these substances as substrates compared with cholesterol and for this reason a detailed study *in vitro* seemed warranted. The compounds examined (cholesteryl-3β-sulphate, cholesteryl fatty acyl esters and cholest-4-ene-3-one) were those which, on the evidence then available, seemed most likely to be precursors of steroid hormones *in vivo*.

Comparison of the oxidation of cholesterol, cholesteryl esters and cholestenone by adrenal cortex mitochondria

Samples of [26-^{14}C]-cholesterol, [26-^{14}C]-cholesteryl-3β-acetate, [26-^{14}C]-cholesteryl-3β-sulphate (sodium salt) and [26-^{14}C]-cholest-4-ene-3-one were purified by thin-layer chromatography and diluted to the same specific activity by addition of appropriate amounts of the pure unlabelled compounds. The substances were incubated under identical conditions with fresh mitochondria prepared from adrenal cortex and the [5-^{14}C]-isocaproic acid formed was extracted and counted. A series of such experiments were carried out and, though the results varied considerably from one experiment to another, cholesterol was

always more readily oxidised than the derivatives (table 12), in spite of the presence of large amounts of endogenous cholesterol.

The oxidation of cholesteryl-3β-linolenate was compared with oxidation of cholesterol in a separate series of experiments by incubating [4-^{14}C]-cholesteryl-3β-linolenate and [4-^{14}C]-cholesterol of the same specific activity with the mitochondrial preparations. After incubation, the steroids were extracted by shaking firstly with ethyl acetate, then with ether and finally with dichloromethane. The combined extracts were evaporated to dryness at 50^{o} under a jet of nitrogen, redissolved in small amounts of ethyl acetate and quantitatively chromatographed on thin layers of silica in system II. The various steroid fractions were removed and counted, pregnenolone and progesterone being removed and counted together as a single fraction. The formation of pregnenolone plus progesterone from cholesteryl linolenate was less than that from free cholesterol (table 13). An appreciable amount of free cholesterol was formed in the cholesteryl linolenate incubations and it seems probable that this is the source of much of the pregnenolone and progesterone that was found in such incubations (see below).

Hydrolysis and oxidation of cholesteryl esters by adrenal cortex mitochondrial preparations

Fatty acyl esters.

The experiments with cholesteryl linolenate described above suggest that cholesteryl fatty acyl esters may be hydrolysed to free

Table 12

Oxidation of cholesterol derivatives by adrenal cortex mitochondria compared with oxidation of free cholesterol

Experiment number	Substrate	Substrate Concentration (μM)	Duration of incubation (hrs.)	% Conversion to [^{14}C]-isocaproic acid		Net % conversion compared to cholesterol *
				denatured enzyme	'active' enzyme	
1	[26-^{14}C]-	1.7	3	0.02	0.96	4.4
2		51.5	3	0.11	2.01	15.0
3	cholestenone	1.7	1	0.02	0.38	6.3
4		51.5	1	0.10	1.28	9.3
1	[26-^{14}C]-	1.7	1	0.09	2.98	13.5
2	cholesteryl	1.7	3	0.43	7.78	56.0
3	sulphate	54.6	1	0.05	3.75	24.0
1	[26-^{14}C]-	1.7	3	0.09	4.42	48.0
2	cholesteryl	1.7	1	0.10	3.70	32.0
3	acetate	26.1	1	0.07	3.10	35.0

* [^{14}C]-isocaproic acid formed from [26-^{14}C]-cholesterol = 100%.

Each incubation contained fresh adrenal cortex mitochondria and the malate cofactor system in the standard way and was performed in duplicate. Denatured enzyme was made by heating the mitochondrial preparation at 100^{o} for 30 min. In each experiment incubations were also performed with [26-^{14}C]-cholesterol at the same concentration as substrate.

Table 12

Table 13

Comparison of [4-^{14}C]-cholesteryl-3β-linolenate and [4-^{14}C]-cholesterol as substrates for cholesterol oxidase of fresh adrenal cortex mitochondria

Experiment number	Substrate	Enzyme	Percentage recovery of radioactivity	Products (percentages)		
				Cholesteryl esters	Free Cholesterol	Pregnenolone Progesterone
1	Cholesterol	denatured	95.1	0	99.8	0.2
1		'active'	94.7	0.1	89.1	10.8
2		denatured	87.3	0	99.9	0.1
2		'active'	85.0	0	87.4	12.6
1	Cholesteryl linolenate	denatured	95.7	99.7	0.3	0
1		'active'	92.9	82.4	14.5	3.1
2		denatured	89.8	99.8	0.2	0
2		'active'	86.2	91.8	7.3	1.9

The substrates at the same concentration (5.4 μM) were incubated for 1 hr. with fresh adrenal cortex mitochondria and the malate cofactor system under the standard conditions. The products were extracted and separated into fractions by thin layer chromatography and the table shows the percentage of the total radioactivity recovered in each fraction.

Table 13

cholesterol under the experimental conditions. Thus the possibility arises that hydrolysis of the esters to free cholesterol might precede oxidation of the side-chain. This possibility was investigated.

[4-^{14}C]-Cholesteryl-3β-acetate was incubated for 1 hr. with a mitochondrial preparation under the standard conditions. The products were extracted with ethyl acetate, ether and dichloromethane in turn, and quantitatively chromatographed on thin layers of silica in system II. Zones of the chromatogram corresponding to marker compounds were removed, extracted and assayed for radioactivity. In 1 hr. 50% of the cholesteryl acetate was converted into free sterols of similar mobility to cholesterol and 2.6% into more polar non-esterified sterols. In parallel incubations with heat-denatured enzyme, only 0.6% of the initial radioactive cholesteryl acetate was converted into free cholesterol and 97.5% was recovered as unchanged cholesteryl acetate. In parallel incubations with [26-^{14}C]-cholesteryl acetate at the same specific activity and active enzyme, 3.1% of the cholesteryl acetate was converted into [^{14}C]-isocaproic acid.

A portion of the material extracted from the "steroid acetate" zone was rechromatographed in system I and only 0.5% of the initial radioactivity was found in the pregnenolone-3β-acetate zone. After rechromatography in system I of the products of parallel incubations with denatured enzyme, 0.4% of the initial radioactivity was in the pregnenolone acetate zone; 97.3% of the radioactive esterified sterols was unchanged cholesteryl acetate.

These results with the results of table 13 indicate that cholesteryl

fatty acyl esters are not oxidised directly to pregnenolone esters and that formation of C_{21}-steroids and isocaproic acid occurs only after hydrolysis of the esters by a cholesterol esterase in the mitochondrial preparations.

Cholesteryl sulphate.

Although it is reported that steroid sulphatases are microsomal and inhibited by inorganic phosphate (Roy, 1956; Burstein & Dorfman, 1963) and the incubations of cholesteryl sulphate with adrenal cortex mitochondrial preparations were performed in the presence of inorganic phosphate (table 12), it is clearly important to establish whether cholesteryl sulphate can be metabolised directly by adrenal cortex mitochondria.

Therefore, [4-^{14}C]-cholesteryl-3β-sulphate was incubated for 1 hr. under the standard conditions (5.9 mM phosphate) with fresh adrenal cortex mitochondria and the products were extracted firstly with n-butanol and then with dichloromethane and quantitatively chromatographed in system III. After chromatography, appropriate zones were removed, extracted and assayed for radioactivity. Only 0.12% of the total radioactivity was recovered as free sterols while in parallel incubations with heat-denatured enzyme, 0.30% of the total radioactivity was recovered as free sterols. 98.1% of the initial radioactivity was recovered as sterol sulphates. Thus, under these conditions, the adrenal cortex does not contain an active cholesterol sulphatase (see also below).

A second part of the steroid extract was evaporated to dryness

under nitrogen and then treated with 5% (w/v) trichloroacetic acid in dry dioxane for 24 hrs. at room temperature in the dark (Anderson, Briggs & Haslewood, 1964). Ethyl acetate was added and the mixture was extracted with 1N-NaOH and with water. Separate control experiments established that this technique quantitatively solvolysed cholesteryl sulphate without detectable side reactions (<0.1%). When the solvolysed product was chromatographed in system II, it was found that 6.7% of the total activity corresponded to pregnenolone and other more polar steroids; the rest was free cholesterol. Parallel incubations with heat-denatured enzyme, similarly extracted, solvolysed and chromatographed, gave only 0.23% of the total radioactivity outside the cholesterol zone. In parallel incubations with [26-^{14}C]-cholesteryl sulphate of the same specific activity the yield of [^{14}C]-isocaproic acid was 7.5%.

These results suggest that cholesteryl sulphate is converted directly into more polar steroid sulphates such as pregnenolone sulphate by the adrenal cortex mitochondrial preparations.

A comparison of cholesterol and cholesteryl sulphate as substrates for cholesterol oxidase of adrenal cortex

Although the experiments described above show that cholesteryl-3β-sulphate as well as cholesterol itself can be oxidised directly by adrenal cortex mitochondrial preparations, they give no information on the relative efficiencies of the two substances as substrates.

This is largely because mitochondrial enzyme preparations were used.

These contain large amounts of endogenous cholesterol, equivalent to a concentration in the incubations of 200 - 450 μM, and also cholesteryl fatty acyl esters which may have been hydrolysed to free cholesterol under the incubation conditions. The greatest concentrations of added cholesterol which were used were of the order of 100 μM. Bovine adrenal cortex also contains cholesteryl sulphate (Drayer et al. 1964); on the basis of the quantities reported by them (1.5 mg./Kg. wet weight of cortex) the concentration of cholesteryl sulphate in the incubations described in this work would be about 1.5 μM, assuming that all the cholesteryl sulphate was in the mitochondria. Furthermore, although the total endogenous cholesterol and cholesteryl sulphate concentrations in the incubations may be known, it is uncertain to what extent the endogenous steroids are available for metabolism. A further complication arises because the products of the oxidation of cholesterol by adrenal cortex are known to inhibit the oxidation itself whereas the products of cholesteryl sulphate oxidation do not (see below).

For these reasons, experiments were carried out in which the oxidation of cholesterol and of cholesteryl sulphate was compared in a variety of tissue preparations using a variety of concentrations of the added steroids and in which the initial rates of oxidation of the two substrates were measured.

The [26-^{14}C]-steroids at three different concentrations were incubated for 0, 10 and 20 minutes with two (or sometimes three) concentrations of enzyme in each experiment and the [^{14}C]-isocaproic acid formed was extracted and counted. In some experiments three concentrations

of steroid and two concentrations of enzyme were used but incubations were performed in duplicate and for 0 or 15 minutes only. Experiments established that the rate of oxidation of cholesterol or cholesteryl sulphate by all the preparations used was linear for about 30 minutes (see for example figure 7).

From the results of these experiments, the initial rates of oxidation of the [26-^{14}C]-steroid were calculated and these were used to derive K_m-values by the graphical method of Lineweaver and Burk (1934). In all such calculations the endogenous, unlabelled substrate in the incubations was neglected; for this reason it is misleading to attach any great significance to the absolute values obtained using fresh mitochondria or the sonicated supernatant and the constants obtained are therefore referred to as 'apparent K_m-values'. In experiments with acetone-ether dried powders there was no endogenous steroid and therefore the constants obtained are true K_m-values.

K_m-values, true or apparent, were obtained for cholesterol and for cholesteryl sulphate using fresh mitochondria, the 105,000 g supernatant of sonicated mitochondria (sonicated supernatant), acetone-ether dried powders of whole mitochondria and acetone-ether dried powders of the sonicated supernatant. Examples of the graphs obtained are shown in figures 8 and 9. As expected, a wide range of values was obtained, especially with fresh mitochondria, and these are collected in table 14. Using acetone-ether powders made from whole mitochondria it was found that there was a somewhat variable time-lag of 10 - 20 minutes

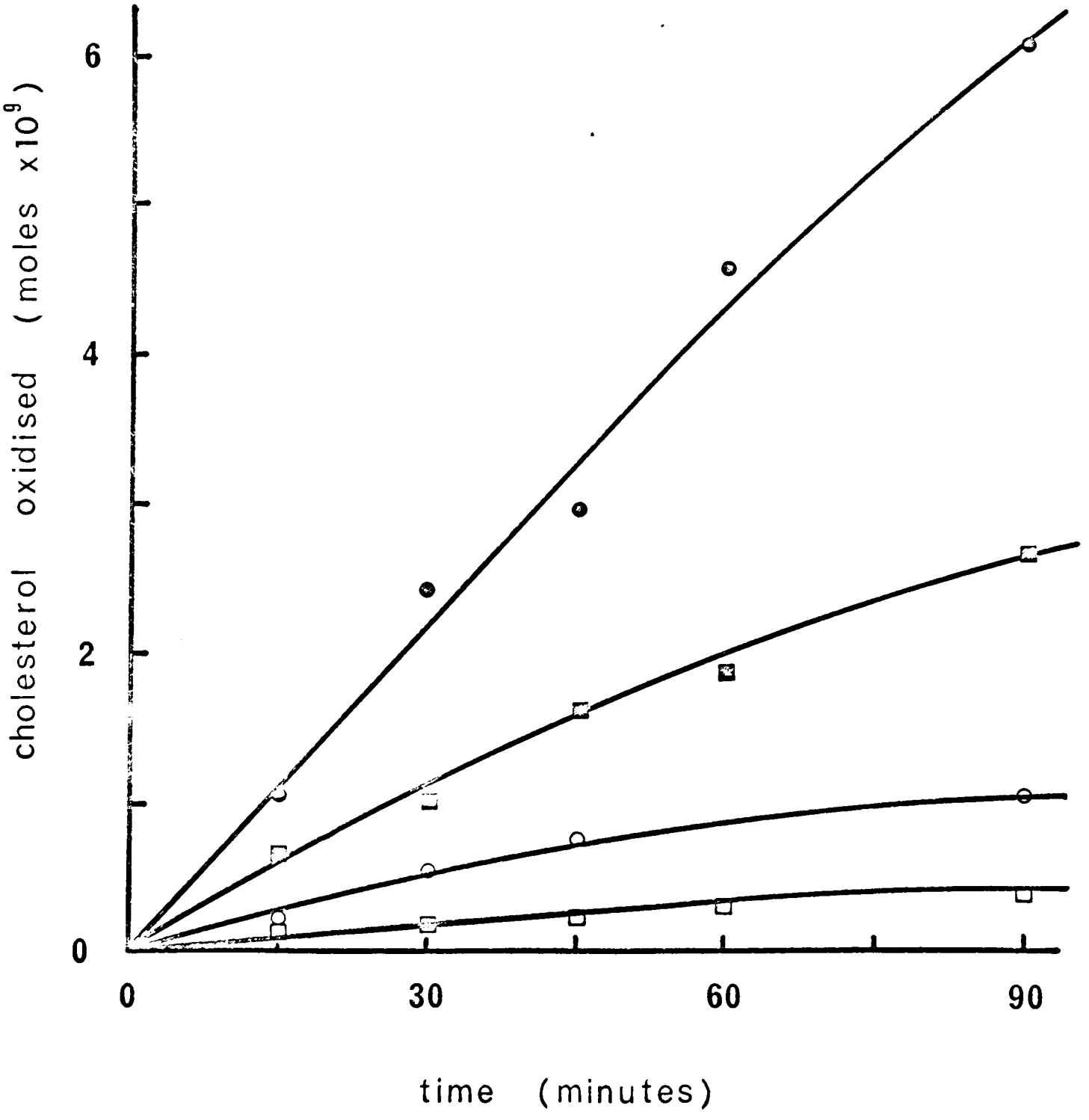


Figure 7

The time course of [26-^{14}C]-cholesterol oxidation by adrenal cortex mitochondria

concentration of added cholesterol :- □ 0.83μM ○ 2.9μM ■ 6.6μM ● 18.1μM

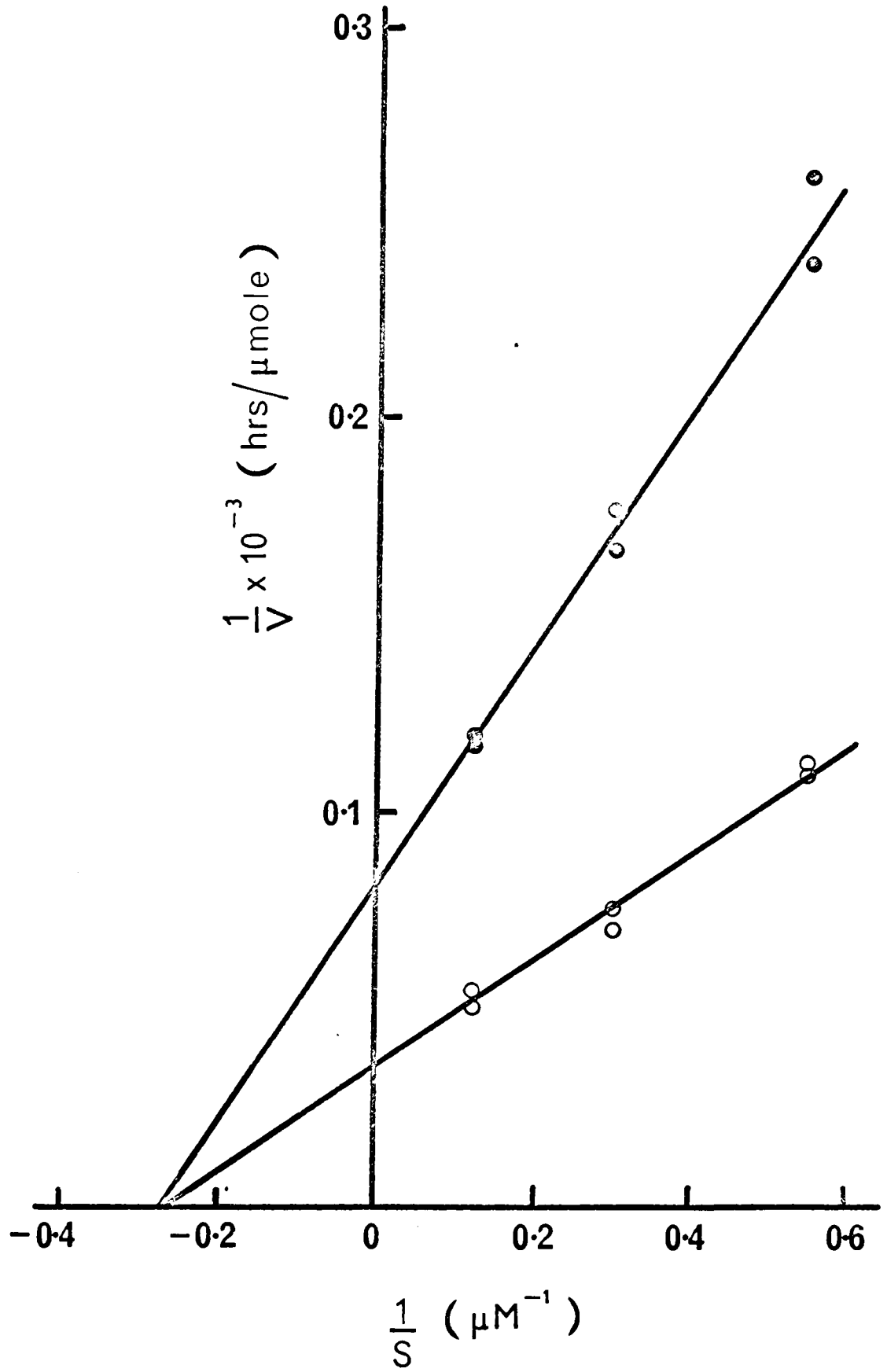


Figure 8

Lineweaver-Burk plot for the oxidation of cholesterol by acetone-ether powder of sonicated supernatant

$K_m = 3.7 \pm 0.1$ µM $\qquad V_{max} = 4.4 \pm 0.3$ mµmole/hr/mg.

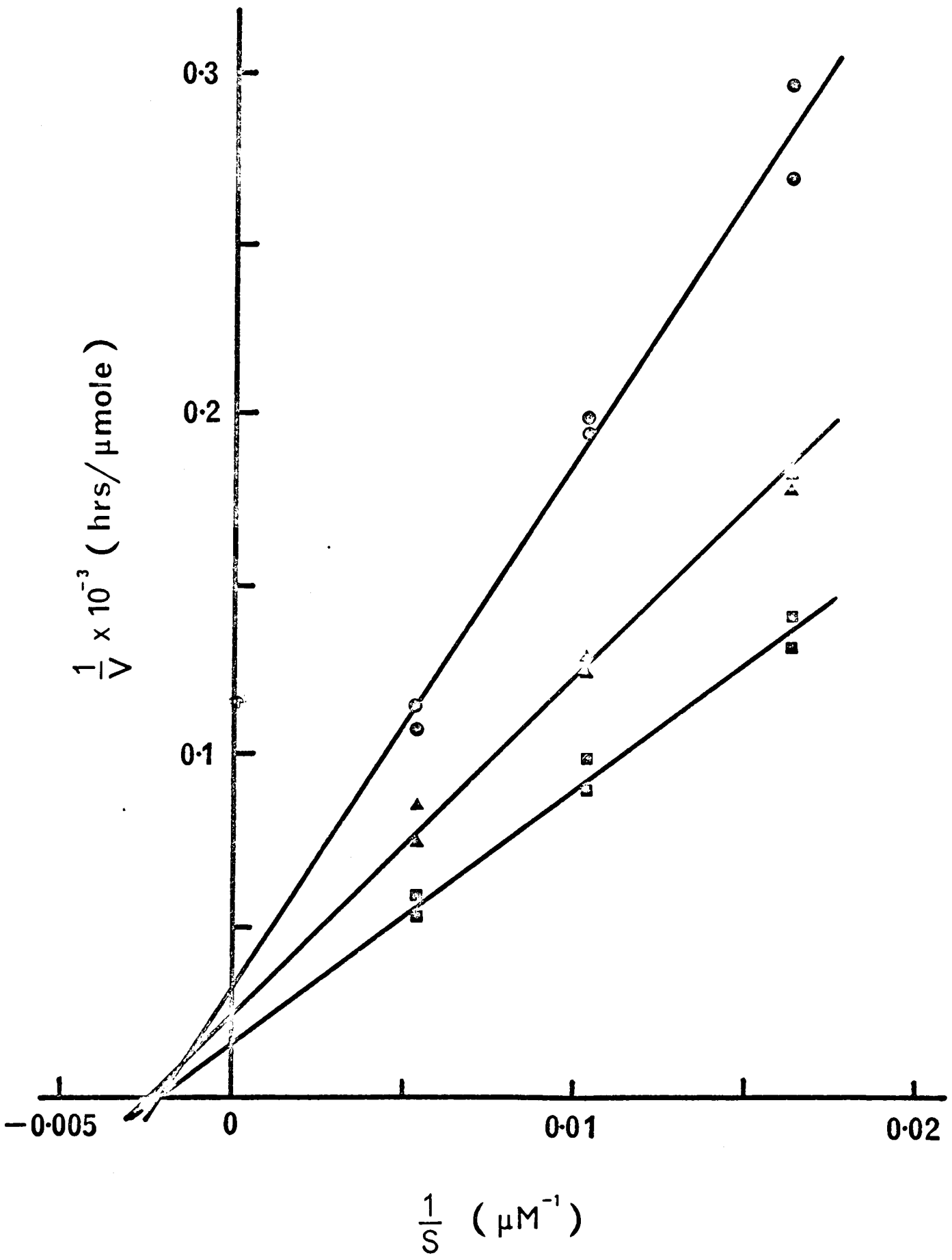


Figure 9

Lineweaver-Burk plot for the oxidation of cholesteryl-3β-sulphate by acetone-ether powder of sonicated supernatant

K_m = 460 ± 40 µM V_{max} = 8.5 ± 2 mµmole/hr/mg.

before any [^{14}C]-isocaproic acid could be detected (figure 10). A similar time-lag was observed using [26-^{14}C]-cholesterol or [26-^{14}C]-cholesteryl sulphate. No such time-lag was ever observed using fresh mitochondrial preparations, the sonicated supernatant or acetone-ether powders of the sonicated supernatant (for example see figure 7). Because of this time-lag, which may represent the time taken to eliminate an inhibitor, initial rates were difficult to measure with any confidence using acetone-ether powders of whole adrenal cortex mitochondria.

As has been already stated, no great significance can be attached to absolute values for K_m obtained from studies using tissue preparations that contain endogenous steroids. Equally, it is difficult to know to what extent the results obtained using acetone-ether powders reflect the situation in vivo. Nevertheless the experiments with the acetone-ether dried preparations of the sonicated supernatant are uncomplicated by the presence of unlabelled endogenous steroids and for this reason the K_m-values of 1 - 4 μM for cholesterol and of 470 - 510 μM for cholesteryl sulphate may be considered the most reliable guide to the relative efficiencies of the two substances as substrates for cholesterol oxidase. On this basis cholesterol is greatly preferred to cholesteryl sulphate.

The maximum rates of oxidation ($V_{max.}$) obtained from the Lineweaver-Burk plots of the results with acetone-ether powders of the sonicated supernatant were for cholesterol, 3 - 5 mμmoles/hr./mg. of powder, and for cholesteryl sulphate, 5 - 10 mμmoles/hr./mg. of powder. This may indicate that only one enzyme system is present that can utilise both

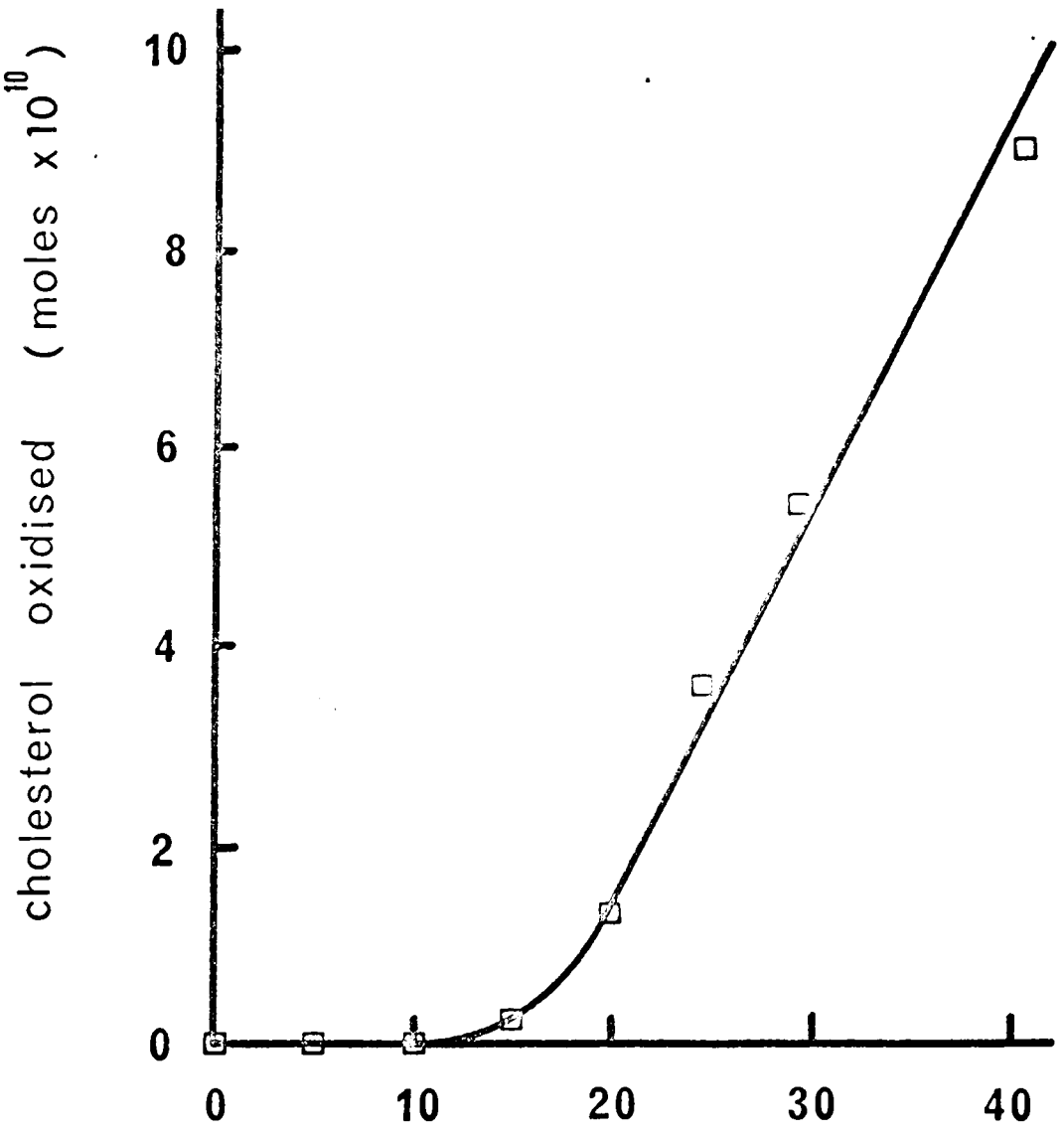


Figure 10

The time course of [26-^{14}C]-cholesterol oxidation by acetone-ether powder of whole mitochondria

(added cholesterol concentration 7.7μM)

cholesterol and cholesteryl sulphate while having a much greater affinity for cholesterol.

Cholesteryl-3β-sulphatase in adrenal cortex

Sulphatases are found in mammalian liver, adrenal, ovary, testis and placenta and, in lesser amounts in other tissues (Burstein & Dorfman, 1963; Warren & French, 1965; Nicol & Roy, 1965). The aryl sulphatase of ox liver is microsomal (Nicol & Roy, 1965) and Burstein and Dorfman (1963) found that dehydroepiandrosterone-3β-sulphatase of liver and testis was microsomal also, but no reports could be found of the intracellular location of sulphatase in adrenal cortex nor of the presence of a sulphatase acting on cholesteryl sulphate.

Although in the experiments described above no hydrolysis of cholesteryl sulphate was detected, they were performed in the presence of inorganic phosphate which inhibits sulphatase (Roy, 1956). It therefore seemed important to examine the intracellular distribution of any cholesteryl sulphatase in adrenal cortex in order to assess the possible importance of cholesteryl sulphate as a precursor of steroid hormones *in vivo*.

Subcellular fractions were prepared from adrenal cortex using the standard technique except that in the medium used for homogenisation and resuspension of subcellular components the phosphate was replaced with tris. Similarly, phosphate buffer in the cofactor mixture was replaced with tris. Each of the subcellular fractions was incubated

in the standard way with freshly rechromatographed [4-^{14}C]-cholesteryl-3β-sulphate, alone and separately with added sodium phosphate buffer pH 7.4. Heat-denatured controls for each fraction were incubated in parallel. After 1 hr. at 37° the incubation mixtures were transferred to stoppered tubes, the pH was adjusted to 9 and the steroids were extracted with hexane:ethanol 9:1 (v/v) and then twice with ether. Cholesterol (2 mg.) and cholesteryl sulphate (2 mg.) were added as carriers and the extracts were evaporated to dryness under a stream of nitrogen. The residue was dissolved in 2 ml. of acetone:ethanol 3:1 (v/v) and 3 ml. of 0.5% digitonin in 50% aqueous ethanol (an approximately threefold excess) was added. After mixing and standing overnight at room temperature in the dark, the digitonide was collected by centrifugation, washed with three separate portions of acetone:ethanol 1:1 (v/v) and then dissolved in hot methanol:water 9:1 (v/v) and quantitatively transferred to glass vials for liquid scintillation counting. The radioactivity in the digitonide was taken as a measure of the production of free 3β-hydroxy-sterols from cholesteryl sulphate. The results are shown in table 15.

In another similar experiment the extracts were chromatographed on thin layers of silica in system II, in which cholesteryl sulphate is immobile, and the whole area beyond R_f 0.1 was extracted and counted to measure the total non-sulphated steroids. Essentially similar results were obtained as by the digitonin method.

The microsomal fraction contained the most sulphatase activity and

Table 15

Cholesteryl sulphatase in subcellular fractions of adrenal cortex

Subcellular fraction	Free cholesterol formed from cholesteryl-3β-sulphate	
	counts/sec. per mg. protein	% conversion
mitochondrial fraction	2.6	0.87
mitochondrial fraction + phosphate (10 mM)	2.0	0.67
heat-denatured mitochondrial fraction	1.9	0.64
microsomal fraction	6.8	2.3
microsomal fraction + phosphate (10 mM)	2.3	0.77
heat-denatured microsomal fraction	2.1	0.70
supernatant fraction	0.94	0.31
supernatant fraction + phosphate (10 mM)	1.0	0.34
heat-denatured supernatant fraction	0.91	0.30

The subcellular fractions were prepared and incubated by the standard procedure but with phosphate replaced by tris-HCl. The table shows the production of digitonin precipitable [^{14}C]-steroids from [4-^{14}C]-cholesteryl-3β-sulphate (1.4 μM), in one of three similar experiments. The denatured fractions were prepared by heating at 100^{o} for 30 min.

this was almost completely inhibited by 10 mM inorganic phosphate. The mitochondrial fraction also contained a very small amount of sulphatase but the cytoplasmic fraction contained no sulphatase. Thus cholesteryl-3β-sulphatase closely resembles the other sulphatases which have been described.

The effect of ATP, inorganic phosphate and inorganic sulphate on cholesterol oxidase activity

If sulphation or phosphorylation are important or obligatory steps in the oxidation of free cholesterol to steroid hormones by adrenal cortex mitochondria, it might be expected that the presence of sulphate or phosphate, especially in the presence of ATP, would stimulate the production of steroid hormones and that this would be detectable by the increased production of isocaproic acid.

Omission of ATP from the normal incubation mixture or depletion of ATP by the addition of an ATP-trap (hexokinase plus glucose) caused some stimulation of [26-^{14}C]-cholesterol oxidation to [^{14}C]-isocaproic acid (table 16). These results may be due to swelling of the mitochondria, which is inhibited by ATP, resulting in increased permeability to substrate and/or cofactors (see Hirschfield & Koritz, 1964), but in any case they do not suggest an obligatory role for ATP in the oxidation process.

Adrenal cortex mitochondria were prepared in the standard way except that in the medium used for homogenisation and resuspension of the

Table 16

The effect of ATP on cholesterol oxidation

by adrenal cortex mitochondria

Incubation conditions	[^{14}C]-isocaproic acid formed			
	Experiment 1		Experiment 2	
	counts/sec.	% conversion (average)	counts/sec.	% conversion (average)
Normal (denatured enzyme)	0.43	0.07	2.56	0.06
	0.52		0.39	
Normal (active enzyme)	63.3	8.7	281.4	11.6
	59.7		275.1	
No ATP	80.4	11.4	327.7	13.9
	80.9		340.9	
Hexokinase (0.1 mg.) & glucose (10 mM) added, no ATP	81.2	11.6	324.6	13.8
	83.1		335.3	

[26-^{14}C]-Cholesterol (0.86 μM) was incubated for 1 hr. with adrenal cortex mitochondria prepared in the usual way. The cofactor system was the standard malate cofactor system except that ATP was omitted where shown. Denatured enzyme was the product of heating the mitochondrial preparation at 100° for 30 min.

mitochondria phosphate had been replaced by tris. These phosphate-free mitochondrial preparations were incubated with [26-^{14}C]-cholesterol and a cofactor system identical to the standard malate cofactor system except that the phosphate had been replaced by tris and it was found that under these conditions addition of inorganic phosphate caused slight stimulation of cholesterol oxidation; this effect was also noted in the absence of ATP (table 17). These results suggest that phosphorylation is not an important step in the oxidation of cholesterol *in vitro*.

Inorganic sulphate had no effect on cholesterol oxidation by adrenal cortex mitochondria either in the presence or the absence of ATP or inorganic phosphate (tables 17 and 18). Sulphation of cholesterol therefore seems unlikely to be an obligatory step in the oxidation of cholesterol *in vitro*.

Table 17

The effect of inorganic phosphate and sulphate on cholesterol oxidation by adrenal cortex mitochondria

Additions and omissions	[^{14}C]-isocaproic acid formed	
	counts/sec.	% conversion (average)
None (denatured enzyme)	5.37	0.6
	8.16	
None (active enzyme)	162.1	6.5
	155.5	
PO_4^{3-} (10 mM) added	169.1	7.1
	176.5	
SO_4^{2-} (10 mM) added	160.0	6.3
	148.0	
PO_4^{3-} (10 mM) added, ATP omitted	173.9	7.1
	172.2	
SO_4^{2-} (10 mM) added, ATP omitted	159.7	6.5
	158.7	

Adrenal cortex mitochondria were prepared in the usual way except that in the supplemented sucrose used for homogenisation and resuspension, the phosphate buffer had been replaced by tris. The cofactor system used was the standard malate cofactor system except that the phosphate in it had been replaced by tris. Thus, except where shown, the incubations were performed in the absence of inorganic phosphate. The table shows the [^{14}C]-isocaproic acid formed from [26-^{14}C]-cholesterol (0.83 μM) in 1 hr.

Table 18

The effect of inorganic sulphate on cholesterol oxidation by adrenal cortex mitochondria

Additions	[^{14}C]-isocaproic acid formed	
	counts/sec.	% conversion (average)
None (denatured enzyme)	2.01	0.08
	1.79	
None (active enzyme)	254.7	10.8
	238.4	
SO_4^{2-} (10 mM)	230.2	10.4
	249.8	

Adrenal cortex mitochondria were prepared and incubated with the malate cofactor system in the standard way. The table shows the [^{14}C]-isocaproic acid formed from [26-^{14}C]-cholesterol (0.83 μM) in 1 hr. Denatured enzyme was prepared by heating the mitochondrial preparation at 100^{o} for 30 min.

RESULTS

3) Inhibitors of the cholesterol oxidase of adrenal cortex.

Inhibition of cholesterol oxidase by cholesteryl esters

Cholesteryl esters were incubated with fresh adrenal cortex mitochondria and the malate cofactor system and were found to inhibit the oxidation of [26-^{14}C]-cholesterol to [^{14}C]-isocaproic acid. Table 19 shows the results obtained in one such experiment. In a series of four such experiments, it was established that the order of effectiveness as inhibitors of cholesterol oxidation was:- cholesteryl phosphate, cholesteryl acetate, cholesteryl sulphate, cholesteryl linolenate, cholesteryl oleate. Cholesteryl chloride was without effect. Sodium lauryl sulphate was also without effect and therefore the effects of cholesteryl sulphate and cholesteryl phosphate are probably not unspecific detergent effects (see also the lact of effect of pregnenolone sulphate, below).

Apparent K_i-values were determined for some of these esters by measuring the yield of [^{14}C]-isocaproic acid after 0, 10 and 20 minute incubations at four different inhibitor concentrations and three different concentrations of [26-^{14}C]-cholesterol in each experiment. Initial rates were measured and the apparent K_i-value and the nature of the inhibition found by the graphical method of Dixon (1953). A typical plot obtained with these cholesteryl esters is shown in figure 11. In all cases the inhibition was competitive in character. The following apparent K_i-values were obtained using fresh mitochondrial preparations:- for cholesteryl -3β-sulphate 110 $\pm$ 10 μM; for cholesteryl-3β-acetate 65 $\pm$ 15 μM; for dihydrogen cholesteryl-3β-phosphate 28 $\pm$ 5 μM. The

Table 19

Inhibition of cholesterol oxidase by cholesterol esters

Additions	% Inhibition (average)
Dihydrogen cholesteryl-3β-phosphate	37
Cholesteryl-3β-acetate	32
Cholesteryl-3β-sulphate (sodium salt)	30
Cholesteryl-3β-linolenate	23
Cholesteryl-3β-oleate	19
Cholesteryl-3β-chloride	0
Sodium lauryl sulphate	1

The esters, at 180 μM, were incubated for 30 min. with [26-^{14}C]-cholesterol and adrenal cortex mitochondria in the presence of the malate cofactor system. The table shows the percentage inhibition of [^{14}C]-isocaproic acid formation. The incubations were performed in duplicate and average values are shown here.

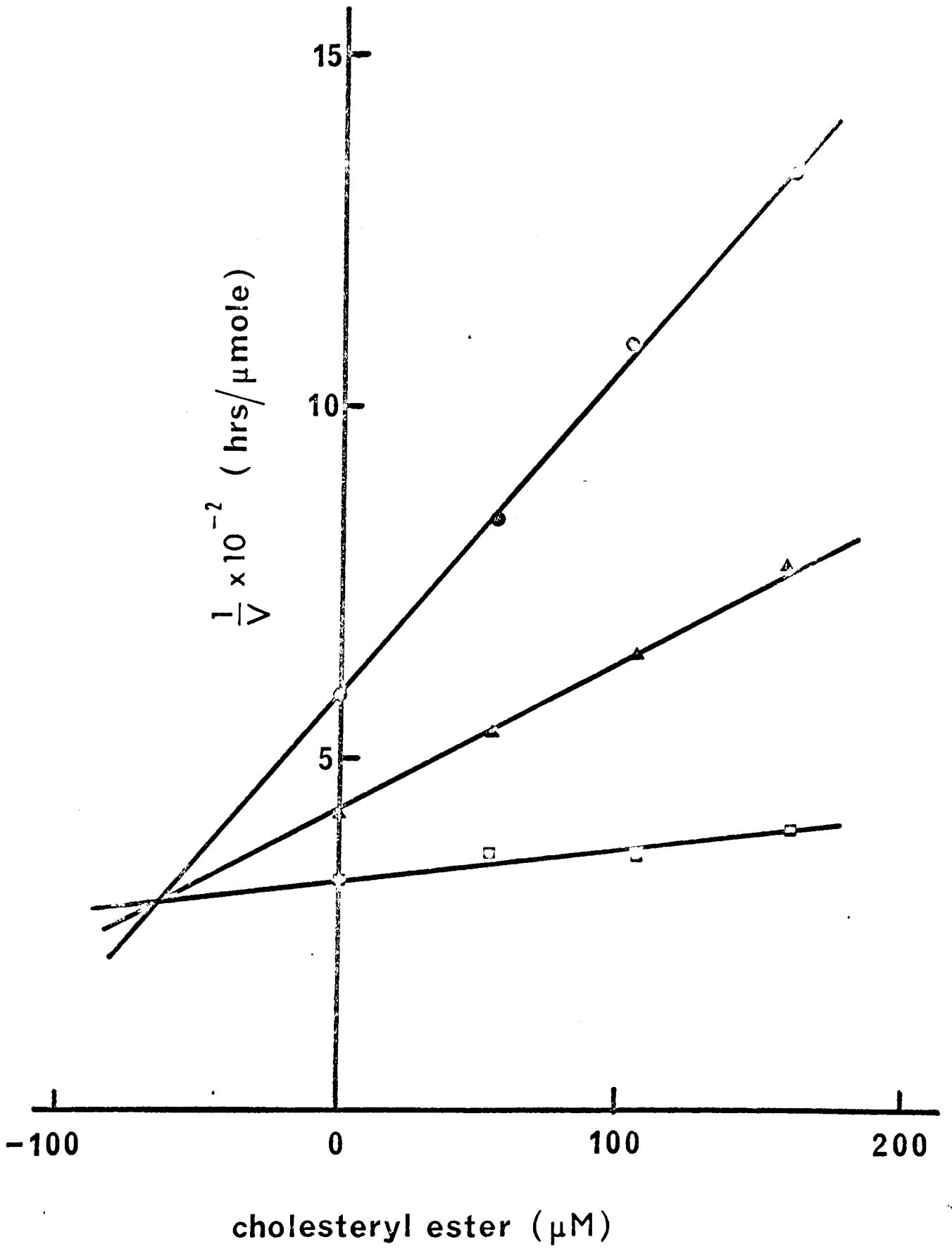


Figure 11

Dixon plot showing competitive inhibition of cholesterol oxidation in an adrenal cortex mitochondrial preparation caused by a cholesteryl ester (cholesteryl-3β-acetate, $K_i = 63 \pm 3$ μM)

concentration of added cholesterol :- ● 2.39μM; ▲ 4.66μM; ■ 7.68μM;

experiments were repeated using the sonicated supernatant acetone-ether powder preparation. The apparent K_i-values obtained were 90 ± 10 μM for cholesteryl-3β-sulphate and 50 ± 10 μM for dihydrogen cholesteryl-3β-phosphate. The inhibition characteristics were in both cases competitive.

It is probable that for the fatty acyl esters the inhibition observed in these *in vitro* experiments is at least partly due to their hydrolysis to free cholesterol. This would result in the lowering of the specific activity of the added labelled cholesterol and would cause an apparent decrease in the rate of cholesterol oxidation. The competitive inhibition by cholesteryl sulphate which was observed under conditions such that the cholesterol sulphatase of adrenal cortex would be inhibited (see above), suggests that cholesteryl sulphate is an alternative substrate for cholesterol oxidase.

Inhibition of cholesterol oxidase by its end-products

Pregnenolone and 20α-hydroxy-cholesterol are products of the oxidation of cholesterol by the adrenal cortex cholesterol oxidase system and both have been shown in this work to inhibit the production of [^{14}C]-isocaproic acid from [26-^{14}C]-cholesterol.

Figure 12 shows the reduction in the yield of [^{14}C]-isocaproic acid at various concentrations of pregnenolone. Kinetic experiments were performed in the same way as for cholesterol esters (above) and the results were analysed by the graphical method of Dixon (1953). A typical

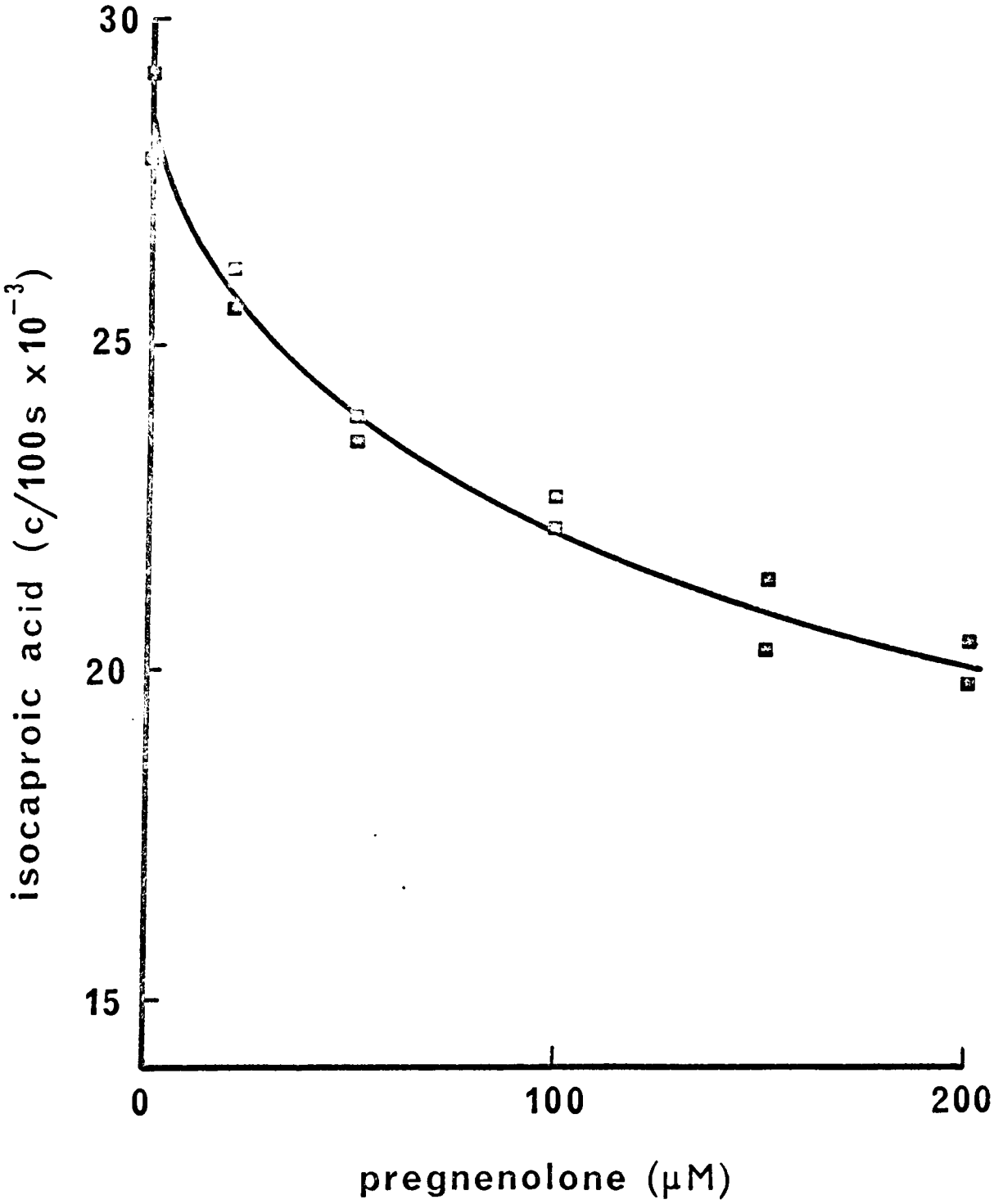


Figure 12

The effect of pregnenolone on [26-^{14}C]-cholesterol oxidation by adrenal cortex mitochondria

concentration of added cholesterol 0.83μM; incubation time 1hr.

plot for the effect of pregnenolone is shown in figure 13 and it clearly indicates the non-competitive character of the pregnenolone inhibition, in contrast to that of the cholesterol esters (figure 11). Apparent K_i-values were calculated from the results of experiments carried out with fresh mitochondria, with fresh sonicated supernatant and with reconstituted acetone-ether powders of the sonicated supernatant. These values together with the concentrations of endogenous pregnenolone in these experiments are shown in table 20. The calculations neglected the endogenous cholesterol and pregnenolone which were present in the experiments with fresh mitochondria and the sonicated supernatant but the acetone-ether powders contain no endogenous steroids and so it is probable that the values obtained with the latter are closest to the true K_i. The similarity between the value of the true K_i (80 μM) and the concentration of endogenous pregnenolone in these experiments (50 μM) suggests that this feed-back inhibition by pregnenolone may be effective *in vivo*.

20α-Hydroxy-cholesterol also inhibited cholesterol oxidase. Kinetic experiments were performed with 20α-hydroxy-cholesterol in the same way as for cholesteryl esters and pregnenolone and in all the experiments, 20α-hydroxy-cholesterol was a non-competitive inhibitor of cholesterol oxidation.. The apparent K_i-value obtained using fresh adrenal cortex mitochondria was 17 ± 5 μM. Using the acetone-ether dried sonicated supernatant the value obtained was 10 ± 5 μM (3 values).

To study the effects of 20α-hydroxy-cholesterol and pregnenolone

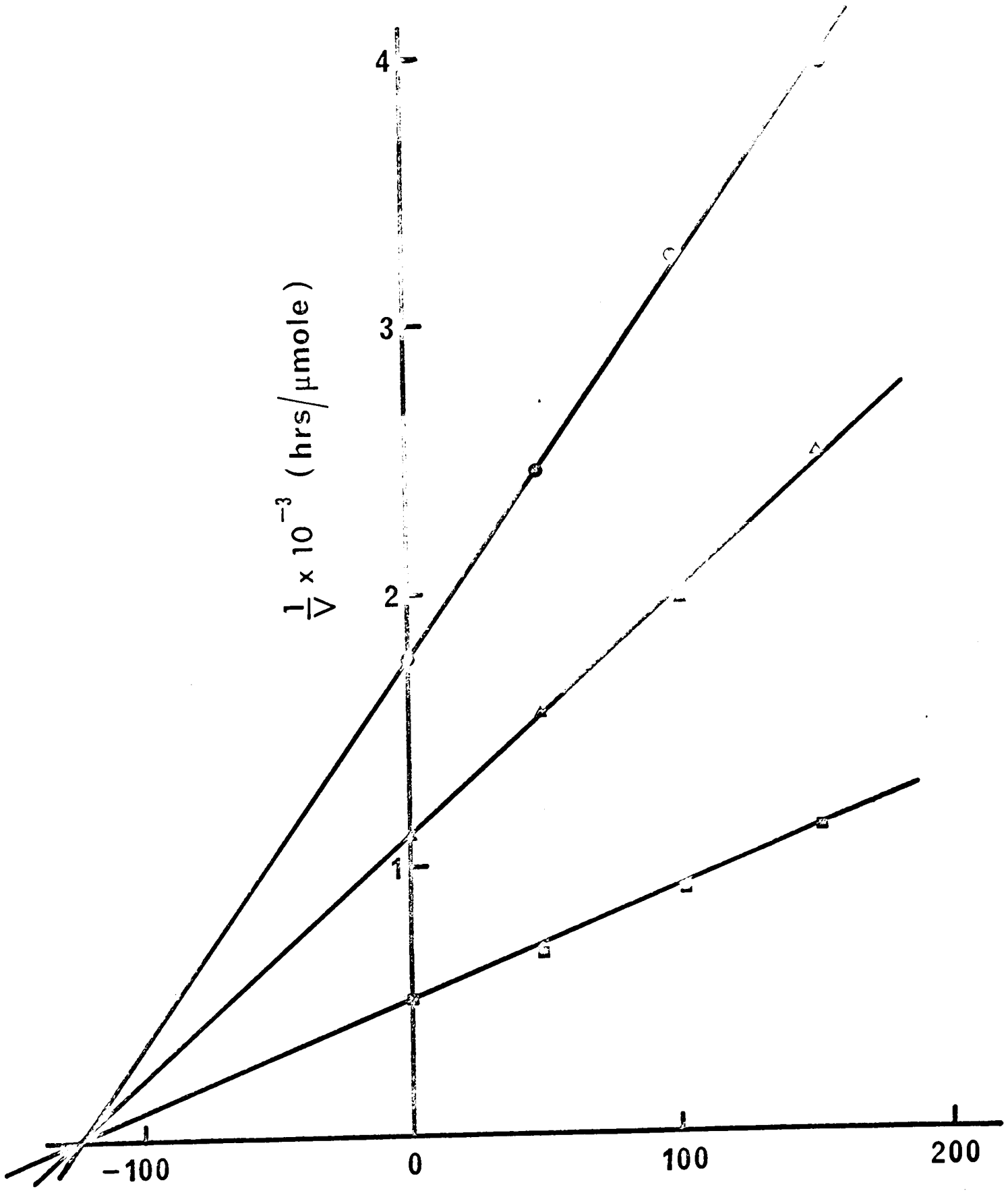


Figure 13

Dixon plot showing non-competitive inhibition of cholesterol oxidation in an adrenal cortex mitochondrial preparation by pregnenolone

concentration of added cholesterol :- ○ 0.82μM; ▲ 1.91μM; ■ 2.93μM;

$K_i = 125 \pm 5$ μM

Table 20

K_i-values for the inhibition of cholesterol oxidation by pregnenolone in various adrenal cortex preparations

Preparation	K_i-value	Number of values	Approximate concn. of endogenous pregnenolone (μM)
Fresh mitochondria	130 ± 10	3	50
Sonicated supernatant	70 ± 10	2	10
Acetone-ether powder of sonicated supernatant	80 ± 15	4	0

Initial rates of [^{14}C]-isocaproic acid formation from [26-^{14}C]-cholesterol were measured and the K_i-values calculated by the graphical method of Dixon (1953). Pregnenolone was determined by the method described in the experimental section.

in more detail, [4-^{14}C]-cholesterol was incubated with fresh adrenal cortex mitochondria or with the reconstituted acetone-ether powder of the sonicated supernatant, in the presence of unlabelled 20α-hydroxy-cholesterol (35 μM), pregnenolone (100 μM), or both together. The products were extracted with ether and dichloromethane and chromatographed on thin layers of silica in system II. In no case could significant amounts of [^{14}C]-20α-hydroxy-cholesterol be detected although the amount of [^{14}C]-pregnenolone produced was reduced compared with control incubations containing no 20α-hydroxy-cholesterol or pregnenolone. The lack of accumulation of 20α-hydroxy-cholesterol suggests that pregnenolone and 20α-hydroxy-cholesterol inhibit the 20α-hydroxylase but not the 22-hydroxylase or the side-chain cleaving enzyme.

The effect of pregnenolone-3β-esters on cholesterol oxidase

When pregnenolone-3β-esters were incubated with [26-^{14}C]-cholesterol and fresh adrenal cortex mitochondria in the presence of the malate cofactor system, no significant inhibition of [^{14}C]-isocaproic acid formation was observed (table 21). 20α-Hydroxy-cholesterol-3β-acetate at 190 μM caused only 25% inhibition although the apparent K_i-value of 20α-hydroxy-cholesterol under these conditions was found to be 17 μM. This suggests that 20α-hydroxy-cholesteryl-3β-acetate itself is not inhibitory but that the inhibition was caused by free 20α-hydroxy-cholesterol produced from the ester by hydrolysis. Under these conditions cholesteryl esters have been shown to be hydrolysed (above). However

Table 21

The effect of pregnenolone esters on cholesterol oxidase

Addition	Net [^{14}C]-isocaproic acid formed	
	counts/sec.	% inhibition
None	59.34	0
Pregnenolone	28.44	52
Pregnenolone palmitate	56.91	4
Pregnenolone acetate	58.33	2
Pregnenolone sulphate	60.92	0
20α-hydroxy-cholesteryl-3β-acetate	44.59	25

The table shows the production of [^{14}C]-isocaproic acid from [26-^{14}C]-cholesterol in 30 min. incubations with adrenal cortex mitochondria under the standard conditions (malate cofactor system) in the presence of pregnenolone esters at 190 μM. The results are averages of duplicates.

the pregnenolone acetate and palmitate do not seem to have been similarly hydrolysed.

Inhibition of cholesterol oxidation by other steroids

In order to gain some additional information on the structural requirements of an inhibitor of cholesterol oxidase, a number of steroids were incubated with adrenal cortex mitochondria in the presence of the malate cofactor system and the production of [^{14}C]-isocaproic acid from [26-^{14}C]-cholesterol was measured. The results of table 22 show that a number of steroids besides pregnenolone can inhibit cholesterol oxidation. The structures of the compounds used in these experiments are shown in figure 14.

The most potent inhibitor was 25-oxo-27-nor-cholesterol, a synthetic steroid which proved to be as effective as 20α-hydroxy-cholesterol and much more effective than pregnenolone. Some kinetic studies were performed with 25-oxo-27-nor-cholesterol in the same way as with pregnenolone. Using fresh mitochondria the apparent K_i-value for 25-oxo-27-nor-cholesterol was 16 $\pm$ 4 μM and the graphs showed that the inhibition was non-competitive in character (figure 15), like that of pregnenolone. Using the acetone-ether dried sonicated supernatant which contains no endogenous steroids, the K_i-value for 25-oxo-27-nor-cholesterol was 10 $\pm$ 2 μM (non-competitive).

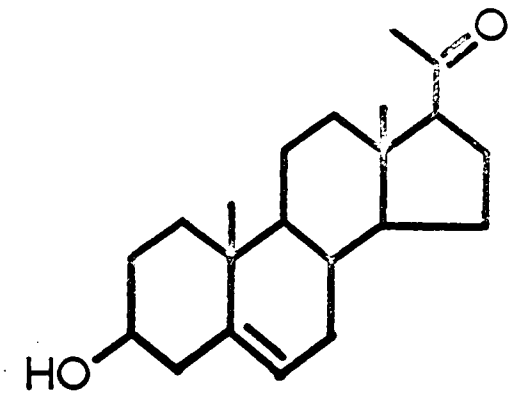
In table 22, 20α-hydroxy-cholesterol appears to be less effective as an inhibitor than 25-oxo-27-nor-cholesterol despite the evidence that

Table 22

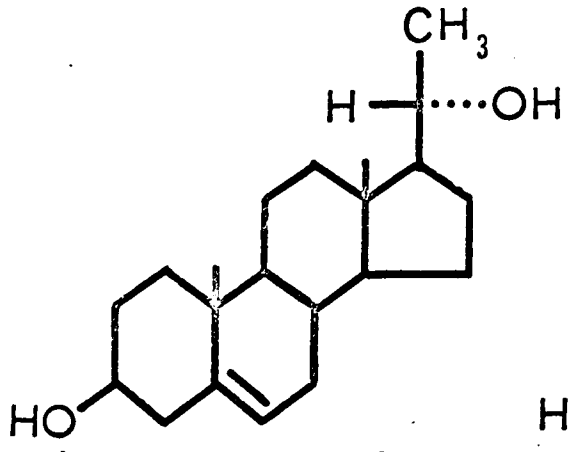
The effect of some other steroids on cholesterol oxidation

Steroid added	% Inhibition of [^{14}C]-isocaproic acid formation from [26-^{14}C]-cholesterol
1) Pregnenolone	19
2) Pregn-5-ene-3β,20α-diol	24
3) 17α-hydroxy-pregnenolone	20
4) Progesterone	18
5) Dehydroepiandrosterone	13
6) 20α-hydroxy-cholesterol	34
7) 20α-hydroxy-cholesteryl-3β-acetate	25
8) 25-oxo-27-nor-cholesterol	70
9) 25-oxo-27-nor-cholesteryl-3β-acetate	54
10) 25-oxo-27-nor-cholesteryl-3β-sulphate	8
11) 25-hydroxy-27-nor-cholesterol	3
12) 27-nor-cholest-4-ene-3,25-dione	11
13) 25-hydroxy-cholesterol	33

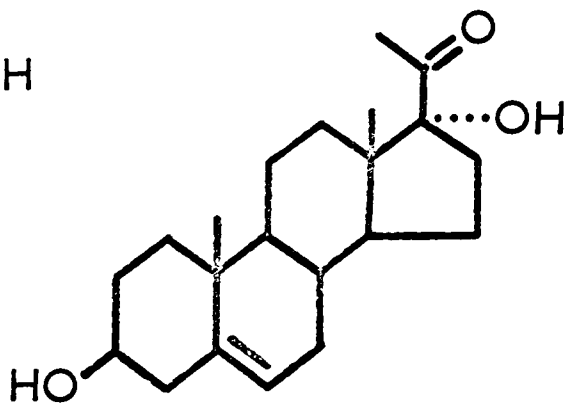
The steroids at concentrations of about 100 μM were incubated with adrenal cortex mitochondria for 1 hr. in the presence of the malate cofactor system. The results are averages of duplicates. The structures of these compounds are shown in figure 14.



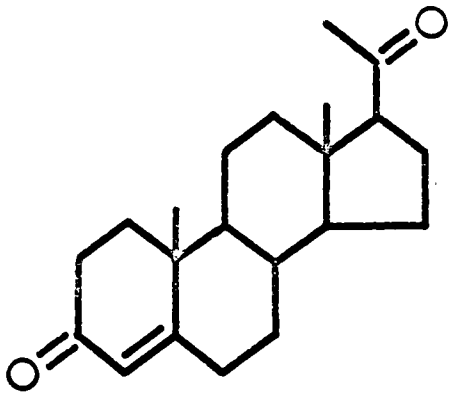
1) pregnenolone



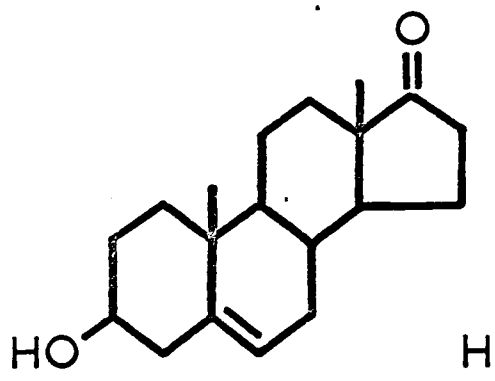
2) pregn-5-ene-3β,20α-diol



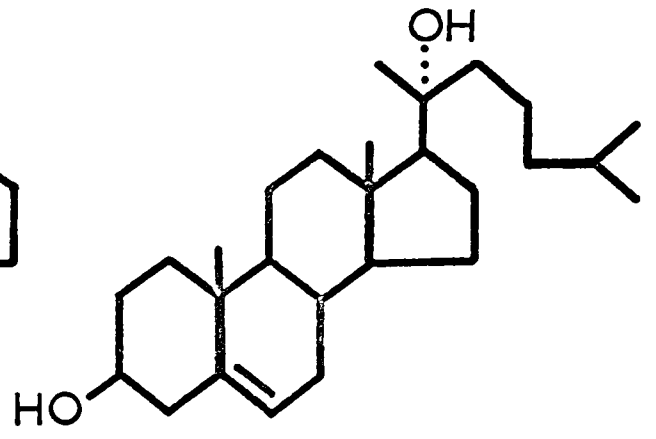
3) 17α-hydroxy-pregnenolone



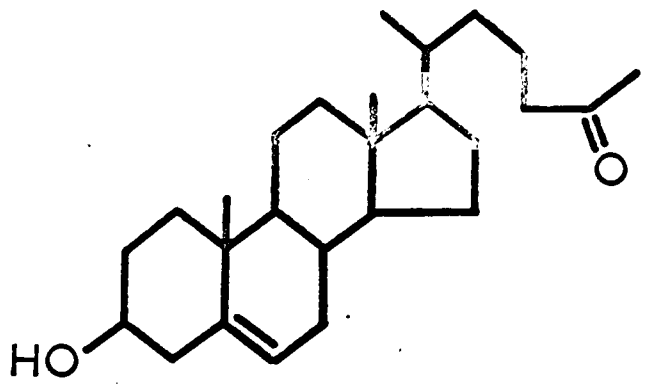
4) progesterone



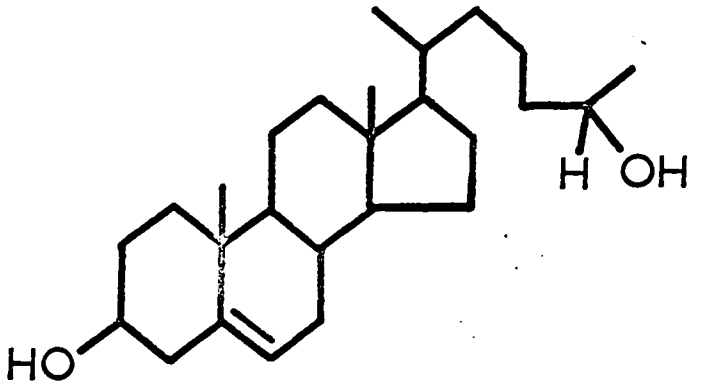
5) dehydroepiandrosterone



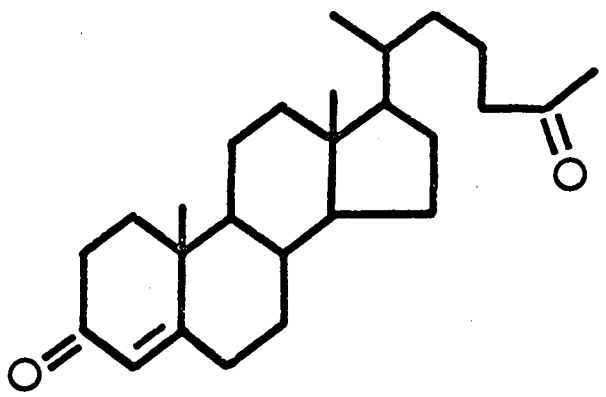
6) 20α-hydroxy-cholesterol



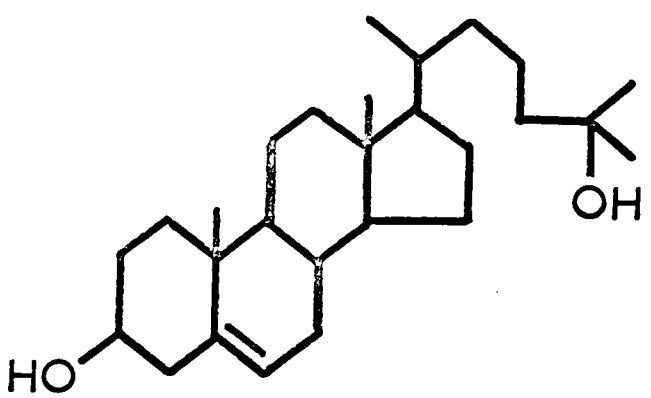
8) 25-oxo-27-nor-cholesterol



11) 25-hydroxy-27-nor-cholesterol



12) 27-nor-cholest-4-ene-3,25-dione



13) 25-hydroxy-cholesterol

Figure 14

Structures of steroids in table 22

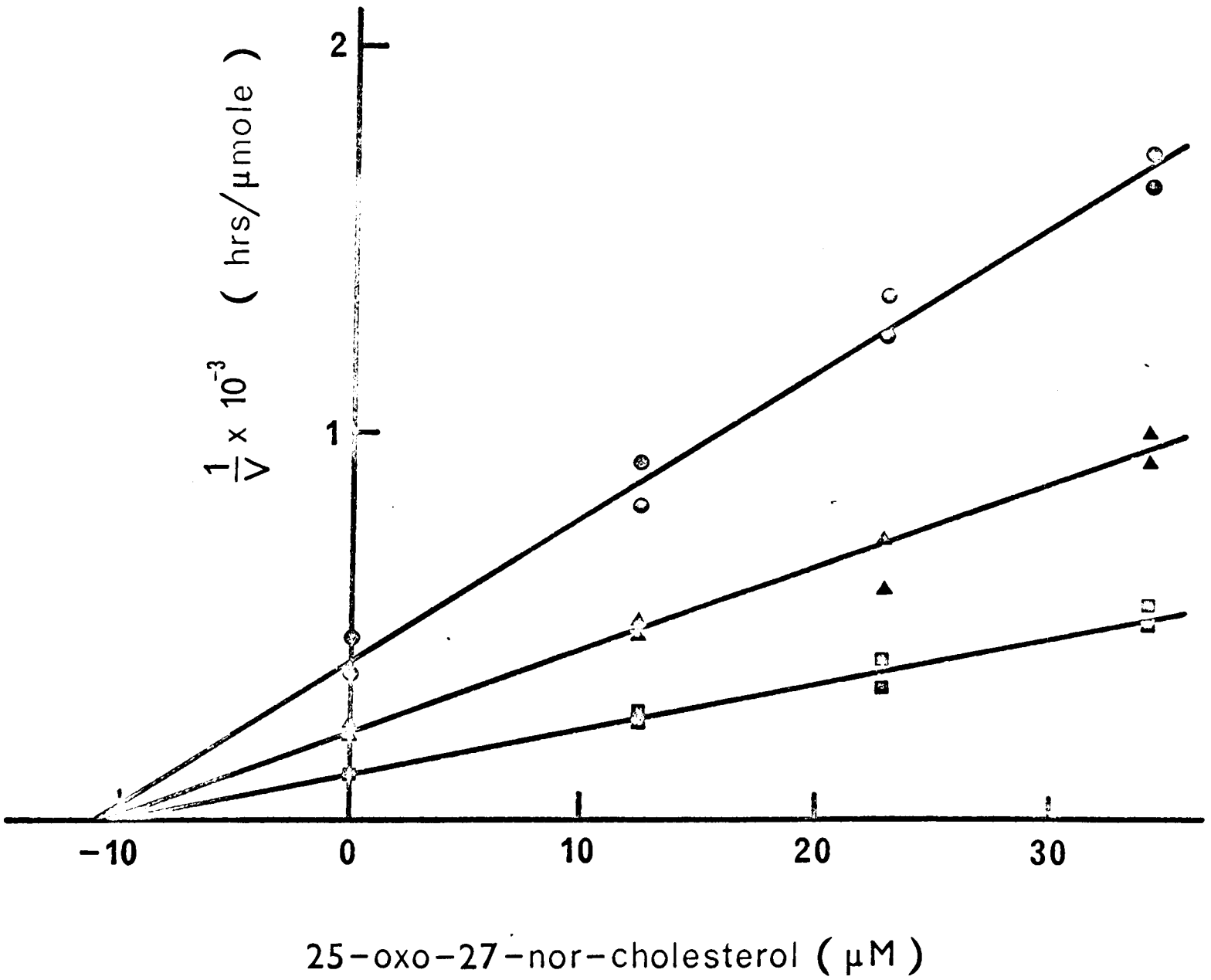


Figure 15

Dixon plot showing non-competitive inhibition of cholesterol oxidation by 25-oxo-27-nor-cholesterol in an experiment using acetone-ether powder of sonicated supernatant (K_i = 10 ± 1 µM)

concentration of added cholesterol:- ● 1.48µM; ▲ 2.62µM; ■ 4.77µM

the K_i-values of the two compounds are very similar. This may be explained by the fact that 20α-hydroxy-cholesterol will be metabolised to pregnenolone under these conditions and in 1 hr., a considerable proportion of the 20α-hydroxy-cholesterol would probably have disappeared. The kinetic studies avoid this problem by using initial rates determined from short-time incubations.

When [4-^{14}C]-cholesterol was incubated with either fresh mitochondria or an acetone-ether powder of the sonicated supernatant preparation in the presence of 25-oxo-27-nor-cholesterol (30 μM), no labelled intermediates (e.g. 20α-hydroxy-cholesterol) accumulated. This indicates that 25-oxo-27-nor-cholesterol probably acts like pregnenolone in inhibiting 20 α-hydroxylase, but not subsequent enzymes.

Stimulation of cholesterol oxidation by steroid carboxylic acids

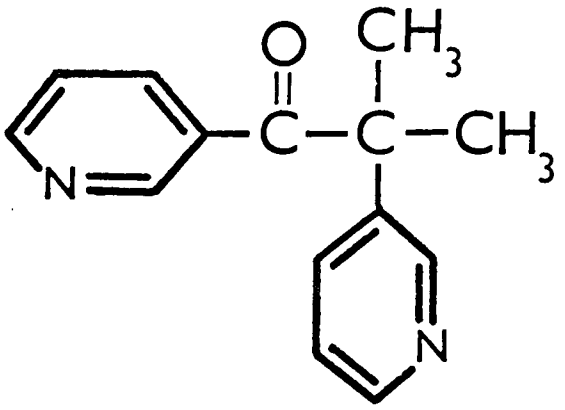
In an attempt to define further the structural requirements for inhibition of cholesterol oxidase some experiments were performed with steroid carboxylic acids. When [26-^{14}C]-cholesterol was incubated for 1 hr. with adrenal cortex mitochondrial preparations in the presence of certain steroid carboxylic acids at concentrations of about 100 μM, the yield of [^{14}C]-isocaproic acid was consistently greater than in parallel control incubations in the absence of these acids (table 23). The structures of these acids are shown in figure 16. 3β-hydroxy-chol-5-enoic acid (100 μM) increased isocaproic acid formation by approximately 50% over the controls. Lithocholic acid (3α-hydroxy-5β-cholanic acid) and

Table 23

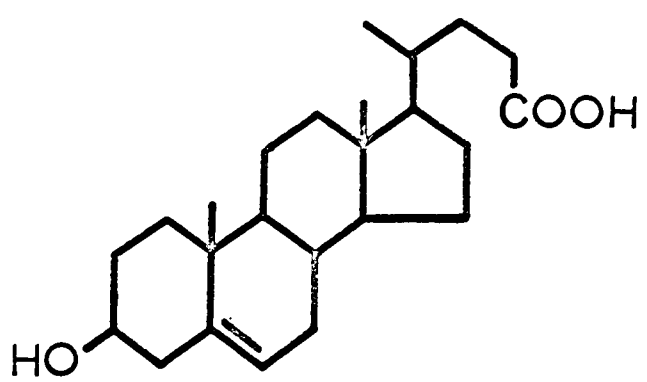
The effect of steroid carboxylic acids on mitochondrial cholesterol oxidation

Additions	[^{14}C]-isocaproic acid formed from [26-^{14}C]-cholesterol	
	counts/sec.	% stimulation
None	59.71	0
1) 3β-Hydroxy-chol-5-enoic acid	92.28	54
2) Lithocholic acid	67.39	13
3) 3β-Hydroxy-22,23-bisnor-chol-5-enoic acid	66.26	11
4) 3β-Acetoxy-22,23-bisnor-chol-5-enoic acid	61.69	3
5) 3β-Hydroxy-androst-5-ene-17β-carboxylic acid	60.40	1

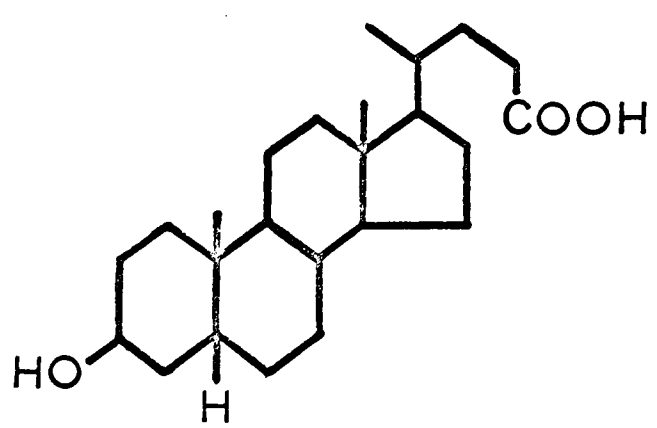
The structures of these acids are shown in figure 16. They were present at about 100 μM in incubations of [26-^{14}C]-cholesterol with adrenal cortex mitochondria and the malate cofactor system. The results are the averages of duplicates and essentially similar results were observed in another experiment.



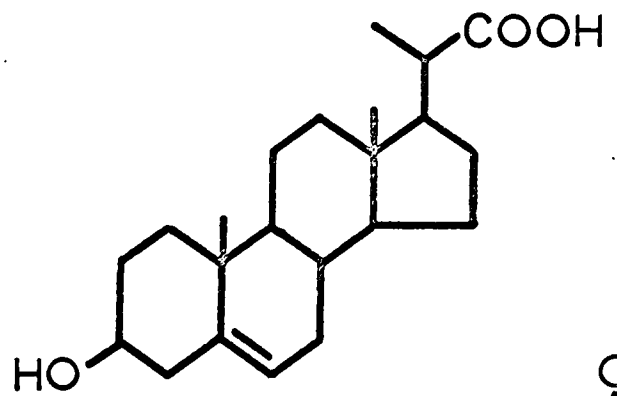
Su 4885



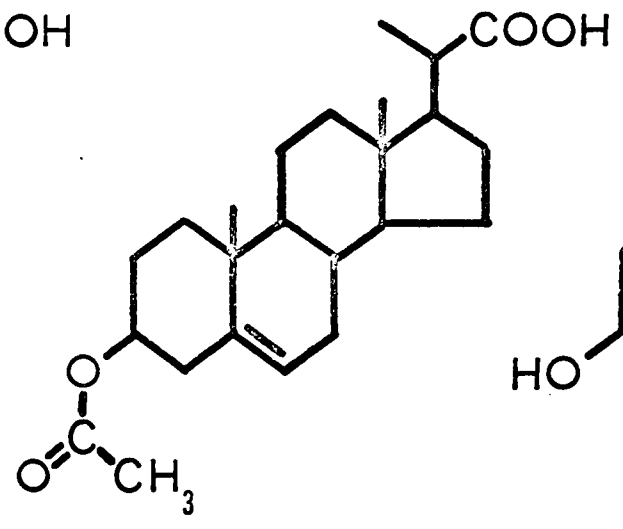
1) 3β-hydroxy-chol-5-enoic acid



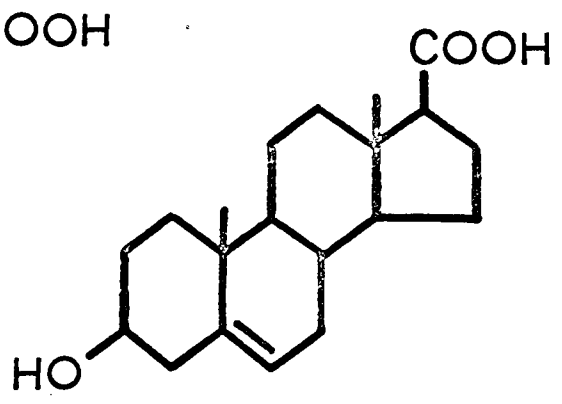
2) lithocholic acid



3) 3β-hydroxy-22,23-bisnor-chol-5-enoic acid



4) 3β-acetoxy-22,23-bisnor-chol-5-enoic acid



5) 3β-hydroxy-androst-5-ene-17β-carboxylic acid

Figure 16

Structures of Su 4885 and the steroid acids in table 23

3β-hydroxy-22,23-bisnor-chol-5-enoic acid also stimulated isocaproic acid production but to a lesser extent; 3β-acetoxy-22,23-bisnor-chol-5-enoic acid and 3β-hydroxy-androst-5-ene-17β-carboxylic acid had no effect on cholesterol oxidation at this concentration.

No satisfactory explanation of these results was discovered. Such steroid acids and their salts have some detergent action and lithocholic acid is of course a bile acid, so this effect may have been due to swelling of the mitochondria resulting in increased availability to the enzyme of substrate or cofactors. However ATP was present in the incubations which is reported to inhibit mitochondrial swelling (Hirschfield & Koritz, 1964), and moreover, sodium lauryl sulphate at 180 μM was found to have no effect on cholesterol oxidation by adrenal cortex mitochondria (table 19). Lee and Whitehouse have found that bile acids inhibit cholesterol oxidation by liver mitochondria (Lee & Whitehouse, 1963).

Inhibition of cholesterol oxidation by Su 4885

Su 4885 (Metopirone, Metyrapone, 2-methyl,1,2-di-(pyrid-3-yl)-propan-1-one), whose structure is given in figure 16, is well known as a steroid hydroxylation inhibitor, though it is usually thought of as a specific 11β-hydroxylase inhibitor (Saroff, Slaunwhite, Costa & Sandberg, 1963). Incubation of [26-^{14}C]-cholesterol with adrenal cortex mitochondria in the presence of Su 4885 resulted in reduced yields of [^{14}C]-isocaproic acid (figure 17). Similar incubations were carried out

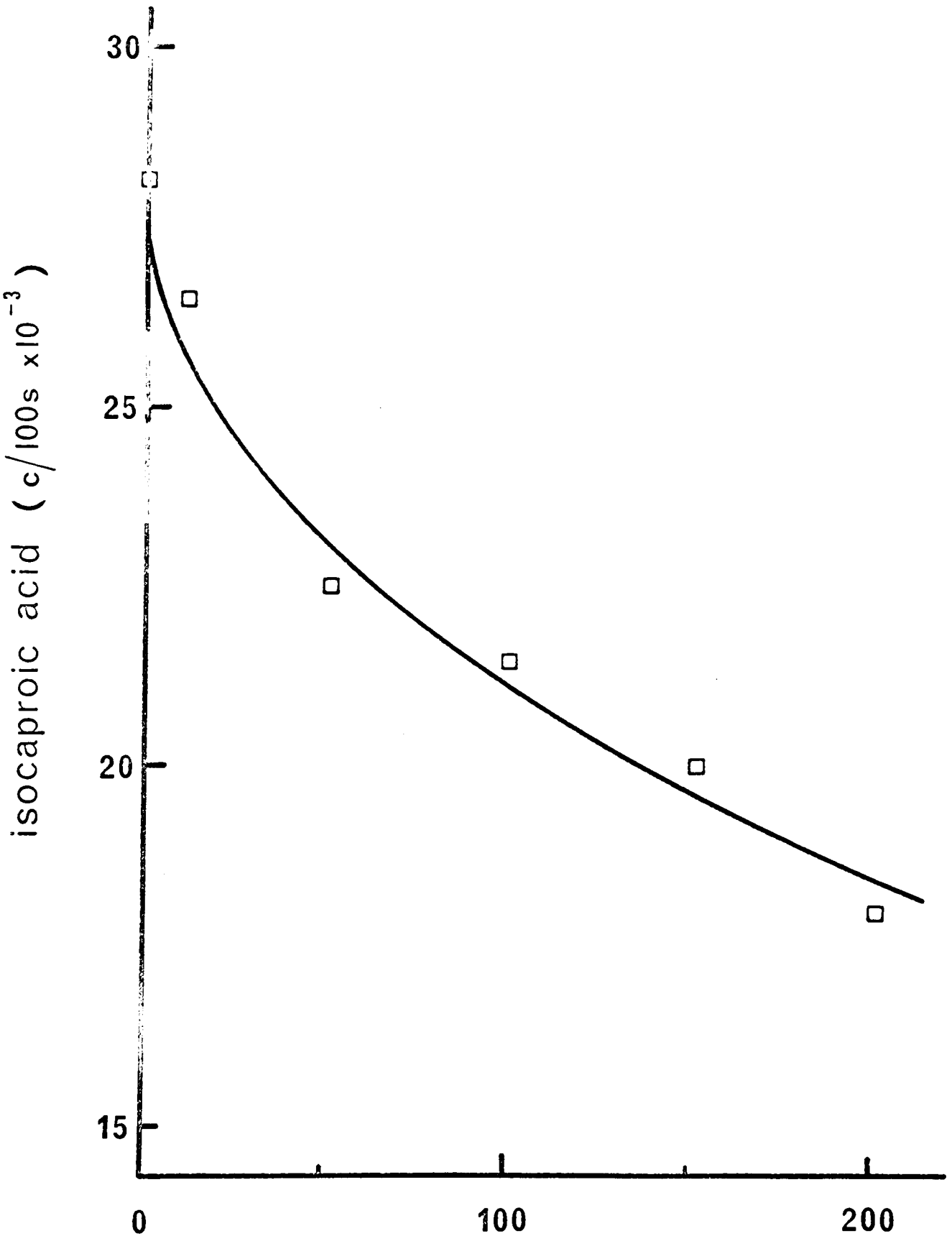


Figure 17

The effect of Su 4885 on cholesterol oxidation by adrenal cortex mitochondria

cholesterol concentration 0.83μM incubation time 1 hr.

with [4-^{14}C]-cholesterol as substrate in the presence of Su 4885 at about 200 μM and the [4-^{14}C]-steroid products were extracted and chromatographed on thin layers of silica in system II. The yield of pregnenolone was almost halved when Su 4885 was present but no trace of accumulated [^{14}C]-20α-hydroxy-cholesterol or other intermediates was found. This suggests that Su 4885 probably acts on the steroid 20α-hydroxylase.

Inhibition of cholesteryl sulphate oxidation

Freshly rechromatographed [26-^{14}C]-cholesteryl-3β-sulphate was incubated with fresh adrenal cortex mitochondria in the presence of pregnenolone and other known inhibitors of cholesterol oxidation. The incubations contained inorganic phosphate which inhibits any cholesterol sulphatase which may have been present (see above). The steroids with free 3β-hydroxyl groups, pregnenolone, 20α-hydroxy-cholesterol and 25-oxo-27-nor-cholesterol, all inhibited the conversion of [26-^{14}C]-cholesteryl sulphate to [^{14}C]-isocaproic acid but pregnenolone-3β-sulphate and 25-oxo-27-nor-cholesteryl-3β-sulphate did not (table 24). Comparison of these results with those of tables 21 and 22 suggests that the inhibitor specificity of the enzyme system oxidising cholesteryl sulphate is the same as that oxidising free cholesterol and supports the theory that only a single enzyme system is concerned.

Table 24

Inhibition of the oxidation of cholesteryl sulphate

Additions	% Inhibition of [^{14}C]-isocaproic acid formation from [26-^{14}C]-cholesteryl-3β-sulphate
Pregnenolone	36
Pregnenolone-3β-sulphate	0
20α-Hydroxy-cholesterol	65
25-Oxo-27-nor-cholesterol	62
25-Oxo-27-nor-cholesteryl-3β-sulphate	3.1

The steroids at 150 μM were incubated in duplicate for 1 hr. with fresh adrenal cortex mitochondria and the malate cofactor system. The substrate was [26-^{14}C]-cholesteryl-3β-sulphate (24.1 μM) and the [^{14}C]-isocaproic acid formed was extracted and counted. The results are the averages of two separate experiments, each in duplicate.

The effect of microsomes on mitochondrial cholesterol oxidation

Once cholesterol has been converted into pregnenolone by the mitochondria, the most important subsequent reaction in corticosteroid biosynthesis is conversion of pregnenolone into progesterone and this reaction is catalysed by microsomal enzymes (Cheatum et al. 1967). Pregnenolone must therefore at some stage be transferred from the mitochondria to the microsomes and thus it would be expected that any process which accelerated the removal of pregnenolone from the mitochondria would stimulate mitochondrial cholesterol oxidation by reducing the feed-back inhibition of pregnenolone. Therefore experiments were performed to see whether any stimulatory effect of microsomes on mitochondrial cholesterol oxidation could be demonstrated in vitro.

Washed mitochondria and microsomes were prepared from fresh adrenal cortex in the usual way; in some experiments part of the mitochondrial fraction was sonicated and then centrifuged at 105,000 g to obtain the sonicated supernatant. [26-^{14}C]-Cholesterol was incubated with various combinations of mitochondria, microsomes and sonicated supernatant in the presence of the appropriate cofactor system (see experimental section) and the [^{14}C]-isocaproic acid formed was extracted and counted. The results are shown in table 25.

Contrary to expectations, microsomes caused considerable inhibition of cholesterol oxidation by both the mitochondria and the sonicated supernatant preparations. The effect was additive to the effect of pregnenolone and was also observed with microsomes which had

Table 25

The effect of microsomes on cholesterol oxidation

Incubation conditions	Experiment 1		Experiment 2	
	Net % Conversion	% Inhibition	Net % Conversion	% Inhibition
Mitochondria alone	5.3	0	19.0	0
Mitochondria + pregnenolone	2.7	49	10.1	47
Mitochondria + microsomes	4.35	19	13.4	30
Mitochondria + microsomes + pregnenolone	2.5	53	8.6	55
Sonicated supernatant alone	14.0	0	42.7	0
Sonicated supernatant + pregnenolone	9.0	36	28.4	33
Sonicated supernatant + microsomes	5.5	61	32.7	23
Sonicated supernatant + microsomes + pregnenolone	3.1	78	20.7	52
Sonicated supernatant + denatured microsomes	2.8	80	21.8	49
Microsomes alone	1.2	--	2.1	--

Subcellular fractions were prepared by the standard technique and incubated for 30 min. with [26-^{14}C]-cholesterol in the presence of 2 ml. of the appropriate cofactor system (see experimental section). The amount of each fraction used was that corresponding to 1.5 ml. of the cell-free (1,200 g) supernatant and the incubations were made up to 5.1 ml. with supplemented sucrose. The pregnenolone concentration was 102 μM in experiment 1 and 106 μM in experiment 2.

been denatured by heating at 100° for 30 minutes. The denatured microsomal preparation was in fact a more effective inhibitor than the untreated preparation.

It seems possible that this inhibitory effect could be caused by the presence in the microsomes of cholesterol and pregnenolone. The cholesterol could dilute the added labelled cholesterol and so cause an apparent reduction in the amount of the labelled steroid oxidised whereas the pregnenolone could affect the cholesterol oxidase by the feed-back inhibitory mechanism. In any case it is evident that no proper study of the specific effect of product removal by microsomes can be made by this method.

RESULTS

4) Cholesterol oxidation by human term placenta.

Cholesterol oxidase in human term placenta

It has been estimated that the human term placenta produces about 250 mg. of progesterone per day at term (Pearlman, 1957) and although the biosynthesis of oestrogens and other hormones have been extensively studied in placenta (Diczfalusy & Troen, 1961; Hagerman, 1964; Diczfalusy, 1964) the route of biosynthesis of progesterone in placenta was, until quite recently, very poorly documented. In a preliminary communication, Solomon, Lenz, Van de Wiele & Lieberman (1954) reported that labelled pregnenolone and progesterone were produced when two placentae were perfused with [^{14}C]-cholesterol, but this work was never published in full (although mentioned in a book (Solomon, 1960)), perhaps because only two placentae were used and the conversion of cholesterol was very low indeed. Shimizu *et al.* (1961) gave a single instance of the conversion of [22-^{14}C]-20α-hydroxy-cholesterol into [^{14}C]-isocaproic acid in a placental homogenate but it was not until 1965 that any detailed study of cholesterol oxidation and pregnenolone and progesterone formation in placenta appeared (Morrison *et al.* 1965).

Experiments performed in this Department by Miss P. Bardsley and later by myself (Bardsley, 1962; Raggatt, 1964) had demonstrated that cholesterol could be oxidised to pregnenolone and isocaproic acid *in vitro* but the evidence was in several respects unsatisfactory. While cholesterol oxidase of adrenal cortex is mitochondrial (Halkerston *et al.* 1961), experiments with placental preparations suggested that the

cholesterol oxidase of this organ was cytoplasmic and, again in contrast to the enzyme of adrenal cortex, was not inhibited by pregnenolone. Further, the yield of pregnenolone and other C_{21}-steroids in these experiments seemed far too small to account for the massive amounts of progesterone which the placenta is generally believed to produce. A more detailed study of the localisation and properties of placental cholesterol oxidase thus seemed warranted.

The techniques of preparation of the subcellular fractions, of incubation of these with [^{14}C]-cholesterol and of extraction of the products are described in the experimental section. The technique used for the extraction of isocaproic acid gave much more reliable results than the techniques used in the earlier work and, as in the work on the adrenal cortex, the production of [^{14}C]-isocaproic acid from [26-^{14}C]-cholesterol was taken as a measure of the conversion of cholesterol into C_{21}-steroids by cleavage of the cholesterol side-chain.

The intra-cellular location and cofactor requirements of placental cholesterol oxidase

Using the malate cofactor system (malate + NAD + ATP + $MgCl_2$) as described for the studies with the adrenal cortex, a system which had also been used with placental preparations by Shimizu *et al.* (1961), most of the enzyme activity was found in the 105,000 g supernatant fraction, in agreement with the earlier work (table 26). However it was striking that the total activity of the cell fraction was extremely low;

Table 26

Intracelluler distribution of placental cholesterol oxidase:

Results obtained using the malate cofactor system

Subcellular fraction		[^{14}C]-isocaproic acid counts/sec. (average)	% conversion of cholesterol
Homogenate:	denatured	2.61	0.14
	active	24.11	1.3
Mitochondria:	denatured	1.79	0.10
	active	3.94	0.22
Microsomes:	denatured	3.37	0.18
	active	4.05	0.22
105,000 g supernatant:			
	denatured	2.28	0.13
	active	27.02	1.48

The subcellular fractions were prepared from a fresh human term placenta in supplemented sucrose and were incubated for 1 hr. with [26-^{14}C]-cholesterol (0.83 μM) and the malate cofactor system as described in the experimental section. The results show the [^{14}C]-isocaproic acid produced by 'active' (untreated) fractions and by fractions which had been denatured by heating at 100° for 30 min. The results are averages of duplicates.

in many incubations less than 1% of the cholesterol was converted in 1 hr. Attempts were made to increase the activity by substituting succinate, citrate, fumarate or α-ketoglutarate for malate but without success; in fact, malate was slightly preferred. No evidence was obtained that NADH or NADPH were more effective than malate + NAD as cofactors for cholesterol oxidation by the supernatant fraction, even when dialysed (table 27). In previous experiments 0.1 M sodium phosphate buffer (pH 7.4) had been used for homogenisation of the placental tissue; when placental tissue was prepared in 0.25 M sucrose, 0.44 M sucrose or supplemented sucrose, the 105,000 g supernatant preparation appeared to contain less enzymic activity than when the tissue was prepared in the phosphate buffer (table 28).

This failure to find any conditions in which the activity of the placental enzyme system could be raised, led to a study of the effect of adding an endogenous NADPH-generating system that had been used with adrenal cortex preparations. The system used (glucose-6-phosphate + NADP + glucose-6-phosphate dehydrogenase) is described in detail in the experimental section. With this system the intra-cellular distribution of cholesterol oxidase of term placenta was once more examined. The activity of the 105,000 g supernatant fraction, hitherto considered the fraction richest in cholesterol oxidase, was unchanged, but the activity in the mitochondrial fraction was greatly increased as was the total enzymic activity (table 29). This experiment was repeated using different placentae, and also using subcellular fractions prepared in

Table 27

Cofactor requirements of cholesterol oxidase in 105,000 g supernatant of human term placenta

Additions	[^{14}C]-isocaproic acid counts/sec. (average)	% conversion of cholesterol
None	2.74	0.11
NADPH (1.25 mM)	17.52	0.73
NADH (1.25 mM)	14.97	0.62
Malate (3 mM), NAD (1.25 mM) ATP (1.25 mM)	18.03	0.75
Succinate (3 mM)	14.90	0.62
Citrate (3 mM)	10.14	0.42
Fumarate (3 mM)	17.48	0.73
α-Keto-glutarate (3 mM)	9.66	0.40

Placental 105,000 g supernatant was prepared in 0.1 M sodium phosphate buffer pH 7.4 and then dialysed overnight against 0.01 M sodium phosphate buffer pH 7.4. The dialysed enzyme was incubated with [26-^{14}C]-cholesterol (0.83 μM) and $MgCl_2$ (1.5 mM) with the additions shown. The results are averages of duplicates and show the [^{14}C]-isocaproic acid formed in 1 hr.

Table 28

The effect of homogenisation in various media on the cholesterol oxidase activity of 105,000 g supernatant of human term placenta

Homogenisation medium	[^{14}C]-isocaproic acid formed counts/sec. (average)	% conversion of cholesterol
Supplemented sucrose	28.91	1.12
0.44 M sucrose	31.44	1.22
0.25 M sucrose	29.92	1.16
0.1 M phosphate buffer pH 7.4	35.47	1.32

The 0.44 M and 0.25 M sucrose media contained sodium phosphate buffer (10 mM) pH 7.4 and the 105,000 g supernatant was prepared in the various media as described in the experimental section. The preparations were incubated with [26-^{14}C]-cholesterol (0.83 μM) and the malate cofactor system and the table shows the [^{14}C]-isocaproic acid produced in 1 hr. The results are averages of duplicates.

Table 29

Intracellular distribution of placental cholesterol oxidase: Results obtained using the glucose-6-phosphate dehydrogenase cofactor system

Subcellular fraction		[^{14}C]-isocaproic acid counts/sec. (average)	% conversion of cholesterol
Mitochondria:	denatured	7.59	0.78
	active	52.62	5.5
Microsomes:	denatured	5.04	0.52
	active	17.85	1.84
105,000 g supernatant:			
	denatured	5.24	0.54
	active	1.11	0.11

The subcellular fractions were prepared in supplemented sucrose and incubated with the glucose-6-phosphate dehydrogenase cofactor system as described in the experimental section. The table shows the [^{14}C]-isocaproic acid formed from [26-^{14}C]-cholesterol (0.42 μM) in 1 hr. The results are averages of duplicates.

0.25 M sucrose and in 0.1 M sodium phosphate buffer (pH 7.4); in each case the bulk of the cholesterol oxidase activity was in the mitochondrial fraction. It was also confirmed that whereas the glucose-6-phosphate dehydrogenase cofactor system was capable of supporting cholesterol oxidation, the malate cofactor system was not (see for example table 30). Acetone-ether powders made from placental mitochondria were also highly active using the glucose-6-phosphate dehydrogenase cofactor system. Because preformed NADPH was able to substitute for the NADPH-generating system to a considerable extent, whereas other pyridine nucleotides were not (table 30) the effect of the glucose-6-phosphate dehydrogenase cofactor system seemed to be due solely to the NADPH which it produced. In the absence of ATP the mitochondria may have been permeable to pyridine nucleotides to a considerable extent (Hirschfield & Koritz, 1964).

The reason for the previous confusion over the intra-cellular location of the placental cholesterol oxidase now seems clear. The NADPH-generating system in placental mitochondria as prepared in this study does not seem to be active, in contrast to that in adrenal cortex mitochondria prepared in the same way. In consequence, the only active cholesterol oxidase detectable using the malate cofactor system was that which had 'leaked' from the mitochondria into the cytoplasm and thus had access to the cytoplasmic NADPH-generating enzymes. This also accounts for the decrease in the activity of the 105,000 g supernatant fraction when the tissue was homogenised in isotonic or hypertonic sucrose, in which mitochondria would have been less damaged than in the

Table 30

Cofactor requirements of the cholesterol oxidase in mitochondria of human term placenta

Additions	[^{14}C]-isocaproic acid counts/sec. (average)	% conversion of cholesterol
None	3.06	0.13
NADH (1.25 mM)	75.12	3.2
NADPH (1.25 mM)	181.4	7.8
NAD (1.25 mM)	2.67	0.12
NADP (1.25 mM)	4.97	0.21
NAD (1.25 mM) + malate (3 mM) + ATP (1.25 mM)	12.84	0.55
NADP (1.25 mM) + G6P (3 mM) + G6PDH (0.1 mg.)	214.7	9.3

Mitochondria were prepared in supplemented sucrose as described in the experimental section and incubated for 1 hr. with [26-^{14}C]-cholesterol (0.83 μM). Incubations contained the additions shown and also $MgCl_2$ (1.5 mM) and phosphate buffer pH 7.4 (10 mM). The results are averages of duplicates.

hypotonic phosphate buffer.

Feed-back inhibition of placental cholesterol oxidase by pregnenolone

Previous attempts to demonstrate inhibition of cholesterol oxidation by pregnenolone using the placental 105,000 g supernatant fraction and the malate cofactor system had been unsuccessful, suggesting that the placental enzyme system was different from that of adrenal cortex (Raggatt, 1964). However, using fresh mitochondrial preparations and acetone-ether dried mitochondrial preparations, inhibition by pregnenolone at a variety of concentrations was clearly demonstrated when the glucose-6-phosphate dehydrogenase cofactor system was used to generate NADPH (table 31, figure 18). The small amount of cholesterol oxidase in the 105,000 g supernatant fraction also seemed to be inhibited by pregnenolone but in these experiments the percentage conversion of cholesterol was so small that the experimental error was large enough to make the conclusion uncertain.

Table 31

Inhibition of placental cholesterol oxidase by pregnenolone

Experiment number	Enzyme	Homogenisation medium	Pregnenolone (μM)	[^{14}C]-isocaproic acid counts/sec. (average)
1	mitochondria	supplemented sucrose	0	52.62
1	"	"	204	33.09
2	mitochondria	0.25 M sucrose	0	341.72
2	"	"	198	153.93
3	mitochondria	0.1 M phosphate buffer pH 7.4	0	375.83
3	"	"	178	291.33
4	mitochondria	0.25 M sucrose	0	50.23
4	"	"	123	32.76
4	"	"	246	29.55
4	"	0.1 M phosphate buffer pH 7.4	0	51.77
4	"	"	123	42.82
4	"	"	246	31.73
5	105,000 g supernatant	0.1 M phosphate buffer pH 7.4	0	5.54
5	"	"	248	3.26
6	mitochondrial acetone-ether powder dissolved in supplemented sucrose		0	529.9
			205	142.9

The table shows the production of [^{14}C]-isocaproic acid from [26-^{14}C]-cholesterol (approx. 0.8 μM) in 1 hr. incubations using the glucose-6-phosphate dehydrogenase cofactor system. The results are averages of duplicates.

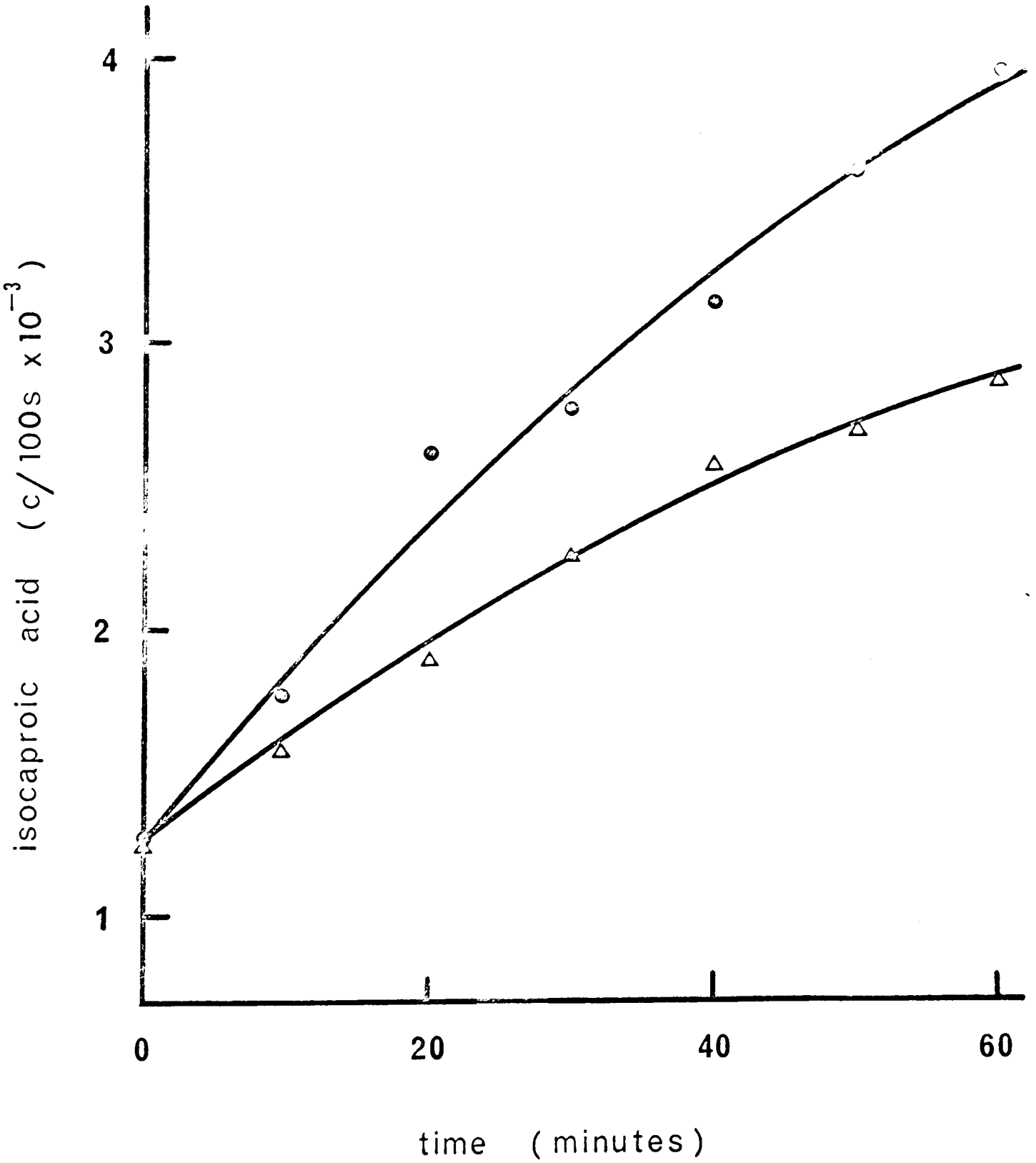


Figure 18

The effect of pregnenolone on the time course of cholesterol oxidation by a mitochondrial preparation of human term placenta

● no pregnenolone

△ pregnenolone concentration 49.3μM

DISCUSSION

Discussion

Generation of NADPH required for cholesterol oxidation

The first part of this work is concerned with the establishment of the conditions *in vitro* under which cholesterol is oxidised by cell fractions of bovine adrenal cortex. The results show that, in agreement with previous reports (e.g. Halkerston *et al.* 1961), cholesterol oxidase is a mitochondrial enzyme system which oxidises cholesterol to pregnenolone and isocaproic acid (table 5). The experiments with soluble preparations of the enzyme (105,000 g supernatant of sonicated mitochondria and acetone-ether powders made from this supernatant) confirm that the cofactor required by this enzyme is NADPH and that for such soluble preparations this cofactor can be provided by the action of yeast glucose-6-phosphate dehydrogenase (table 3).

With fresh, intact mitochondria and presumably *in vivo* also, the situation is more complex. It seems that the most satisfactory explanation of the present findings would be to assume that the NADPH required for the cholesterol oxidase is generated by the action of a mitochondrial transhydrogenase interconverting NADH and NADPH. Such an enzyme has been shown to be present in adrenal cortex mitochondria by Peron *et al.* (1966) and is presumably the mitochondrial type of transhydrogenase described by Kaplan, Colowick, Neufeld and Ciotti (1953),

rather than the oestrogen-activated, cytoplasmic transhydrogenase described by Villee (1960) and by Talalay and Williams-Ashman (1962). It may thus be postulated that the efficiency of the Krebs-cycle intermediates in promoting cholesterol oxidation by adrenal mitochondria is due to their ability to enter the mitochondria and generate NADH therein for subsequent transhydrogenation. The relative failure of added preformed pyridine nucleotides to support cholesterol oxidation (table 2) could be explained by their inability to enter the mitochondria freely (Lehninger, 1953; Bücher & Klingenberg, 1958), though, since NAD in the presence of malate was more effective than either NAD or malate alone (table 2), the mitochondria must have been permeable to NAD to some extent. Although no generation of reduced pyridine nucleotide in the incubation medium was detected when intact mitochondria were incubated with malate and NAD in vitro, this perhaps indicates only that the nucleotides are not normally released into the incubation medium.

The possible role of malic enzyme as a direct generator of NADPH in vivo requires some consideration. Like NADP-linked isocitrate dehydrogenase, NADP-linked malic enzyme is present in the cytoplasm in most tissues (Plaut, 1963; Kun, 1963) and it is difficult to see how any NADPH generated in the cytoplasm could be used directly for cholesterol oxidation in the mitochondria. In the present study however, some malic enzyme activity was associated with the mitochondria. Since the mitochondria were only washed once after isolation, this activity could be due to contamination with the cytoplasmic malic enzyme. Nevertheless

it is also possible that this enzyme may be localised, at least in part, within the mitochondria in adrenal cortex and in these circumstances, it could clearly play a part in the generation of intra-mitochondrial NADPH.

Glucose-6-phosphate dehydrogenase is also NADP-linked and in most tissues is a cytoplasmic enzyme (Noltman & Kuby, 1963). However in the adrenal cortex, it was not detected in the microsomal plus cytoplasmic fraction (16,000 g supernatant) under conditions in which it could readily be demonstrated in washed mitochondria and in soluble preparations made from these (table 7). Although the mitochondrial location of this enzyme is surprising in view of its supposed function in most tissues *in vivo*, the findings were quite clear. It is impossible to know how far a mitochondrial glucose-6-phosphate dehydrogenase could function *in vivo* in the generation of intra-mitochondrial NADPH, but the possibility certainly cannot be excluded.

Insofar as glucose-6-phosphate dehydrogenase in the mitochondria may be a source of the NADPH required for cholesterol oxidation, some regulation of steroid hormone biosynthesis might be achieved by virtue of the inhibition of its activity by steroids (table 7; McKerns & Kaleita, 1960). However, the observed activity of the enzyme was halved approximately by adding pregnenolone at 84 μM (table 7), whereas the amount of pregnenolone in adrenal cortex mitochondria would correspond to a concentration of 40 - 60 μM under the incubation conditions. McKerns and Kaleita (1960) report 60% inhibition of adrenocortical

glucose-6-phosphate dehydrogenase at a pregnenolone concentration of 50 μM. Thus *in vivo*, the activity of mitochondrial glucose-6-phosphate dehydrogenase may be significantly reduced by the pregnenolone present. However as already mentioned, it seems probable that there are other sources of NADPH which are not affected by pregnenolone and it thus seems unlikely that pregnenolone could have any regulatory effect on steroid hormone biosynthesis by virtue of its effect on glucose-6-phosphate dehydrogenase, unless this enzyme is the sole source of the NADPH required.

In summary, the NADPH required for cholesterol oxidation by adrenal cortex mitochondria may be obtained *in vivo* either through transhydrogenase action or by the activity of mitochondrial NADP-linked dehydrogenases. In the present studies with intact mitochondria, oxidation appeared to be greatest in the presence of NAD and malate and it has been presumed that transhydrogenase action was mainly responsible for the generation of the required NADPH. With soluble preparations of cholesterol oxidase, a supply of NADPH was ensured by using a preparation of yeast glucose-6-phosphate dehydrogenase.

Placental mitochondria, prepared and incubated under conditions exactly similar to those used for adrenal cortex mitochondria, seemed relatively unable to use malate as a source of NADPH for cholesterol oxidation (table 30), although just as for adrenal cortex mitochondria, NADPH was required. If this observation reflects the situation *in vivo*, then the placenta may differ from the adrenal cortex in being susceptible

to regulation by control of the supply of NADPH. However, the term placenta is an organ whose physiological function has ended and which has been deprived of its blood supply for perhaps as long as thirty minutes before it can be cooled to 0°. It is thus possible that some inactivation of NADPH-generating enzymes may have occurred during this time and that this could account for the above observation. If general degeneration of this tissue, including its subcellular components, had already begun, the evident ability of preformed NADPH to enter the placental mitochondria (table 30), in contrast to adrenal cortex mitochondria, could also be explained. Nevertheless, no difference between placentae delivered normally and those delivered by Caesarean section was observed and thus the above explanation is not wholly satisfactory.

Interaction between steroids and proteins

A recurring problem in the field of steroid biochemistry is the uncertainty of our knowledge of the effectiveness of steroids because of their interactions with proteins. In the experiments described in this thesis a stabilising effect of proteins, both of adrenal cortex and of serum, on cholesterol dispersed in aqueous media was observed and cholesterol was also found to be extensively 'taken up' in a partly reversible manner by both fresh and boiled mitochondria prepared from adrenal cortex. The nature of the interactions between steroids and proteins has been investigated in some detail (for a review see Westphal,

1962) and two types have been distinguished: a non-specific interaction between steroids and albumin or lipoproteins and a much more specific interaction of steroids with 'transcortin', the protein which is believed to transport corticosteroids in the blood-stream (Yates and Urquhart, 1962). Cholesterol, having only one 'polar' group, the 3β-hydroxyl group, is more strongly bound to lipoproteins than to albumin, whereas for more polar steroids, the reverse is true (Avigon, 1959).

Considered in the context of the present work, the occurrence of steroid-protein interactions makes it difficult to decide what are the effective concentrations of steroids in the different incubation systems used. Moreover, it is clearly difficult to relate an effect observed at a particular steroid concentration *in vitro* to conditions *in vivo* for similar reasons. Although this has been attempted on some occasions in this work, in the hope of gaining some information on possible effects *in vivo*, the above considerations should be borne in mind.

The interaction of steroids and proteins may explain some otherwise puzzling reports of stimulatory effects of fresh and boiled proteins from a variety of sources, on mitochondrial steroid hydroxylation *in vitro* (Tomkins, Curran & Michael, 1958; Peron & Koritz, 1960; Makoff & Roberts, 1964; Nakamura, Otsuka & Tamaoki, 1965). However, it should be noted that the effect of the protein-like substance which Farese (1967) has reported to be induced by the action of ACTH seems to be in a different category.

Possibly related to steroid-protein interactions was the finding in the present work that protein in the form of microsomes or cell cytoplasmic fraction was able to stimulate the release of the pregnenolone formed from cholesterol from the mitochondria (table 11). It was somewhat surprising to find in these experiments that the microsomes showed no tendency to take up the pregnenolone, although the enzymes which catalyse the conversion of pregnenolone into progesterone, the most important product of pregnenolone metabolism in the adrenal cortex, are microsomal (Cheatum et al. 1967). If pregnenolone had been rapidly converted into more polar steroids than progesterone and then released again into the incubation medium, these experiments would have failed to detect any accumulation of pregnenolone in the microsomes. However, formation of polar steroids, e.g. corticosteroids, was not rapid under the conditions of these experiments. A further difficulty in attempting to relate the present findings to conditions in vivo derives from the finding that the distribution of pregnenolone formed from labelled cholesterol was different in an unfractionated homogenate and in a preparation in which the subcellular fractions had been reassembled. In the unfractionated homogenate rather more pregnenolone was detected in the microsomes (table 11).

The presence of protein capable of binding pregnenolone, or other products of mitochondrial steroid hydroxylations, could conceivably stimulate the hydroxylations by removal of the products; in the case of the mitochondrial oxidation of cholesterol to pregnenolone, removal of

the pregnenolone could reduce the feed-back inhibition as suggested by Koritz (1966b). However, in the experiments described here, microsomes were found to have a strong inhibitory effect on oxidation of labelled cholesterol by both mitochondria and sonicated supernatant (table 25). This effect is attributed either to the dilution of the labelled cholesterol by the unlabelled cholesterol in the microsomes or to pregnenolone in the microsomes inhibiting cholesterol oxidation by the feed-back mechanism. Whilst it remains possible, as Koritz has suggested, that microsomes may act *in vivo* to remove pregnenolone from the mitochondria and hence reduce the feed-back inhibition, the possibility that microsomal pregnenolone may inhibit by the feed-back mechanism also exists. In this case, the rate of utilisation of pregnenolone by microsomal enzymes could be a feature of the regulation of cholesterol oxidation.

Alternative substrates to cholesterol for cholesterol oxidase

The results that have been reported in this work of experiments with fatty acyl esters of cholesterol show that these substances can act as precursors of steroid hormones only by first being hydrolysed to free cholesterol. No evidence was obtained that such esters were direct precursors of hormones or that the cholesterol side-chain could be cleaved while the 3β-hydroxyl group was esterified with a fatty acid. Cholesteryl fatty acyl esters are reported to form a considerable proportion of the total adrenocortical cholesterol (Riley, 1963; Goodman,

1965) although most of the esters appear not to be associated with the microsomes or mitochondria (table 10b). Cholesterol esterase activity was shown to be present in the adrenal cortex mitochondria used in these experiments and there is thus no reason why these substances could not act as a store of precursor cholesterol in the adrenal cortex.

It has been reported that administration of ACTH causes hydrolysis of cholesterol esters in the adrenal cortex *in vivo* (Griffiths *et al.* 1963; Goodman, 1965) and thus the action of ACTH on the biosynthesis of steroid hormones could be twofold: firstly to cause hydrolysis of cholesteryl esters resulting in an increase in the size of the pool of precursor cholesterol and secondly, to cause stimulation of cholesterol oxidation by the mediation of 3',5'-cyclic AMP as described in the Introduction (page 11).

The competitive inhibition of cholesterol oxidation that was observed with fatty acyl esters can best be explained by assuming that rapid hydrolysis of the esters to free cholesterol occurred. This would result in the lowering of the specific activity of the added labelled cholesterol and thus a decrease in the production of labelled products which would occur with change in the K_m (true or apparent).

The very small extent to which cholest-4-ene-3-one was metabolised by adrenal cortex mitochondrial preparations (table 12) makes it clear that this compound cannot be an important precursor of steroid hormones. Nevertheless, a small but detectable amount of the cholestenone was observed consistently to be metabolised to acidic products, presumably

isocaproic acid. Shimizu (1965b) has reported that 20α-hydroxy-cholest-4-ene-3-one was converted into progesterone in very small yield by a preparation of adrenal cortex. It does seem therefore, that oxidation and isomerisation of ring A of C_{27} steroids can precede side-chain cleavage although Kowal *et al.* (1964) have reported that the enzymes of bovine adrenal cortex and corpus luteum which convert Δ^5,3β-hydroxy-steroids into Δ^4,3-ketones had no effect on cholesterol. In contrast to adrenal cortex, cholestenone is preferred to cholesterol as a substrate for the liver enzymes which cleave the side-chain of C_{27} steroids to yield, eventually, bile acids and carbon dioxide (Stevenson & Staple, 1962; Raggatt *et al.* 1965).

Cholesteryl-3β-sulphate, although it resembles the cholesteryl fatty acyl esters in being a competitive inhibitor of cholesterol oxidation is a different case. The incubations in these experiments were performed in the presence of sufficient inorganic phosphate to completely inhibit the sulphatase that was present (page 65). Moreover, in contrast to the experiments with the fatty acyl esters, the steroid products were also sulphate esters, the chief one being identified, though not rigorously, as pregnenolone-3β-sulphate. (Rigorous identification would require crystallisation to constant specific activity. It was found that, with the solvents used, sodium pregnenolone-3β-sulphate was fairly readily solvolysed on repeated recrystallisation.) It can be concluded therefore that cholesteryl sulphate can be metabolised *in vitro* to pregnenolone sulphate without formation of intermediary free sterols.

This agrees with the results of *in vivo* experiments (Calvin *et al.* 1963; Roberts *et al.* 1964).

Further studies were performed to gain information on the relative efficiencies of cholesterol and cholesteryl sulphate as substrates for the cholesterol oxidase system of adrenal cortex. For a reaction for which product inhibition had been demonstrated and where the enzyme preparation contained large amounts of endogenous steroids, not all of which were necessarily metabolisable, the only way to compare the two substrates seemed to be to use a steroid-free, soluble enzyme preparation and to measure initial rates and to perform a simple kinetic analysis. Such experiments were carried out with the acetone-ether powder made from the 105,000 g supernatant of sonicated mitochondria. The results showed that the K_m for cholesterol (about 2 μM) was much less than that for cholesteryl sulphate (about 500 μM), indicating that cholesterol was the preferred substrate. Even with whole mitochondria and with sonicated supernatant, in which there were the complications of endogenous substrate, the apparent K_m-values were never greater than 55 μM with cholesterol whereas they were still of the order of 500 μM with cholesteryl sulphate. If the concentration of cholesteryl sulphate in the adrenal gland is as low as the figures of Drayer *et al.* (1964) suggest, then, presuming that the K_m-values can be extrapolated to conditions *in vivo*, it seems clear that cholesteryl sulphate cannot be a quantitatively important source of steroid hormones.

Steroid hormones have very great physiological effects in very

small quantities and so the above considerations do not necessarily mean that cholesteryl sulphate is of no physiological importance. It has been demonstrated only fairly recently (MacDonald, Chapdelaine, Gonzalez, Gurpide, Vande Wiele & Lieberman, 1965) that the adrenal cortex produces dehydroepiandrosterone-3β-sulphate which has androgenic properties. Dehydroepiandrosterone sulphate has been shown to be produced from cholesteryl sulphate and from pregnenolone sulphate *in vivo* without hydrolysis of the ester linkage (Calvin *et al.* 1963; Roberts *et al.* 1964) and Baulieu and Carpechot (1965) have emphasised the relative importance *in vivo* of the direct metabolism of dehydroepiandrosterone sulphate to other androgen sulphates without hydrolysis compared with metabolism after hydrolysis to the free 3β-hydroxy-steroid. Cholesteryl sulphate could thus be a precursor of a series of steroid sulphates including a number of physiologically active adrenal androgens.

It is interesting that many of the reports of the metabolism of steroids by this sulphate pathway are concerned with experiments using cancerous tissue or in patients with adrenal cancers (e.g. Calvin *et al.* 1963; Roberts *et al.* 1963; Calvin & Lieberman, 1964). There is also a report of changes in the secretion rate of dehydroepiandrosterone sulphate and changes in the ratio of secreted dehydroepiandrosterone sulphate to free dehydroepiandrosterone in a number of different types of adrenal cancer, compared with the values found in normal patients (Kono, Yoshimi, Tatsumi, Osako, Hiramori & Miyake, 1966). Thus the possibility is raised that the enzymes of the sulphate pathway are among those affected

in virilising adrenal cancer. Changes in the relative importance of the steroid sulphate and free steroid pathways may account for the evidence obtained for an alternative pathway of steroid hormone formation, not *via* cholesterol or pregnenolone, in patients with adrenal cancer (Ungar & Dorfman, 1957; Burstein & Dorfman, 1962).

The fact that the maximum initial rates for cholesterol oxidation and for cholesteryl sulphate oxidation were of the same order and the similarity between the inhibitor specificities for the two substrates suggest that we are dealing not with two enzyme systems but with only one, capable of metabolising two substrates. However, if this is so, then the production of steroid sulphates *in vivo* should be closely integrated with the production of free steroids, providing that the amount of the appropriate substrate is not limiting. The small amounts of cholesteryl sulphate in bovine adrenal cortex suggest that, unlike cholesterol, the former may be in short supply. In the cases where the ratio of sulphated hormone formation to free hormone formation is changed, it is possible that the production of cholesteryl sulphate is changed also. If there were some other regulatory mechanism which is specific for the sulphate pathway, this could best be investigated in patients with such abnormalities.

If cholesteryl sulphate is a true precursor of steroid hormones *in vivo*, then it is necessary to consider how cholesteryl sulphate could arise in the adrenal cortex. There appear to be only two studies of the enzymic sulphurylation of cholesterol, both by Banerjee and Roy (1966, 1967)

and in these an extract of guinea-pig liver was used. Cholesteryl sulphate has been detected in blood (Drayer & Lieberman, 1965) and it may thus be formed in the liver and transported to the adrenal cortex in the bloodstream. However, the sulphurylation of other Δ^5,3β-hydroxy-steroids is very well documented and takes place in human and bovine adrenal cortex as well as in liver and other tissues. The early work on sulphokinases was by Lipmann and his collaborators and more recently Boström and his collaborators have published a great deal on this topic (e.g. Robbins & Lipmann, 1956; Boström, Franksson & Wengle, 1964; Holenberg & Rosen, 1965).

Sulphatase, the enzyme hydrolysing sulphate esters, was shown in the present work to be active in the microsomes of bovine adrenal cortex and to be inhibited by inorganic phosphate (table 15). It thus resembles the steroid sulphatases of liver and testis (Burstein & Dorfman, 1963) and the aryl sulphatase of liver (Nicol & Roy, 1965). It may compete with cholesterol oxidase for the cholesteryl sulphate *in vivo* but the fact that the cholesterol oxidase is mitochondrial whilst the cholesterol sulphatase is microsomal makes this rather unlikely. The small amount of sulphatase detected in the mitochondrial fraction of adrenal cortex (table 15) is probably accounted for by the contamination of the mitochondria with microsomes which no doubt arose from the use of Halkerston's method of centrifugation (Halkerston *et al.* 1961) in which the 1,200 g - 16,000 g fraction is taken as the mitochondrial fraction.

Although the experiments were not very extensive, no evidence was

obtained in this work to support the view that sulphurylation or phosphorylation is an obligatory or quantitatively important step in cholesterol oxidation. The effects of ATP and inorganic phosphate that were observed (tables 16 & 17) seem better explained in terms of swelling of the mitochondria. The omission of ATP might result in greater swelling of the mitochondria (Lehninger, 1962; Hirschfield & Koritz, 1964) and thus greater permeability of the mitochondrial membrane to substrate and cofactors and hence increased cholesterol oxidation. Similarly, phosphate is reported to cause mitochondrial swelling (Raaflaub, 1953; Lehninger, 1962) and this could explain the slight stimulatory effect of inorganic phosphate on mitochondrial cholesterol oxidation. However, phosphate has also been reported to stimulate soluble cholesterol oxidase preparations (Satoh et al. 1966).

Cholesteryl phosphate itself was found to be a competitive inhibitor of cholesterol oxidation in mitochondrial preparations (page 75). This suggests either that it can be an alternative substrate, like cholesteryl sulphate, or that it is hydrolysed to free cholesterol under the experimental conditions, like cholesteryl fatty acyl esters. Owing to the failure of attempts to synthesise labelled cholesteryl phosphate by a number of methods (page 22), no further experiments with this ester could be carried out.

Feed-back inhibition of cholesterol oxidase by pregnenolone

Pregnenolone, the principal product of cholesterol oxidation by

the mitochondria of endocrine organs, inhibits the oxidation itself non-competitively in fresh mitochondrial preparations and in acetone dried preparations of both adrenal cortex and term placenta (tables 20 & 31). Koritz (1964) has also reported non-competitive inhibition by pregnenolone of cholesterol oxidation in adrenal cortex mitochondria acetone powders but he gives no quantitative data.

The values of the K_i for added pregnenolone that were obtained (about 80 μM) may be compared with the measured amount of endogenous pregnenolone in the adrenal cortex mitochondrial preparations (40 - 60 μM). Although the effective pregnenolone concentration *in vivo* is uncertain, this comparison suggests that pregnenolone may be a physiological inhibitor. In addition, pregnenolone in other parts of the cell may inhibit cholesterol oxidation in the mitochondria as has been suggested earlier for microsomal pregnenolone. The amount of pregnenolone in the microsomes corresponded to a concentration of about 40 μM and that in the supernatant to a concentration of about 20 μM making a total of about 110 μM (see table 10c).

20α-Hydroxy-cholesterol also inhibits cholesterol oxidation non-competitively but the amounts of this compound that are present in the cell are believed to be so small that, although more potent than pregnenolone, 20α-hydroxy-cholesterol does not seem likely to be of physiological importance as a regulator.

When cholesterol oxidation was inhibited by pregnenolone, by 20α-hydroxy-cholesterol, by the synthetic steroid 25-oxo-27-nor-cholesterol

or by the non-steroidal hydroxylation inhibitor Su 4885, no intermediates accumulated. Many workers in this field have similarly failed to trap intermediates although Solomon *et al.* (1956) had some success. This is commonly interpreted as indicating that 20α-hydroxylation is the slow step in cholesterol oxidation to pregnenolone while subsequent steps are faster and furthermore that the intermediates remain bound to the enzyme for all three reactions performed by cholesterol oxidase. The regulatory step would thus appear to operate at the 20α-hydroxylation stage.

A very recent paper (Ichii *et al.* 1967) has provided striking confirmation of this conclusion. Having devised a soluble adrenal cortex enzyme system in which 20α-hydroxy-cholesterol could be trapped, these workers were able to measure 20α-hydroxylase activity by determining the 20α-hydroxy-cholesterol used up by further metabolism. They found that the 20α-hydroxylase was inhibited by pregnenolone but that the 22-hydroxylase and side-chain cleaving enzyme were not. They report 70% inhibition of the 20α-hydroxylase by 70 μM pregnenolone which would suggest that the K_i for pregnenolone was about 50 μM. They further report that the K_m for the 20α-hydroxylation of cholesterol was 5 μM. Comparison of these figures with the results obtained in the work in this thesis (tables 14 & 20) provides independent confirmation of the validity of the kinetic constants and the conclusion that 20α-hydroxylation is the rate determining step and the step subject to feed-back inhibition.

That added pregnenolone, 20α-hydroxy-cholesterol and 25-oxo-27-nor-

cholesterol are non-competitive inhibitors of cholesterol oxidation suggests strongly that these compounds are exerting their effects by interaction with a site on the enzyme other than that used for binding the substrate, despite the fact that all these compounds, like cholesterol itself are Δ^5,3β-hydroxy-steroids. The observation that these compounds exert their effects on the soluble cholesterol oxidase in the acetone-ether powders made from the sonicated supernatant suggests that the effect of these compounds is primarily on the enzyme itself and is not due to an indirect effect for example on the mitochondrial membrane.

A number of other steroids have been shown to inhibit cholesterol oxidation to various degrees (tables 21, 22 & 23). The interpretation of these experiments is complicated however, by the possible further metabolism of the added substances that may have taken place and to be certain that any of the compounds could have any effect in vivo, it would be necessary to perform kinetic analyses as were performed for pregnenolone. Nevertheless, from an examination of the structures of the compounds tested (figure 14) it would seem that a free 3β-hydroxyl group and an oxygen function in the side-chain are usually parts of the structure of such inhibitors. The specificity of the inhibitor binding site is indicated by the fact that 25-hydroxy-cholesterol is a potent inhibitor but 25-hydroxy-27-nor-cholesterol is completely ineffective. This indicates also that the chemically unreactive methyl groups of the side-chain are not biochemically inert. However the effectiveness of

25-oxo-27-nor-cholesterol shows that the situation is not simple. The latter compound is of particular interest as it may be regarded as an analogue of pregnenolone since both these compounds have methyl-ketone groups in the side-chain. Similarly, 25-hydroxy-cholesterol may be regarded as an analogue of 20α-hydroxy-cholesterol as both have in the side-chain tertiary alcohol groups that are surrounded by a methyl group and a methylene group (i.e. a 2-hydroxy-propyl group in the side chain). The reason for the greater efficacy of these two compounds, compared to their naturally occurring analogues may be their greater lipophilic character. The importance of lipophilic character in determining pharmacological activity amongst compounds with the requisite chemical groupings has been well documented (Sexton, 1963; Whitehouse, 1964; Parker, 1965). As far as is known, 25-oxygenated derivatives of cholesterol are not involved in steroid hormone metabolism and thus these compounds might be effective tools for the further investigation of pregnenolone feed-back inhibition, or even might form the basis of useful drugs for the suppression of total steroid hormone production.

The relationship of cholesterol oxidation *in vitro* to steroid hormone production *in vivo*

As the cholesterol oxidase of bovine adrenal cortex has been examined in some detail *in vitro* it is of interest to compare the measured rates of cholesterol oxidation with a cow's daily corticosteroid production. Although there seem to be no direct measurements of the

adrenocortical output of the cow, probably because of the magnitude of the practical difficulties, it is possible to make an order of magnitude estimate that a fully grown cow such as were the source of the adrenals used in this work, produces of the order of 100 mg. of corticosteroids per day. This estimation is very approximate and is based on the corticosteroid production rate in a number of domestic animals (dogs, cats, rodents and sheep) and in man, which is in the range 120 - 1200 µg/Kg. body weight/day (Dorfman & Ungar, 1955). Thus, for a cow weighing about 500 Kg. the range of secretion rate would be 60 - 600 mg./day, i.e. of the order of 100 mg./day.

In vitro, and using sonicated mitochondria prepared from cow adrenal cortex, the rate of oxidation of added cholesterol was 2.62 mµ-mole/hr./ml. (table 5). Although the cholesterol content of this particular preparation was not measured, in a large number of experiments performed over the course of two years, the cholesterol content of mitochondrial preparations, prepared in the standard way described in the experimental section, lay in the range 100 - 300 µg/ml. (table 10a). Thus assuming that all the cholesterol in the preparation was oxidised at the same rate as the added cholesterol, the total rate of cholesterol oxidation in this experiment was 510 - 1700 mµmoles/hr./incubation, or 425 - 1420 mµmoles/hr./g. of cortex. For a cow with two adrenal glands, each with a cortex weighing about 5 g., the total cholesterol oxidation rate in the adrenal cortex would be 100 - 340 µmoles/day. For corticosteroids with a molecular weight of about 350, this represents a

daily corticosteroid production of 35 - 120 mg. These very approximate calculations suggest that the cholesterol oxidation observed *in vitro* was of the same order as that required to account for corticosteroid production *in vivo*.

The intramitochondrial site of adrenocortical cholesterol oxidase

The increase of cholesterol oxidase activity that was observed when the mitochondria were sonicated (table 5) suggests that some of the enzyme in the intact mitochondria was not available to the added cholesterol. Equally, though, the activity of the unsonicated preparations shows that some of the enzyme was available to added substrate. Although the mitochondria were prepared under fairly mild, hypertonic conditions, it seems likely that there were some damaged structures in the preparations which could account for this. It is suggested, therefore, that cholesterol oxidase is primarily located within the mitochondrion, rather than on the outer membrane. Such a location would also explain the lack of effect as a cofactor of added NADPH (table 2) which would not be able to penetrate intact mitochondria and reach the enzyme.

The apparently reduced efficacy as a substrate of cholesterol absorbed on the mitochondria (table 9) can also be explained in terms of an intramitochondrial location for cholesterol oxidase. Cholesterol is known to be bound to proteins and lipoproteins (Westphal, 1962) and to exchange with the cholesterol in cell and mitochondrial membranes

(Hagerman & Gould, 1951; Graham & Green, 1967). The bound cholesterol in the experiments of table 9, i.e. that sedimentable with the mitochondria, was probably bound to the outer membrane of the mitochondria and this cholesterol appeared to be less readily converted to steroid hormones than the cholesterol remaining in solution, suggesting that the enzyme cholesterol oxidase was not in the outer membrane but inside.

The Krebs-cycle enzymes which, it has been suggested, may be concerned in supplying the NADPH required for cholesterol oxidation, appear to be located in the matrix of the mitochondrion (Lehninger, 1962). Moreover, in contrast to the classical electron-transport chain that appears to be very firmly attached to the mitochondrial membranes (Lehninger, 1962), the P_{450} electron-transport chain, which is believed to oxidise the NADPH used for cholesterol oxidation (see Introduction, page 10), is released from adrenal cortex mitochondria on sonication (Simpson & Boyd, 1966). On present evidence therefore, it seems reasonable to propose that the cholesterol oxidase is situated within the outer membrane of the mitochondrion.

If this hypothesis is true, then it may be asked how the substrate cholesterol arrives at this site. The adrenal cortex is rich in cholesterol (table 10a) and in cholesterol esters which can be hydrolysed to free cholesterol as has been shown already, and it can also synthesise cholesterol, (Girner, Dvornik & Gosselin, 1964; Billiar, Oriol-Bosch & Eik-Nes, 1965). The substrate cholesterol may also be carried to the adrenal intramitochondrial site of cholesterol oxidation from the blood-

stream and it may be that specific transport of cholesterol across sub-cellular barriers is necessary. If such transport occurs then a protein or lipoprotein seem likely candidates for the role of carrier. Farese (1967) has shown that a protein-like substance is synthesised in the adrenal cortex after stimulation by ACTH and that this effect is mediated by 3',5'-cyclic AMP. The protein-like substance was able to stimulate cholesterol oxidation in mitochondrial preparations from adrenal cortex which had not been treated with ACTH and so it could be that Farese's factor is the specific substance that carries cholesterol to the site of its oxidation within the mitochondrion. Similar considerations must also apply to the products of cholesterol oxidation which are eventually released into the blood-stream and Farese's factor could equally be that concerned in transport of hormones from the site of production to their next destination. The interesting thing about Farese's factor is that, in contrast to the protein effects described above, it was specifically induced by ACTH. If no specific carrier exists and all or most of the cell cholesterol is available for oxidation to steroid hormones, then regulation by feed-back inhibition by the products would be even more important.

ABSTRACT

Sterol Metabolism in Extra-hepatic Tissues

It is currently believed that cholesterol is the principal precursor of steroid hormones in endocrine organs and that it is converted to hormones *via* pregnenolone. The cholesterol molecule is first hydroxylated at C-20, and then at C-22 to yield 20α,22ξ-dihydroxy-cholesterol and this is then cleaved between C-20 and C-22 to yield pregnenolone and isocaproic acid.

The enzyme system catalysing this series of reactions (cholesterol oxidase, E.C. 1.1.3.6) has been investigated in some detail using tissue preparations of bovine adrenal cortex and human term placenta. Cholesterol oxidase in both the adrenal cortex and the placenta is associated with the mitochondria but a method was devised for bringing it into solution by means of sonication. Mitochondria, soluble preparations of mitochondria and soluble, steroid-free extracts of acetone-dried preparations have been used as the source of the enzyme in these experiments. A reliable method of assaying cholesterol oxidase by measuring the [5-^{14}C]-isocaproic acid produced from [26-^{14}C]-cholesterol was devised and thin-layer chromatographic methods were developed for the separation and identification of the steroid products. An apparatus was devised for the quantitative recovery of the steroids from the thin-layer chromatograms,

and this was also utilised in a method of extraction, separation and assay of the endogenous cholesterol, cholesteryl esters and pregnenolone in adrenal cortex subcellular fractions. The method was capable of detecting 7 μg of cholesterol, 16 μg of cholesteryl ester and 2 μg of pregnenolone, using pure samples of the steroids.

In the course of this investigation the following compounds were prepared:- sodium cholesteryl-3β-sulphate, pyridinium cholesteryl-3β-sulphate, sodium [4-^{14}C]-cholesteryl-3β-sulphate, sodium [26-^{14}C]-cholesteryl-3β-sulphate, sodium pregnenolone-3β-sulphate, sodium 25-oxo-27-nor-cholesteryl-3β-sulphate, [4-^{14}C]-cholesteryl-3β-acetate, [26-^{14}C]-cholesteryl-3β-acetate, [26-^{14}C]-cholest-4-ene-3-one, 27-nor-cholest-4-ene-3,25-dione, dihydrogen cholesteryl-3β-phosphate, diphenyl cholesteryl-3β-phosphate, cholesteryl-3β-chloride, pregnenolone-3β-palmitate, 20α-hydroxy-cholesterol, 20α-hydroxy-cholesteryl-3β-acetate and pregnenolone-3β-tetrapyranyl ether.

Evidence was obtained that NADPH is required for cholesterol oxidation in adrenal cortex mitochondria and in preparations derived from the mitochondria and that in mitochondria, this may arise by the action of transhydrogenase (E.C. 1.6.1.1) on the NADH formed from Krebs-cycle intermediates. However, adrenal cortex mitochondria as prepared in this work were shown to contain NADP-linked malic enzyme (E.C. 1.1.1.40) and NADP-linked glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49); in most tissues these enzymes are found mainly in the cytoplasm but if in the adrenal cortex they are associated with the mitochondria, as the

results suggest, then they also could give rise to mitochondrial NADPH. The glucose-6-phosphate dehydrogenase of adrenal cortex (but not of yeast) was shown to be inhibited by pregnenolone but not by cholesterol. However, by comparison of the magnitude of this inhibition and the measured pregnenolone content of the adrenal cortex preparations, it was concluded that pregnenolone was unlikely to be a physiological regulator of cholesterol oxidase through its effect on glucose-6-phosphate dehydrogenase and the NADPH supply.

Cholesterol was dispersed in aqueous incubation media using N,N-dimethyl formamide and the cholesterol dispersed in this way was found to be absorbed by the mitochondria. This absorption was partly reversible and the amount absorbed increased with the concentration of the added cholesterol. Absorbed cholesterol appeared to be less readily converted into steroid hormones than free cholesterol.

The principal steroid product of cholesterol oxidation by adrenal cortex mitochondria, and by extracts made from mitochondria, was pregnenolone, but some progesterone was also formed, especially in experiments with whole mitochondria. The pregnenolone formed was partly retained in the mitochondria and partly released into the extra-particulate fluid; it was not taken up by the microsomes although the enzymes which convert pregnenolone into progesterone are known to be microsomal.

The C_6-product of cholesterol oxidation by adrenocortical preparations was isocaproic acid. This isocaproic acid was oxidised to carbon dioxide by the mitochondria under the conditions used but to such

a slight extent that it did not affect the use of the production of isocaproic acid as a measure of cholesterol oxidase activity.

The substrate specificity of cholesterol oxidase was investigated: cholesteryl-3β-sulphate, cholest-4-ene-3-one, cholesteryl-3β-acetate and cholesteryl-3β-linolenate were compared with cholesterol and were found to be less readily oxidised. Evidence was obtained that cholesteryl fatty acyl esters are not oxidised directly to pregnenolone esters but are first hydrolysed to free cholesterol by an esterase and subsequently oxidised to pregnenolone. It is suggested that cholesteryl fatty acyl esters form a reserve of cholesterol which can be metabolised, after hydrolysis, to steroid hormones.

Cholesteryl-3β-sulphate was oxidised directly to pregnenolone-3β-sulphate under the experimental conditions employed and no free pregnenolone or other steroids were formed. It is suggested that oxidation of cholesteryl-3β-sulphate forms part of an alternative pathway of steroid hormone biosynthesis in adrenal cortex, but no evidence was obtained that sulphurylation or phosphorylation were obligatory steps in cholesterol oxidation.

Cholesteryl-3β-sulphate and free cholesterol were compared as substrates for cholesterol oxidase. Kinetic studies in a variety of tissue preparations indicated that free cholesterol was the preferred substrate with a K_m of 1 - 4 μM whereas the K_m for cholesteryl-3β-sulphate was about 500 μM.

Cholesterol sulphatase was detected in adrenal cortex and was found to be microsomal. It was inhibited by inorganic phosphate and

therefore phosphate was used in experiments designed to measure cholesteryl-3β-sulphate oxidation.

The inhibitor specificity of cholesterol oxidase was investigated. Cholesteryl-3β-esters (phosphate, sulphate, acetate, oleate and linolenate) inhibited cholesterol oxidation competitively but it is suggested that inhibition by fatty acyl esters was due to production of free cholesterol by esterase activity. The products of cholesterol oxidation, pregnenolone and 20α-hydroxy-cholesterol inhibited cholesterol oxidation non-competitively. The K_i for pregnenolone was 80 μM and for 20α-hydroxy-cholesterol 10 μM. Evidence was obtained that feed-back inhibition by pregnenolone may be a physiological mechanism for the control of cholesterol oxidation and steroid hormone formation and that this effect is exerted directly on the enzyme cholesterol oxidase.

A number of other steroids inhibited cholesterol oxidation and among these was 25-oxo-27-nor-cholesterol, a synthetic steroid which was more potent than pregnenolone (K_i 16 μM, non-competitive). These results suggest that a 3β-hydroxyl group as well as an oxygen function in the side chain are important structural characteristics of an inhibitor of cholesterol oxidase. Cholesterol oxidase was also inhibited by Su 4885 (2-methyl,1,2-di-(pyrid-3-yl)-propan-1-one), a synthetic hydroxylation inhibitor.

When cholesterol oxidation was inhibited by pregnenolone, 20α-hydroxy-cholesterol, 25-oxo-27-nor-cholesterol or Su 4885, no trace of accumulated intermediates was detected. This supports the theory that

20α-hydroxylation is the first and rate-limiting step of cholesterol oxidation.

Certain steroid carboxylic acids (3β-hydroxy-chol-5-enoic acid, 3α-hydroxy-chol-5-enoic acid and 3β-hydroxy-22,23-bisnor-chol-5-enoic acid) stimulated cholesterol oxidation but 3β-hydroxy-androst-5-ene-17α-carboxylic acid and 3β-acetoxy-22,23-bisnor-chol-5-enoic acid did not.

The oxidation of cholesteryl-3β-sulphate by adrenal cortex mitochondria was inhibited by pregnenolone, 20α-hydroxy-cholesterol and 25-oxo-27-nor-cholesterol but not by pregnenolone-3β-sulphate or 25-oxo-27-nor-cholesteryl-3β-sulphate. This similarity to the inhibitor specificity of cholesterol oxidation suggests that there is only one enzyme system oxidising both cholesterol and cholesteryl-3β-sulphate. This theory is supported by the similarity between the maximum rates of oxidation of cholesterol and cholesteryl-3β-sulphate by soluble, steroid-free cholesterol oxidase.

In view of the necessity for pregnenolone made in the mitochondria to be transported to the microsomes for conversion to progesterone, it seemed possible that microsomes might increase the rate of cholesterol oxidation by mitochondrial preparations by reducing the feed-back inhibition. However microsomes were found to inhibit cholesterol oxidation by mitochondria and this effect was additive to that produced by pregnenolone. Heat-denatured microsomes were more effective than fresh microsomes for this and so it is suggested that the results can

best be explained by the pregnenolone in the microsomes inhibiting cholesterol oxidation by the feed-back mechanism or by the cholesterol in the microsomes diluting the added labelled cholesterol.

Evidence is presented which suggests that cholesterol oxidase is located within the outer membrane of the mitochondrion. Cholesterol oxidation was also investigated in human term placenta. The experiments were directed towards discovering whether placenta contained cholesterol oxidase and if so whether it was similar to that of adrenal cortex. The ability of placental preparations to oxidise cholesterol was demonstrated and the cholesterol oxidase was shown to be mitochondrial and to require NADPH. However, unlike adrenal cortex mitochondria, placental mitochondria did not appear to possess the ability to produce sufficient NADPH to support cholesterol oxidation and it was necessary to add a NADPH-generating system. If theseresults reflect the capability of the placenta *in vivo* to generate NADPH for cholesterol oxidation then the supply of NADPH may be regulatory in placenta, unlike the adrenal cortex.

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