X

THE OESTRADIOL DEHYDROGENASES OF AVIAN LIVER

A Thesis submitted for the Degree of

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ERRATA.

Page 58. Table 8. Row 5. Headed "Oestrone mg./tube". The figure should read 0.0391 not 0.391.

Page 109. Line 4. "In" instead of "On".

Appendix C. Headed "Dialysis". Bottom line on page - there should be no hyphen in deionised.

Acknowledgments. Line 10. "American Society, Inc." should read "American Cancer Society, Inc."

INTRODUCTION

Sterols and steroids are relatively complex molecules which contain a number of asymmetric centres; numerous species are able to synthesise such molecules and transform them to a wide variety of related compounds. Thus many of the enzymes concerned in steroid hormone metabolism offer unique opportunities as model systems for the study of substrate specificity.

Although steroid hormones share certain basic structural characters the possession of specific conformations and configurations provides a physico-chemical basis for the observed differences in physiological action. In this discussion only certain aspects of the enzymic transformations of 17α-oestradiol, 17β-oestradiol and oestrone will be considered. These phenolic steroids are closely related by simple oxidation-reduction reactions, viz.

17α-oestradiol $\rightleftharpoons$ oestrone $\rightleftharpoons$ 17β-oestradiol

The isolation and partial purification of a human placental 17β-oestradiol dehydrogenase by Langer and Engel (1958) and subsequent kinetic studies by Langer, Alexander and Engel (1959) showed that this enzyme

1. had an absolute steric requirement for the 17β-hydroxyl group
2. required that the steroid substrate must possess a highly planar ring A or B or both for significant reactivity

3. interacted with the entire steroid surface.

These findings were largely confirmed by Adams, Jarabak and Talalay (1962) using enzyme preparations of much higher specific activity.

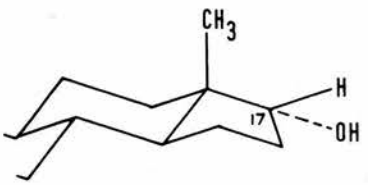
It was therefore of interest to extend these observations using a 17α-oestradiol dehydrogenase, thereby providing a more complete system for the investigation of effects of substrate structure upon reaction kinetics. Such a model would also facilitate the experimental approach to studies of the mechanism(s) of these enzyme reactions.

There was no published report of a purified preparation of a 17α-oestradiol dehydrogenase when this work was undertaken in October, 1962. Thus it was necessary to find a source of that enzyme; tissues of those animals known to metabolise or to excrete free or conjugated 17α-oestradiol seemed the most suitable starting material.

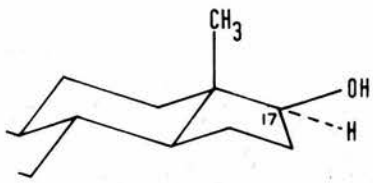
Although the configuration of the hydroxyl group at carbon-17 was accurately inferred by Fieser and Fieser (1949) it was not established until 1950 (Gallagher and Kritchevsky). There was much confusion in the literature prior to these dates and in several later publications. The correct designation is used throughout this review; where early authors assigned the α-configuration to the β-epimer (and vice versa), this has

Fig. 1

THE CONFIGURATION OF THE HYDROXYL GROUP AT CARBON 17



"17 α - OESTRADIOL"
OESTRA - 1,3,5 (10) - TRIENE-3, 17α - DIOL
m.p. 223°
$[\alpha]_{589\,m\mu}^{18°}$ = + 53·5 (C 0·9 ALCOHOL)



"17 β - OESTRADIOL"
OESTRA - 1,3,5 (10) - TRIENE-3, 17 β - DIOL
m.p. 178°
$[\alpha]_{589\,m\mu}^{20-25°}$ = + 79 ± 2 (C 0·7 ALCOHOL)

ONLY THE C AND D RINGS ARE DEPICTED IN THIS PROJECTION

been changed in the interests of uniformity.

'17α-Oestradiol' will be used to denote that epimer with the higher melting-point (m.p.) and the less positive optical rotation in which the hydroxyl group at carbon-17 is trans to the methyl group at carbon-13. Fig. 1.

The literature relevant to the metabolism of 17α-oestradiol will be reviewed under five headings:-

I. Animals which metabolise or excrete free or conjugated 17α-oestradiol

II. Studies with animal tissues in vitro

III. The histochemical demonstration of 17α-hydroxysteroid dehydrogenases

IV. Other 17-hydroxysteroid dehydrogenases

V. The mechanism of action of the 17-hydroxysteroid dehydrogenases

I. Animals which metabolise or excrete free or conjugated 17α-oestradiol

The horse

The first isolation of 17α-oestradiol was reported briefly by Wintersteiner and Hirschmann (1937) and more fully by Hirschmann and Wintersteiner (1938). They used the non-ketonic phenolic fraction of pregnant mares' urine as starting material. Twenty-three years later Pigoń, Lunaas and Velle (1961) demonstrated the presence of 17α-oestradiol in stallion urine. Quantitative chemical determinations of urinary oestrogens in thirty-six stallions from 7 - 37 months of age showed that only insignificant amounts were excreted during the first two years of life. At that time there was a marked increase

in the output of oestrone, 17β-, and 17α-oestradiol. Only trace amounts of oestrogens were found in the urine of ten fully-grown geldings.

Ruminants

Unconjugated 17α-oestradiol has also been found in ruminants. Klyne and Wright (1956, 1957) showed that this steroid was excreted in the urine of the pregnant cow and of the pregnant goat. Velle (1958a) found that the main phenolic steroids in pregnant cows' urine were oestrone and 17α-oestradiol. The urine of the newborn calf was also shown to contain the α-epimer (Velle, 1958b) and the partial metabolic transformation of 17α-oestradiol to oestrone was demonstrated in the young calf of both sexes (Velle, 1958c).

Further isolations of 17α-oestradiol have been made from bovine meconium (Velle, 1957), from ovine and caprine meconium (Velle, 1958d), from equine meconium (Velle and Pigon, 1960) and from bovine placental extracts along with 17β-oestradiol and oestrone (Gorski and Erb, 1959). 17α-Oestradiol has been found in the amniotic fluid of the cow (Rommel, 1964) and most recently Adlercreutz and Luukkainen (1965) described the presence of the free steroid in the bile of the pregnant cow.

The dog

According to Siegel, Dorfman, Brodey and Friedman (1962), 17α-oestradiol is a major urinary oestrogen in the mature adult dog where the bulk of phenolic steroids appears to be conjugated with glucuronic acid. Metzler, Eleftheriou and Fox (1966) have also reported the presence of unconjugated 17α-oestradiol in the plasma of pedigree bitches of various breeds.

The rabbit

Stroud (1939) reported the isolation of 17α-oestradiol from the urine of rabbits which had been given oestrone. His criteria of identification were m.p. 216°, elementary analysis, and the demonstration of biological activity which was one seventh of that of oestrone in ovariectomised rats. Heard, Bauld and Hoffman (1941) recovered 2.6% oestrone and 12.1% 17α-oestradiol from the urine of the intact oestrous rabbit after giving 300 mg. 17β-oestradiol. The simultaneous administration of progesterone and 17β-oestradiol or oestrone to the intact oestrous rabbit and to the hysterectomised-ovariectomised animal did not alter the excretory pattern. In all cases the recovery of 17α-oestradiol was four to five times as much as that of oestrone. Fish and Dorfman (1942) also found 17α-oestradiol in the urine of

hysterectomised-ovariectomised rabbits, and the reduction of oestrone to 17α-oestradiol in the rabbit has been described by Pearlman and Pearlman (1944).

The monkey

The demonstration of 17α-oestradiol as a major oestrogen in rabbit urine prompted Doisy, Thayer and Van Bruggen (1942) to investigate the excretion of that compound following the administration of 227 mg. 17β-oestradiol to the ovariectomised-hysterectomised monkey. Using a bioassay, only 0.3% of the injected dose was recovered in the urine.

Man

Heard (1944) stated that he had been unsuccessful in attempts to find 17α-oestradiol in human urine and this has been confirmed by others (Watson and Marrian, 1958). According to Schott and Katzman (1964) "Failure to detect 17α-oestradiol in human urine may be due to inadequacy of the methods employed and/or to its rapid metabolic alteration". They looked for 17α-oestradiol in pools of human late pregnancy urine using mild acid hydrolysis, chromatography on silica gel and dilute iron-Kober Reagent B. In one individual, in urine samples subjected to mild hydrolysis, Schott and Katzman found a compound of chromatographic mobility similar to that of 17α-oestradiol in the $CHCl_3$-formamide system. The conditions for hydrolysis appear to be important because

(Velle, 1958b) noted extensive destruction of 17α-oestradiol when Brown's (1955) hydrolytic procedure was used. Schott and Katzman also confirmed Haenni's (1950) observations with regard to the specificity of iron-Kober Reagent B for 17α-oestradiol.

Following the administration of 16α-hydroxyoestrone and 16β-hydroxyoestrone diacetate to a normal man on separate occasions, Nocke, Breuer and Knuppen (1961) first isolated oestriol, 17-epioestriol and 16-epioestriol, then 16-epioestriol, 16,17-epioestriol and oestriol. Their findings confirmed previous incubation experiments with human liver slices (Breuer and Nocke, 1958) and it was concluded that these results showed that a 17α-hydroxy-steroid dehydrogenase was present in man, in addition to an enzyme specific for the 17β-configuration.

The possible importance of 17α-oestradiol as a major metabolite in man is suggested by the isolation of oestra-1,3,5,(10),11-tetraene-3,17α-diol (11-dehydro-17α-oestradiol) from the urine of pregnant women (Luukkainen and Adlercreutz, 1965). This compound was found in the oestradiol-3-methyl ether fraction of Brown (1955) and it was present in amounts equal to or higher than those of 17β-oestradiol.

II. Studies with animal tissues *in vitro*

In the discussion of results from incubation and histochemical studies the assertion is not infrequently made by some authors that e.g. the oxidation of 17α-oestradiol to oestrone is catalysed by a specific 17α-oestradiol dehydrogenase. Such statements are misleading. Experiments with crude homogenates and tissue slices give little information about the specificity of the reactions concerned, and even in the case of some highly purified enzymes (e.g. horse liver alcohol dehydrogenase) the substrate specificities have not yet been established.

Rabbit liver

When a steroid hormone is incubated *in vitro* with a liver preparation a large number of metabolic transformations is possible. These include oxidation-reductions and conjugations (often loosely termed 'detoxication' reactions).

Breuer and Pangels (1960) studied the metabolism of oestrone and both epimers of oestradiol in rabbit liver slices. They found that incubation of oestrone at 37° for 60 min. at pH 7.4 gave 5 - 8 times more 17β- than 17α-oestradiol.

Recent work by Layne and his colleagues at the Worcester Foundation has stimulated further interest in the mechanisms of conjugation of steroid hormones. Jirku and Layne (1965) showed that when [6,7-$^3H_2$] 17β-oestradiol was incubated with rabbit liver homogenates and uridine diphosphate N-acetylglucosamine in the presence of uridine diphosphate glucosiduronic acid, the product was oestradiol-3-glucuronoside-17α-N-acetyl-glucosaminide. This double conjugate was formed in amounts equivalent to about 10% of the total radioactivity. No evidence was found for the transfer of N-acetyl-glucosamine to the 17β-hydroxyl group of oestradiol and there were good indications that conjugation at carbon-3 was necessary before coupling at carbon-17 occurred.

Avian liver

The presence of a 17α-oestradiol dehydrogenase in ammonium sulphate fractions of chicken liver homogenates has been described by Ozon and Breuer (1965). These results will be examined later in this thesis.

Mammalian erythrocytes

Repke and his collaborators have studied the inter-conversion of oestrone and both epimers of oestradiol by the red blood cells of various animal species. In a brief communication, Portius and Repke (1960a) reported the oxidation of 17α-oestradiol to oestrone by

erythrocytes of cattle, sheep and goats. The highest activity of '17α-oestradiol dehydrogenase' was found in bovine red cells. The authors also commented upon the absence of this enzyme from red blood cells of the rabbit and dog, in view of the fact that these animals excrete 17α-oestradiol after the administration of oestrone or 17β-oestradiol.

Lunaas and Velle (1960) also studied the metabolism of oestrone and 17β-oestradiol with washed erythrocytes from mature, non-pregnant females of eleven mammalian species. They found that when oestrone was incubated with bovine red cells the major product was 17α-oestradiol; the β-epimer was present only in trace amounts. The oxidation of 17α-oestradiol to oestrone by bovine red cells was also observed.

Experiments with bovine erythrocytes have also been reported by Axelrod and Werthessen (1960) who showed that 17α-oestradiol was the major product when [16-^{14}C]oestrone was incubated with blood from the pregnant cow. This conversion did not occur with bull's blood or that of the steer.

Bovine cells in tissue culture

The interconversions of the oestradiol epimers and oestrone have been investigated in tissue culture. The first report by Velle and Erichsen (1960) described the formation of oestrone from 17β-oestradiol in bovine

kidney cells; yields from 17α-oestradiol were very much less. Further incubations with cells in sheet culture from bovine amnion, liver and endometrium, and from the testes of young calves and bulls all showed trace amounts of oestrone formed from 17α-oestradiol (Erichsen and Velle, 1960).

III. The histochemical demonstration of 17α-hydroxysteroid dehydrogenases

Baillie, Calman, Ferguson and Hart (1966) claimed to have demonstrated an 'NAD^+-specific 17α-hydroxysteroid dehydrogenase' in human testicular biopsy material using histochemical methods. The activity (described as 'poor') was localised in the Leydig cells when 17α-hydroxy-androst-4-en -3-one and 17α-hydroxy-pregn-4-ene-3,20-dione were used as substrates. The choice of the latter tertiary alcohol as substrate is curious because chemical oxidation of the hydroxyl group at C-17 would necessitate breaking a carbon-carbon bond. This reaction cannot be catalysed by a hydroxysteroid dehydrogenase.

IV. Other 17-hydroxysteroid dehydrogenases

It has been known for many years that yeasts can effect the reduction of oestrone to 17β-oestradiol (e.g. Mamoli, 1938; Wettstein, 1939) and the existence of 17β-hydroxysteroid dehydrogenases has been inferred from *in vitro* experiments with rat and rabbit liver preparations (e.g. Heller, 1940; De Meio, Rakoff, Cantarow and Paschkis, 1948; Coppedge, Segaloff, Sarrett and Altschul, 1948; Pearlman and De Meio, 1949). Similar conclusions have been drawn from *in vivo* experiments in man (e.g. Heard and Hoffman, 1941; Pearlman and Pincus, 1943).

Sweat, Samuels and Lumry (1950) reported the partial purification and characterisation of a 17β-hydroxysteroid dehydrogenase from steer liver which oxidised testosterone to androstenedione, and the results of incubation studies with a wide variety of normal and diseased human digestive, reproductive and endocrine tissues (Ryan and Engel, 1953) stimulated further interest in the enzymic transformations of steroids.

The reduction of oestrone to 17β-oestradiol by human and rabbit erythrocytes was described by Gray and Bischoff (1955) and Markwardt and Repke (1955) showed that reduction of oestrone to 17β-oestradiol by guinea-pig

erythrocytes was linked to the oxidation of glucose-6-phosphate to 6-phosphogluconic acid by glucose-6-phosphate dehydrogenase in the presence of $NADP^+$. Repke and Markwardt (1959) also found that human red cells and those of the rabbit and guinea-pig reduced oestrone to 17β-oestradiol. In their studies of oestrogen metabolism in washed red cell suspensions from eleven non-pregnant female mammalian species, Lunaas and Velle (1960) showed that 17β-oestradiol was partly metabolised to oestrone in man, monkey, horse, sheep, goat, pig, dog, rabbit, guinea-pig and rat. No metabolite was detected in incubations with rabbit red cells. Oestrone was partly transformed to 17β-oestradiol in all species examined except the bovine where the product was 17α-oestradiol.

Portius and Repke (1960a, 1960b) observed that erythrocytes of rat, golden hamster, guinea-pig, rabbit, pigeon, hen, goat, sheep, cow, pig, cat, dog, frog and human were all able to oxidise 17β-oestradiol to oestrone. No details of age, sex or reproductive state of these animals were given and it is possible that a seasonal variation may have accounted for the difference between Portius and Repke's experiments with bovine red cells and those of Lunaas and Velle.

Other transformations which are probably catalysed by 17β-hydroxysteroid dehydrogenases have been demonstrated in bovine cells in tissue culture (e.g. Erichsen and Velle, 1960) and in incubations of fish and amphibian liver (Breuer, Ozon and Mittermayer, 1963) a '17β-oestradiol dehydrogenase' has also been found in association with human and canine spermatozoa (Hathaway and West, 1964). However, few attempts have been made to purify any of the activities cited.

One of the first hydroxysteroid dehydrogenases to be investigated was that induced in Pseudomonas testosteroni by Talalay, Dobson and Tapley (1952). This enzyme which catalyses the reversible oxidation of 3β-hydroxyl groups of C_{19} and C_{21} steroids and which also interconverts 17-keto and 17-hydroxyl groups of C_{18} and C_{19} steroids has been shown to behave as a homogeneous protein of molecular weight, 100,000 (Squire, Delin and Porath, 1964).

Kochakian and his colleagues have provided much information on the 17β-hydroxysteroid (testosterone) dehydrogenases of guinea-pig liver and kidney. These tissues have each been shown to contain two testosterone dehydrogenases, one of which is NAD^+-specific and microsomal, the other is soluble and requires $NADP^+$ as cofactor (Endahl, Kochakian and Endahl, 1958). The activity

of the renal enzymes but not those of the liver appears to be regulated by testosterone (Kochakian and Endahl, 1960). The separation of the guinea-pig liver enzymes by differential centrifugation has been reported by Endahl, Kochakian and Hamm (1960) and confirmed by Villee and Spencer (1960). Experiments with liver and kidney of guinea-pig, rabbit, mouse, rat, hamster and dog revealed marked species and tissue differences between the NAD^+- and $NADP^+$-specific testosterone dehydrogenases (Aoshima and Kochakian, 1963).

The soluble $NADP^+$-dependent 17β-hydroxysteroid (testosterone) dehydrogenase of guinea-pig liver has been purified some two-hundred fold by Joshi, Duncan and Engel (1963). The purified preparations also oxidised saturated C_{19}-3β-hydroxysteroids of the 5α series and saturated C_{19}-17β-hydroxysteroids, and the substrate specificity studies indicated that planarity of the molecule favoured increased reactivity.

Langer and Engel (1958) described the partial purification of a soluble 17β-oestradiol dehydrogenase from human placenta and this enzyme has been extensively purified by Jarabak, Adams, Williams-Ashman and Talalay (1962). Recent work by Purdy, Halla and Little (1964) showed that preparations of this 17β-oestradiol dehydrogenase (of high specific activity) were also able

to reduce progesterone to 20α-hydroxy-4-ene-pregnen-3-one but activity towards this substrate was low.

A twelve-fold purification of an NAD(P)-linked 17β-hydroxysteroid oxido-reductase from rat kidney has been reported by Breuer and Dahm (1964a). These workers have also published a ten-fold purification of a 17β-hydroxysteroid dehydrogenase from normal and hyperplastic human adrenals (Breuer and Dahm, 1964b).

V. The mechanism of action of the hydroxysteroid dehydrogenases

The mechanism(s) of action of the hydroxysteroid dehydrogenases is unknown but information is available concerning the stereospecificity of the reactions which they catalyse. This knowledge is largely derived from the pioneering studies of Vennesland, Westheimer, and their colleagues who showed that dehydrogenases exhibit a dual stereospecificity in respect of a) substrate and b) cofactor. (It follows that these enzymes must also exhibit product stereospecificity).

In this connection yeast alcohol and lactic dehydrogenases were first investigated (Fisher, Conn, Vennesland and Westheimer, 1953; Loewus, Ofner, Fisher, Westheimer and Vennesland, 1953) and it was concluded that these enzymes catalysed a direct stereospecific transfer of hydrogen between substrate and pyridine ring. The site of reduction of NAD^+ was later shown to be position 4 (Pullman, San Pietro and Colowick, 1954). Subsequent observations in mechanistic studies by Mauzerall and Westheimer (1955) and by Loewus, Vennesland and Harris (1955) established that a 1,4-reduction of the nicotinamide ring of the coenzyme occurs in dehydrogenase reactions.

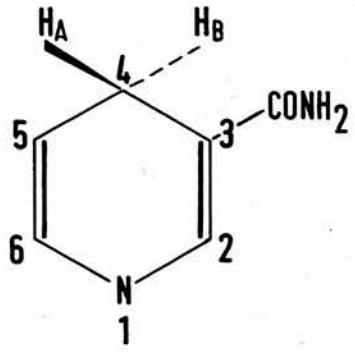
By the use of deuterium, Levy and Vennesland (1957) distinguished two groups of dehydrogenases which transferred the isotope to one or other of the diastereomeric positions of NADH. Those enzymes which had the same coenzyme stereospecificity as yeast alcohol dehydrogenase were said to have 'α-stereospecificity'. Those which used the other position were designated 'β-stereospecific'.

Later investigations have shown that this classification holds for $NADP^+$-linked dehydrogenases (Levy, Talalay and Vennesland, 1962). In this paper the hydrogen atoms attached to position 4 of the reduced nicotinamide ring of the pyridine nucleotides were designated H_A and H_B. H_A dehydrogenases were formerly known as 'α or side 1' enzymes, H_B dehydrogenases were termed 'β or side 2'. This nomenclature was arbitrarily assigned without knowledge of the absolute stereochemistry of the atoms concerned (see Fig. 2).

In the course of their studies on cholesterol biosynthesis the absolute stereochemistry of the hydrogens attached to the meso-carbon atom at position 4 of the dihydronicotinamide ring of the pyridine nucleotide was reported in a preliminary form by Cornforth, Ryback, Popják, Donninger and Schroepfer (1962) and more fully by Cornforth, Cornforth, Donninger, Popják, Ryback and Schroepfer (1966). These workers formulated a general

Fig. 2.

THE STERIC POSITIONS OF H_A AND H_B OF THE DIHYDRONICOTINAMIDE RING OF NADH OR NADPH



(After Cornforth et al. 1966)

rule applicable to all NAD^+- and $NADP^+$-linked dehydrogenases; it states that:-

"All 'A' specific dehydrogenases add hydrogen to that side of the nicotinamide ring in which the numbering of the positions from 1 - 6 appears in an anti-clockwise order. It is the consequence of the steric position of H_A and H_B at position 4 of the dihydronicotinamide ring of NADH or NADPH that when H_A is replaced by a heavy isotope the absolute configuration at position 4 is R and when H_B is similarly replaced the configuration at this position is S". The definition of absolute configuration in terms of 'R' and 'S' is that introduced by Cahn, Ingold and Prelog (1956).

The 17β-hydroxysteroid dehydrogenase of Pseudomonas testosteroni was one of the first enzymes found to transfer hydrogen to the 'B' side of the dihydronicotinamide ring of NADH (Talalay, Loewus and Vennesland, 1955). By incorporating tritium into position 4 of the nicotinamide ring Jarabak and Talalay (1960) showed that the NAD^+-linked 3α- and 3β-hydroxysteroid dehydrogenases of Pseudomonas testosteroni also use side 'B' in the following reactions:-

$$\text{Androsterone} + NAD^+ \longrightarrow \text{androstane-3,17-dione} + NADH + H^+$$

$$\text{Epiandrosterone} + NAD^+ \longrightarrow \text{androstane-3,17-dione} + NADH + H^+$$

The 17β-hydroxysteroid dehydrogenase of human placenta was found to transfer hydrogen to side 'B' when NAD^+ or $NADP^+$ was used as cofactor. The transhydrogenase activity associated with this enzyme was also shown to use side 'B' in the following reaction:-

$$NADH + AcPyAD \longrightarrow NAD^+ + AcPyADH$$

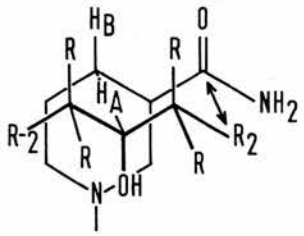
Previously Talalay and Williams-Ashman (1958) and Talalay, Hurlock and Williams-Ashman (1958) had shown that 17β-oestradiol mediated a reversible enzymic transfer of hydrogen between the oxidised and reduced forms of nicotinamide-adenine-dinucleotide and nicotinamide-adenine-dinucleotide phosphate. It was claimed that this activity was catalysed by a single protein from human placenta which also possessed 17β-hydroxysteroid dehydrogenase activity. Conflicting evidence was later presented by Hagerman and Villee (1959) and Villee, Hagerman and Joel (1960). Starting with human placenta these workers separated a 'transhydrogenase' from two 17β-hydroxysteroid dehydrogenases which were respectively NAD^+- and $NADP^+$-specific. The 'transhydrogenase' was free of dehydrogenase activity. The controversy has yet to be resolved. Jarabak et al. (1962) found no alteration in the transhydrogenase to dehydrogenase ratio when they achieved a 2500-fold purification of the 17β-oestradiol dehydrogenase activity.

The hydroxysteroid dehydrogenases so far investigated have been shown to use the 'B' side of the dihydronicotinamide ring but evidence for 'direct transfer' i.e. transfer without any solvent participation, was sought and demonstrated in only one instance, viz. the enzyme-catalysed dehydrogenation of testosterone to androstenedione (Talalay et al. 1955). The significance of cofactor stereospecificity is unknown but Levy and Vennesland (1957) have suggested that it may be of physiological importance.

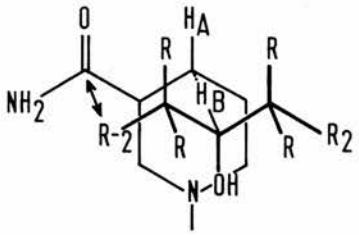
Cori, Velick and Cori (1950) first observed that the rate of oxidation of NADH bound to triosephosphate dehydrogenase by pyruvate in the presence of lactic dehydrogenase was so rapid that the bound NADH appeared to be oxidised without dissociation from the triosephosphate dehydrogenase. This work was confirmed and extended by Nygaard and Rutter (1956) who found that the oxidation by pyruvate of NADH bound to triosephosphate dehydrogenase, in the presence of lactic dehydrogenase could proceed more rapidly under certain conditions than the oxidation of free NADH. Similarly with liver alcohol dehydrogenase faster reactions were observed with NADH bound to triosephosphate dehydrogenase than with the free nucleotide. Loewus, Levy and Vennesland (1956) have suggested that a shift in conformation of the reduced nicotinamide ring might facilitate coupling between NAD^+-linked dehydrogenases of opposite cofactor specificity.

Fig. 3.

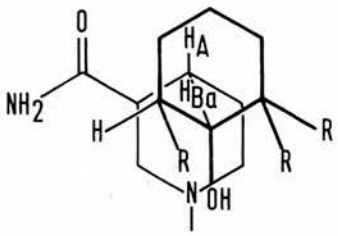
THE STERIC RELATIONSHIP OF SUBSTRATE TO COFACTOR IN THE TRANSITION STATE OF HYDROGEN TRANSFER



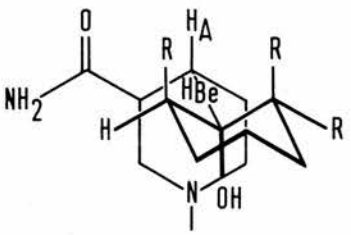
A–COENZYME



B–COENZYME



B–COENZYME a-ENZYME



B–COENZYME e-ENZYME

(AFTER PRELOG, 1963)

The occurrence of coupled dehydrogenase reactions without dissociation of the pyridine nucleotide could provide a means whereby certain metabolic paths would be selected at the expense of others (Levy and Vennesland, 1957).

Concepts of the mechanism(s) of action of dehydrogenases remain highly speculative but Prelog (1963) has attempted to correlate the stereospecificity of certain enzymes with kinetic measurements of the reactions which they catalyse. He studied the ketone-reductases of Curvularia falcata and pig liver which require $NADP^+$ as coenzyme, and the NAD^+-specific horse liver alcohol dehydrogenase.

Prelog stressed the importance of the spatial arrangement of the transition states of the hydrogen transfer in such reactions and his views were as follows. Since the hydrogen transfer occurs between coenzyme and substrate the steric position of both these components must be such that the overlapping of shared electron orbitals should become as great as possible, and the separating of opposing charges and the repelling effects of non-bonded atoms should become as small as possible. According to Prelog these conditions are best fulfilled in transition states which for the transfer of 'A' and 'B' hydrogens are depicted in Fig. 3.

Experiments with about 30 derivatives of cyclohexane, cyclohexanol and decalin of known absolute configuration showed that those substrates in which R_2 is a carbon did not react with enzymes which function with the 'A' side of the coenzyme. In contrast, the substrates in which R_{-2} represents a carbon atom did not react with enzymes which transfer the 'B' hydrogen.

A comparison of reaction rates showed that the so-called 'e'-ketone reductase of C. falcata strongly prefers such substrates in which the hydrogen transferred occupies an equatorial position in cyclohexane, while the 'a'-ketone reductase reacts comparatively quickly with the axial hydrogen. Since both enzymes transfer the sterically equal hydrogen, only the spatial arrangement of the protein is responsible for the difference.

When a lipophilic substrate reacts enzymatically with measurable velocity then the space which it requires during the reaction and especially during the hydrogen transfer is not occupied by the protein nor by particles strongly bound to it such as solvent molecules, ions and inhibitors. A group of substrates whose carbon skeleton was composed of cyclohexane rings in chair form was built up around this unoccupied space. The 2, 3 and 4-methylcyclohexanols and the corresponding cyclohexanones as well as the stereo-isomeric α- and β-decalols and the corresponding decalones formed such a model.

The relative reaction rates or the kinetic constants of the enzymatic oxidations or reductions were determined for these substrates. The carbon skeleton of these and many additional substrate analogues may be likened to parts of a diamond-lattice whose position as opposed to coenzyme, and therefore also to enzyme, is determined by the steric structure of the transition states of the hydrogen transfer.

When Prelog established that a specific substrate reacted with measurable velocity he denoted the corresponding position in a diamond-lattice as 'free'. By the use of a large number of substrates he could deduce (with reservations) which positions were forbidden.

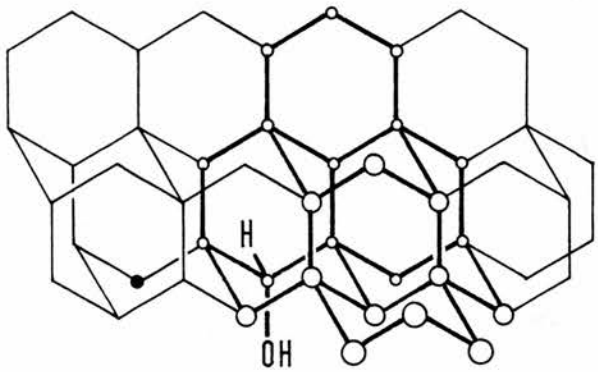
According to this theory each enzyme possesses its own typical diamond-lattice section.

The description of 'free' and 'forbidden' positions in the environment of the coenzyme with the aid of typical diamond-lattice sections confers the following advantages in addition to the possibility of characterising the enzyme.

1. One can sum up the known stereospecificity of the enzyme concerned.

Fig. 4.

THE CHARACTERISTIC DIAMOND-LATTICE SECTION FOR THE a-KETONE-REDUCTASE OF C. falcata.



○"FREE", • "FORBIDDEN".

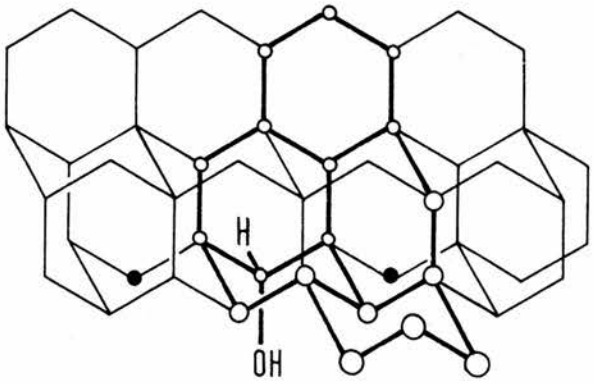
(AFTER PRELOG, 1963)

2. It allows one to predict substrate reactivity. Substrates whose frameworks extended over 'forbidden' positions did not react, whereas those which could be contained in the 'free' space reacted mostly with measurable velocity even when their carbon skeleton was not found to be composed of chair forms of cyclohexane. In the course of investigations on the stereospecificity of horse liver alcohol dehydrogenase Prelog and his colleagues found that this enzyme did not react with many α-decalones and α-decalols. On the basis of the diamond-lattice section of the enzyme it was found that a specific stereoisomer (+)-(1S,9S)-cis-decalol-(1) could be contained in the 'free space' and that this reacted relatively quickly with the enzyme.

3. The diamond-lattice permits one to test various hypotheses concerning the mechanism of action of ketone reductases and alcohol dehydrogenases. One can enlarge and perfect the typical diamond-lattice section by drawing on further substrates. It was Prelog's impression that extension of the 'free' space was possible to the right and in front and not to the left and behind, Figs. 4, 5. It seemed to him that the 'free' space lay in an angle which was formed on the one hand by the surface of the enzyme-coenzyme complex and on the other by a vertical plane impenetrable to the lipophilic substrate. Prelog and his colleagues then theorised that this plane was

Fig. 5.

THE CHARACTERISTIC DIAMOND-LATTICE SECTION
FOR HORSE LIVER ALCOHOL DEHYDROGENASE



o "FREE" • "FORBIDDEN"

(AFTER PRELOG, 1963)

composed of water-molecules which adhered to the polar part of the protein molecule. In ketone-reductases which transfer the 'B' hydrogen, the water-layer and the sterically hindering carboxamide group of the coenzyme lay on the same side of the pyridine nucleus, in alcohol dehydrogenase they were on different sides. This could explain the greater selectivity of the latter enzyme.

The usefulness of the diamond-lattice section of horse liver alcohol dehydrogenase has recently been supported by Waller, Theorell and Sjövall (1965). Using a highly purified preparation of horse liver alcohol dehydrogenase they found that this enzyme showed a high stereospecificity for 3β-hydroxy-5β-cholanic acids, and that the 'A' hydrogen transferred from NADH was the equatorial hydrogen in 3β-hydroxy-5β-cholanic acid. Their findings were in agreement with those of Prelog and Retey (1963, quoted by Waller et al.) that horse liver alcohol dehydrogenase is an 'e' enzyme in that it preferentially transfers the equatorial hydrogen in their cyclohexane, cyclohexanol and decalin series of substrates. This means that in the diamond-lattice model of alcohol dehydrogenase and 3β-hydroxy-5β-cholanic acid that the α-surface of the steroid should face the coenzyme.

It may be significant that the α-surface was invoked as the site of steroid/enzyme interaction in kinetic studies of the bacterial β-hydroxysteroid dehydrogenase

(Marcus and Talalay, 1955) and of the 17β-oestradiol dehydrogenase of human placenta (Langer, Alexander and Engel 1959).

The biological importance of enzyme-catalysed interconversions between hydroxyl and carbonyl groups has been stressed by Prelog (1963). He indicated the ubiquity of the alcohol dehydrogenases and of the aldehyde and ketone reductases. He also emphasised the great diversity of these enzymes in relation to specificity and stereospecificity.

The examples of reversible 17-hydroxysteroid dehydrogenations cited in the foregoing review illustrate the widespread distribution of these reactions in living organisms. It becomes increasingly evident that the hydroxysteroid dehydrogenases are highly specific with respect to the position and configuration of the hydroxyl group in the substrate molecule, and the probability that molecular planarity is an important characteristic of a reactive substrate is of great interest.

Engel (1964) has provided tentative evidence that substituents in the steroid substrate distant from the site of enzymic catalysis exert an effect upon reaction rates. This phenomenon of 'conformational transmission' was first fully defined by Barton, Head and May (1957) in their study of the rates of condensation of some triterpenoid ketones with benzaldehyde. Although there is no complete explanation for this phenomenon, it may well be important to our understanding of steroid-enzyme interactions.

The attempted isolation of a 17α-oestradiol dehydrogenase was therefore considered worthwhile, because this enzyme offers unique opportunities for the study of effects of substrate structure upon reaction kinetics. This enzyme would also permit the investigation of the mechanism(s) of hydroxysteroid dehydrogenations and the elucidation of the nature of the enzyme catalytic site(s).

EXPERIMENTAL AND RESULTS

INTRODUCTION

The many procedures which are available for enzyme purification have been discussed at length by Schwimmer and Pardee (1953) and by Dixon and Webb (1964). The choice and sequence of methods are largely subjective, and much depends on the availability and stability of the activity to be purified and the possession of a rapid means for its determination.

The experiments to be described fall broadly into four groups:-

1. Those concerned with a search for a 17α-oestradiol dehydrogenase and its subcellular location.

2. The development of a convenient assay, the identification of the product and the stoichiometry of the reaction.

3. Methods of enzyme purification.

4. Characterisation of the enzyme.

TISSUE INCUBATIONS

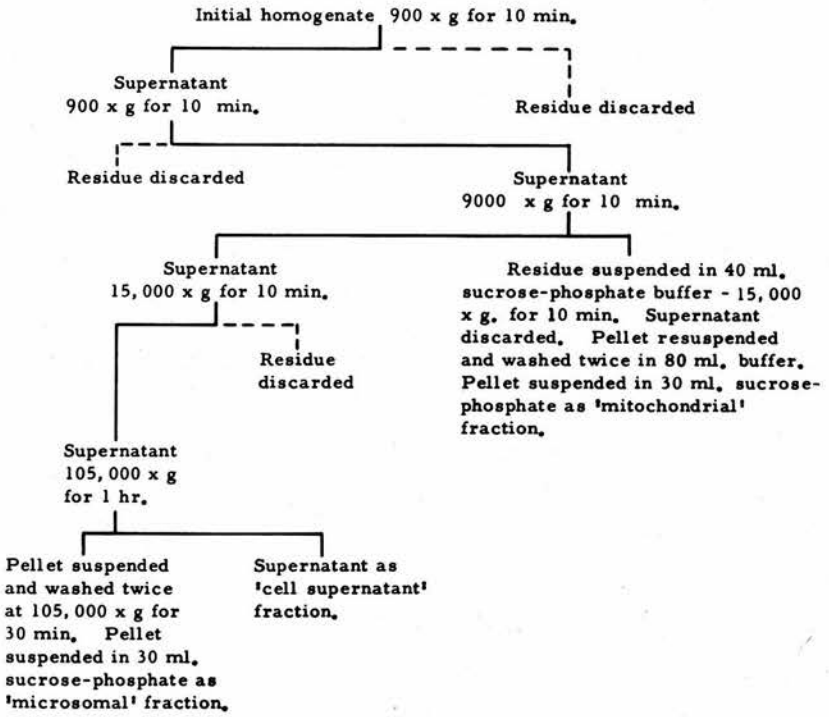
Sheep liver

This could not be obtained less than 36 - 48 hr. old and the tissue was allegedly refrigerated from the time of slaughter. The liver was trimmed free of fat and cut into small pieces before homogenisation in a 'Virtis' homogeniser for 1 min. at reduced speed (60 volts, obtained by means of a variable resistance). Three ml. 0.1M-$NaHCO_3$-Na_2CO_3 buffer, pH 8.5 was used for 1 g. (wet weight) of tissue. The homogenate was centrifuged in a 'Servall' instrument with SS34 Rotor at 34,800 x g for 30 min. at 0°. The residue was discarded and centrifugation was repeated at 34,800 x g for 30 min. Five ml. of this second supernatant was used for each incubation which was carried out in air in a Dubnoff incubator at 37° for 30 min.

The incubation mixtures were cooled quickly to 4°, the pH was adjusted to about 7.4 - 8.0 with N-NaOH, and the steroid reaction products were extracted with three 5 ml. volumes of redistilled methylene chloride. This procedure was used for all tissue incubations. The incubation mixtures plus organic solvent were routinely centrifuged at 3,000 r.p.m. for 10 min. This served to break any emulsion and to separate the aqueous and organic phases. The tissue was compressed into a narrow band at the interface and could be removed as a disc, before pipetting off the organic solvent. The extract was taken to dryness under reduced pressure on a rotary

CHART I

Cell Fractionation by Differential Centrifugation



evaporator. The residues were dissolved in 0.5 ml. redistilled ethanol, and 30 µl. was applied to the origin of a thin layer plate (Appendix A).

Batches of sheep liver showed considerable variation in ability to oxidise 17α- and 17β-oestradiol to oestrone. Whether this was a function of the degree of freshness of the tissue, or represented a seasonal fluctuation in enzyme activity, was not investigated.

Preparations of those animal tissues which were most readily available in the Boston area were examined for 17α- and 17β-oestradiol dehydrogenase activity, using the method described for sheep liver, and a pH range of 7.5 - 9.5. The results were disappointing and are summarised in Tables 1a and 1b.

The only plentiful tissues which remained to be investigated were chicken and turkey liver. The results of these experiments are shown in Tables 2 and 3.

Localisation of enzyme activity in subcellular fractions

Differential centrifugation of sheep, chicken and turkey liver homogenates was carried out in 0.25M sucrose-phosphate buffer, pH 7.2 using 1 g. (wet weight) of tissue for each ml. of buffer, according to Schneider and Hogeboom (1950; Chart I).

Three subcellular fractions were studied in addition to the 'entire' homogenates -

a) 'mitochondrial'

b) 'microsomal'

c) 'cell supernatant'.

Each fraction of chicken liver was examined by phase-contrast microscopy (by courtesy of Dr. Nancy Bucher and Miss Miriam Swaffield).

'Mitochondrial' fraction

No nuclei present, many clumps of mitochondria seen.

'Microsomal' fraction

This was heavily contaminated with mitochondria, but most were removed in subsequent washes.

'Cell supernatant' fraction

A very occasional unidentifiable particle and mitochondrion were seen.

TABLE 1 (a)

Sheep liver which converted 17α- and/or 17β-oestradiol to oestrone

Preparation (5 ml.)	Substrate (5 μmoles)	Conversion to oestrone	Preferred cofactor (25 μmoles)
'Entire' homogenate	17α-oestradiol	+	$NADP^+$
	17β-oestradiol	++	$NADP^+$
'Mitochondria'	17α-oestradiol	0	-
	17β-oestradiol	0	-
'Microsomes'	17α-oestradiol	±	NAD^+ or $NADP^+$
	17β-oestradiol	±	NAD^+ or $NADP^+$
'Cell supernatant'	17α-oestradiol	+	$NADP^+$
	17β-oestradiol	++	$NADP^+$

++ = good conversion
+ = fairly good conversion
± = trace
0 = no conversion

The tissue was allegedly refrigerated for 36 - 48 hours.

Homogenates were prepared using 2 or 3 ml. of buffer for each g. (wet weight) of tissue. The total volume of the incubation mixture was varied from 5.1 - 6.1 ml. according to the volume of solvent in which the substrate was added. No difference in results was found when substrates were added in 0.1 or 1.0 ml. of redistilled ethanol or 1,2-propanediol. Cofactors were added in 0.1 or 1.0 ml. buffer or deionised water. Incubations were carried out at 37° for 30 min. in air in a Dubnoff incubator over a pH range of 7.5 - 9.5 using 0.1M sodium phosphate, tris-HCl, and sodium bicarbonate-carbonate buffers. Similar conditions held for incubations summarised in Tables 1b, 2 and 3. The columns headed 'preferred cofactor' refer to the added nucleotide which gave the better conversion.

TABLE 1 (b)

Fresh mammalian tissues which converted 17α- and/or 17β-oestradiol to oestrone

Species and Tissue	Substrate (5 μmoles)	Conversion to oestrone	Preferred cofactor (25 μmoles)
Lamb liver	17α-oestradiol	0	-
	17β-oestradiol	0	-
Rabbit liver	17α-oestradiol	+	NAD^+ or
	17β-oestradiol	++	$NADP^+$
Calf liver	17α-oestradiol	0	-
	17β-oestradiol	+	NAD^+ or $NADP^+$
Calf kidney	17α-oestradiol	0	-
	17β-oestradiol	0	-

++ = good conversion

\+ = fairly good conversion

0 = no conversion

TABLE 2

The oxidation of 17α- and 17β-oestradiol by chicken liver homogenates

Substrate (10 μmoles)	Cofactor (62.5 μmoles)	Preparation (5 ml.)	Steroid Products
None	None	2nd supernatant	No steroid
17α-oestradiol	NAD^+	2nd supernatant*	0
17α-oestradiol	$NADP^+$	2nd supernatant*	0
17β-oestradiol	NAD^+	2nd supernatant*	0
17β-oestradiol	$NADP^+$	2nd supernatant*	0
17α-oestradiol	NAD^+	2nd supernatant	oestrone + 17β-oestradiol +
17α-oestradiol	$NADP^+$	2nd supernatant	oestrone ++ 17β-oestradiol +
17β-oestradiol	NAD^+	2nd supernatant	oestrone ++ 17α-oestradiol +
17β-oestradiol	$NADP^+$	2nd supernatant	oestrone +++ 17α-oestradiol +

+++ = very good conversion
++ = good conversion
+ = fairly good conversion
0 = no conversion

* The enzyme preparation was boiled prior to incubation.

The homogenate was centrifuged twice at 34,800 x g. The second supernatant was used in these incubations.

TABLE 3

Avian liver homogenates which showed interconversion of 17α-oestradiol, 17β-oestradiol and oestrone

Preparation (5 ml.)	Substrate (5 μmoles)	Steroid products	Preferred cofactor (25 μmoles)
'Entire' homogenate	17α-oestradiol	oestrone +++ and 17β-oestradiol +	$NADP^+$
	17β-oestradiol	oestrone +++ and 17α-oestradiol +	$NADP^+$
	oestrone	17α- and 17β-oestradiol +	NADPH
'Mitochondria'	17α-oestradiol	oestrone +	$NADP^+$
	17β-oestradiol	oestrone +	$NADP^+$
'Microsomes'	17α-oestradiol	oestrone +	NAD^+ or $NADP^+$
	17β-oestradiol	oestrone +	NAD^+ or $NADP^+$
'Cell supernatant'	17α-oestradiol	oestrone ++ and 17β-oestradiol ++	$NADP^+$
	17β-oestradiol	oestrone ++ and 17α-oestradiol ++	NADP

+++ = very good conversion
++ = good conversion
+ = fairly good conversion

These results were obtained with liver from nine-week old laboratory-reared cockerels and with commercially available chicken and turkey livers.

SALT FRACTIONATION

Salt fractionation of the 17α- and 17β-oestradiol dehydrogenase activities in chicken liver homogenates was carried out with crystalline ammonium sulphate. Comparable results were obtained when any of the following grades of $(NH_4)_2SO_4$ were used, Fisher's Certified Reagent, Mann's Special Enzyme Grade or British Drug Houses 'Analar' reagent.

The amount of salt needed to produce the required degree of saturation was calculated from information given by Taylor (1953).

The weight in grams of ammonium sulphate, x, to be added to 100 ml. of solution of saturation S_1, to yield a solution of S_2 is obtained from the equation -

$$x = \frac{0.1\ G\ (S_2 - S_1)}{1 - \frac{VG}{1000}\ S_2}$$

where G = grams of ammonium sulphate in 1 litre of saturated solution

and V = apparent specific volume of ammonium sulphate in a saturated solution.

Because all salt fractionations were carried out at 4 - 5 ° the values of G and V nearest to that temperature were used, i.e. those at 0°. The nomogram published

by Dixon (1953) is open to the criticism that it was based on full saturation at room temperature which was not specified.

Preliminary studies of ammonium sulphate fractionation were carried out with fresh liver or tissue which had been stored for 18 hr. at -10° - -14°. One gram (wet weight) was used for each 3 ml. of the following solution (pH 7.6) which contained nicotinamide (10 mM), cysteine hydrochloride (1 mM) and EDTA (1 mM). The homogenate was prepared in a 'Virtis' at reduced speed (60 volts for 1 min.) and centrifuged at 2520 x g for 30 min. at 0°. The residue was discarded.

The addition of 1 ml. of 0.1M-$CaCl_2$ solution to each 10 ml. of homogenate was found to remove certain impurities, consequently the required volume of aqueous calcium salt was added to the homogenate. The mixture was stirred for 1 hr. at 4 - 5°, then allowed to stand for 1 hr. at that temperature before centrifugation at 2520 x g for 30 min. The residue was discarded and the supernatant was fractionated with ammonium sulphate to give saturations of 15, 30, 50, 75 and 100% at 0°. The salt was added slowly with constant stirring, the pH was maintained at 6.8 by the addition of 3M-NH_4OH and the mixture was stirred for 30 min. after the salt had dissolved. Each fraction was collected by centrifugation.

These precipitates were dissolved in 40 ml. 0.1M-sodium phosphate buffer and 5 ml. volumes were incubated with steroid and cofactor for 30 min. at 37° (Table 4). The steroid reaction products were examined by thin-layer chromatography. Removal of the ammonium sulphate on Sephadex G-25 prior to incubation made no appreciable difference to the results obtained in the presence of salt.

These results were later confirmed by spectrophotometric assay. The homogenate from 1.5 Kg. of fresh tissue was therefore fractionated at 30 and 80% saturation. This procedure concentrated both enzyme activities in the second precipitate (see first method of purification, p. 59).

Further attempts to fractionate the dialysed second precipitate with ammonium sulphate resulted in considerable loss of both activities and this procedure was discontinued.

TABLE 4

The interconversion of 17α-, 17β-oestradiol and oestrone by ammonium sulphate fractions of chicken liver homogenates

Preparation (5 ml.)	Substrate (5 μmoles)	Cofactor (25 μmoles)	Steroid Products
'Entire' liver homogenate	17α-oestradiol	-	oestrone +++ 17β-oestradiol ++
	17β-oestradiol	-	oestrone +++ 17α-oestradiol ++
	oestrone	-	17α-oestradiol ++ 17β-oestradiol ++
0 - 30% or 15 - 30% precipitate	17α-oestradiol	NAD^+	0
	17α-oestradiol	$NADP^+$	oestrone ++ 17β-oestradiol ±
	17β-oestradiol	NAD^+	oestrone +
	17β-oestradiol	$NADP^+$	oestrone + 17α-oestradiol +
	oestrone	NADH	17β-oestradiol +
	oestrone	NADPH	17β-oestradiol + 17α-oestradiol +

(Continued on next page)

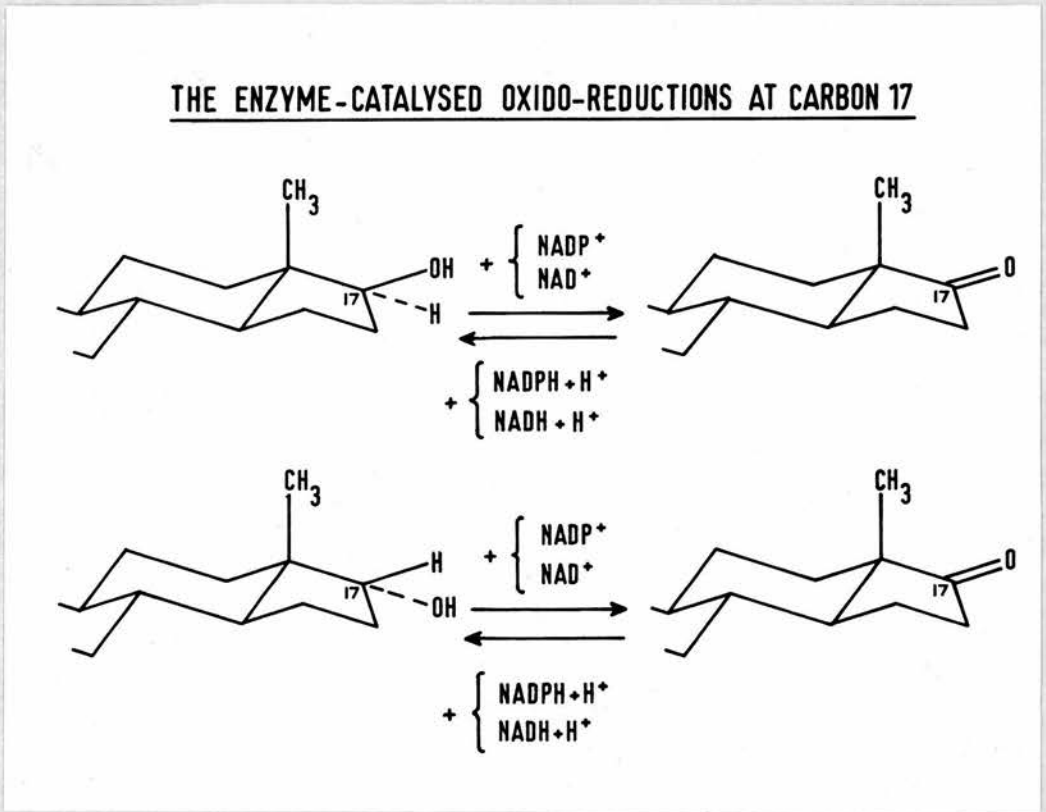
TABLE 4 (contd.)

Preparation (5 ml.)	Substrate (5 μmoles)	Cofactor (25 μmoles)	Steroid Products
30 - 50% precipitate	17α-oestradiol	NAD^+	oestrone ++
	17α-oestradiol	$NADP^+$	oestrone +++ 17β-oestradiol ++
	17β-oestradiol	NAD^+	oestrone ++ 17α-oestradiol +
	17β-oestradiol	$NADP^+$	oestrone ++ 17α-oestradiol ++
	oestrone	NADH	17α-oestradiol ± 17β-oestradiol +
	oestrone	NADPH	17α-oestradiol ++ 17β-oestradiol ++
50 - 75% precipitate	17α-oestradiol	NAD^+	oestrone ±
	17α-oestradiol	$NADP^+$	oestrone ++
	17β-oestradiol	NAD^+	oestrone +++
	17β-oestradiol	$NADP^+$	oestrone +++
	oestrone	NADH	17β-oestradiol +
	oestrone	NADPH	17β-oestradiol ++
75 - 100% precipitate	No conversion with any of the three substrates.		

+++ = very good conversion
++ = good conversion
± = trace
0 = no conversion

Incubations were carried out at 37° for 30 min. at pH 7.0, in air.

Fig. 6.


THE ENZYME-CATALYSED OXIDO-REDUCTIONS AT CARBON 17
CH3
OH
H
17
+ { NADP+ NAD+
+ { NADPH + H+ NADH + H+
O
H
OH

SPECTROPHOTOMETRIC ASSAY

The quantitative determination of activity by a rapid, accurate method is vital for enzyme purification. A spectrophotometric assay was devised which was based upon that used by Langer and Engel (1958) for human placental 17β-oestradiol dehydrogenase. The reactions are depicted in Fig. 6. Only the C and D rings of the oestrogen molecules are shown in a Barton projection.

$NADP^+$ was chosen as cofactor because the yields of oestrone in incubation experiments were generally greater when this nucleotide was added, and because crude enzyme preparations contained an active NAD^+-specific 'alcohol dehydrogenase'. Saturating concentrations of substrate and cofactor, and the pH optima were determined in a number of experiments. (Details are presented in the section headed 'Kinetic Parameters').

The final reaction system is shown in Table 5.

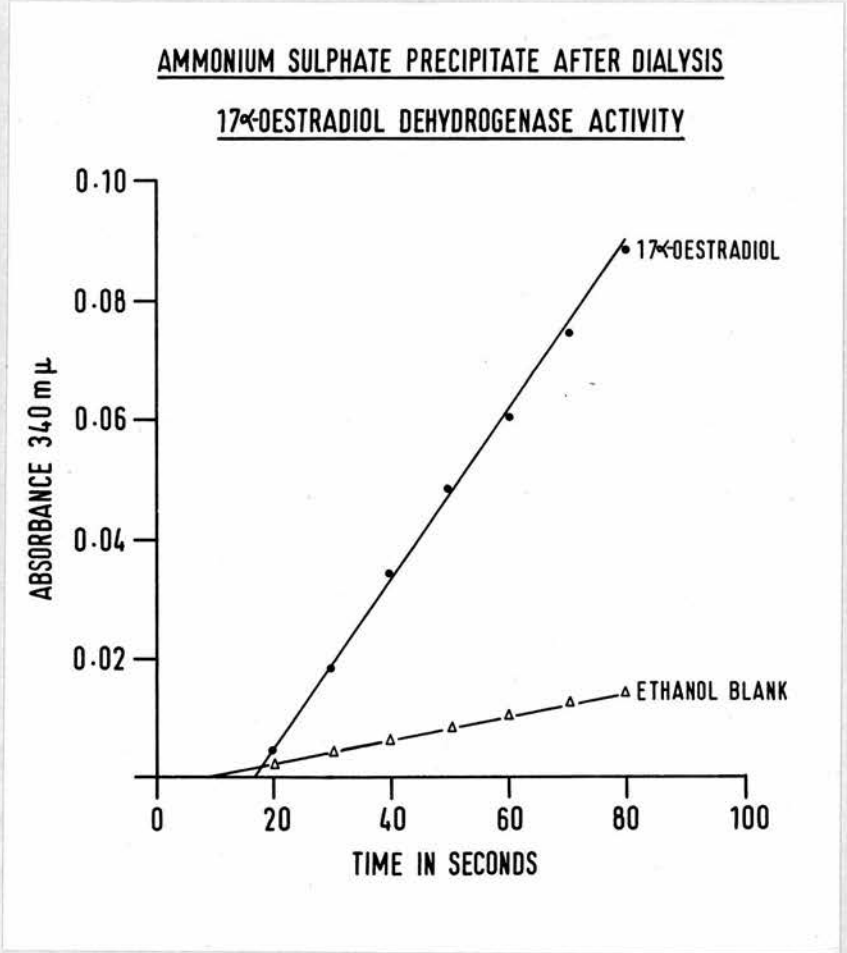
TABLE 5

Routine oxidative assay at 37°

0.2 μmoles steroid in 0.1 ml. ethanol
300 μmoles $NaHCO_3$-Na_2CO_3 buffer pH 9.5 at 20°
0.4 μmoles $NADP^+$ in 0.1 ml. distilled or deionised water
0.3 ml. enzyme preparation
3 ml. total volume with distilled or deionised water, final pH 9.2

When the assay was run at 25° the pH of the sodium bicarbonate-carbonate buffer was 9.2 at 20°.

Fig. 7



An ammonium sulphate precipitate taken between 30 - 80% saturation was stored for 7 weeks at -10° in glycerol-buffer and dialysed against 5 mM-sodium phosphate buffer (pH 7.2) containing EDTA (1 mM) and glycerol (20%, by volume).

The non-diffusible material was centrifuged at 34,800 x g for 20 min. 0.5 ml. Supernatant was assayed under standard conditions at 37°. The specific activity of this preparation was 0.6 milli-units/mg. protein.

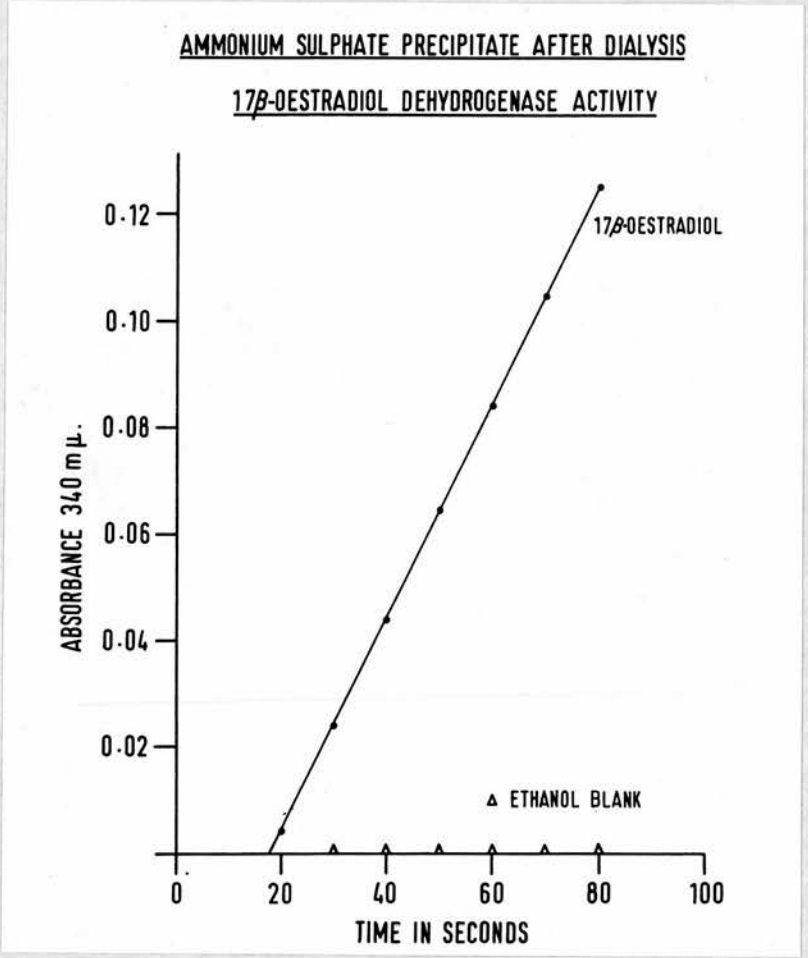
In all instances the reaction was initiated by the addition of $NADP^+$ and the rate of change of absorbance was measured at 340 mμ in a Zeiss PMQ II spectrophotometer or in a Unicam SP800 recording spectrophotometer, against a blank cell containing all components except steroid. In crude homogenates, and in the first ammonium sulphate precipitates after dialysis, there was often considerable non-specific absorbance with enzyme preparation and buffer only. Typical plots of enzyme activities from different batches of liver are shown in Figs. 7 and 8.

When the non-recording Zeiss instrument was used, readings were taken 20 sec. after the addition of cofactor and every 10 sec. thereafter for 1.5 - 2 min. With the recording spectrophotometer, a continuous scan was obtained (for at least 2 min.) following the addition of cofactor. Velocities were calculated from the slopes of the zero order portion of the curves plotted as absorbance against time. These were corrected where necessary for the control absorbance.

Enzyme units

The recommendations of the Report of the Commission on Enzymes of The International Union of Biochemistry (1964) were adopted.

Fig. 8



An ammonium sulphate precipitate taken between 30 - 80% saturation was stored for 8 weeks at -10° in glycerol-buffer and dialysed against 5 mM-sodium phosphate buffer (pH 7.2) containing EDTA (1 mM) and glycerol (20%, by volume).

The non-diffusible material was centrifuged at 34,800 x g for 20 min. 0.5 ml. Supernatant was assayed under standard conditions at 37°. The specific activity of this preparation was 0.9 milli-units/mg. protein.

A unit of 17α- or 17β-oestradiol dehydrogenase activity was defined as that amount which catalyses the conversion of 1 μmole of substrate per minute per millilitre under the specified conditions of assay.

Because of the limited availability of these enzymes all activities in this thesis are reported in milli-units.

In preliminary experiments, and particularly during column chromatography, it was convenient to define a working unit of enzyme activity as that which caused an increase in absorbance of 0.001 at 340 mμ.

'Specific activity' was expressed in milli-units of enzyme per milligram of protein which was determined by the method of Lowry, Rosebrough, Farr and Randall (1951) or by the ratio of absorbance at 280 mμ to 260 mμ (Appendix B).

THE pH DEPENDENCE OF 17α- AND 17β-OESTRADIOL DEHYDROGENASE ACTIVITIES

Preliminary investigations of the pH dependence of 17α- and 17β-oestradiol dehydrogenase activities were carried out as incubations with heat-treated homogenates prepared with 0.1M buffers in the pH range 4.0 - 9.9 (Table 17). In summary, most oestrone was formed at pH 8.4 and 8.9.

The experiments were extended with the development of a spectrophotometric assay. Initial measurements were made with 17β-oestradiol dehydrogenase at 25° in 0.1M buffers from pH 6.4 - 9.8. The pH of the assay mixtures was checked on a Beckman pH meter using a single probe glass electrode and it was found that the buffering power of 0.1M-sodium bicarbonate-carbonate buffer was inadequate above pH 9.0. The experiments were repeated with 1.0M buffers.

Sixty ml. of an ammonium sulphate precipitate taken between 40 and 60% saturation and stored in 5 mM-phosphate buffer containing glycerol (50%, by volume) at -10°, was dialysed for 45 min. against 5 l. of a solution containing sodium bicarbonate (50 mM), EDTA (1 mM) and glycerol (20%, by volume). The pH was 6.8 at 5°. This procedure was repeated. The sodium bicarbonate content of the dialysis solution was reduced to 5 mM and dialysis was continued for two further periods of 45 min. against 5 l. volumes (Appendix C).

The non-diffusible material was centrifuged at 34,800 x g for 30 min. and two ammonium sulphate fractions were taken (equivalent to 30% and 60% saturation at 0°). The 30-60% precipitate was dialysed for 4 hr. against the following mixture: sodium phosphate buffer (5 mM), pH 7.2 EDTA (1 mM) and glycerol (20% by volume). Nineteen ml. of the non-diffusible material was applied to a DEAE-cellulose column (2.2 cm x 19 cm) previously equilibrated with the sodium phosphate-EDTA-glycerol mixture at 5°. The same solution was used for elution and a flow-rate of 4 ml./cm^2/hr. was maintained by L.K.B. 'Minipump' (see p. 60).

The most active fractions were pooled. Spectrophotometric assays were run in triplicate at 25°. Assay mixtures were pooled and stored at 4° for 15 hr. The mixtures were extracted with redistilled methylene chloride after 1 hr. at room temperature (23°) and the steroids were examined by thin-layer chromatography, (Table 6).

TABLE 6

Thin-layer chromatography of spectrophotometric assay mixtures

Assay buffer	pH	Pooled assay mixture (pH)	Oestrone formed
Sodium bicarbonate - carbonate	10.4	10.4	+++
Sodium bicarbonate - carbonate	9.7	9.7	+++
Sodium bicarbonate - carbonate	9.2	9.2	+++
tris-HCl	8.5	8.5	+++
tris-HCl	8.0	8.0	0
Sodium phosphate	7.5	7.5	0

The substrate was 17β-oestradiol, the assay temperature 25°.

+++ = very good conversion
0 = no conversion

In assays carried out at pH 8.0 a linear increase in absorbance at 340 mμ of 0.002 O.D. unit/sec. was noted in the blank and control cuvettes. At pH 7.5 this was increased to 0.006 O.D. unit/sec. On allowing the cuvette contents to stand at room temperature the mixtures became turbid.

The experiment was repeated using an ammonium sulphate precipitate taken at 50% saturation. This was dialysed against the following solution in 5 l. volumes, sodium phosphate buffer (5 mM), EDTA (1 mM), cysteine hydrochloride (1 mM). The pH was 7.2 at 5°. The total period of dialysis was 2 hr. 15 min.

The non-diffusible material was centrifuged at 34,800 x g for 20 min. and the residue discarded. Assays of 17α- and 17β-oestradiol dehydrogenase activities were carried out at 37°, the mixtures at each pH were pooled, and the pH measured. The steroid reaction products were extracted with 3 x 5 ml. volumes of redistilled methylene chloride and examined by thin-layer chromatography, (Table 7).

TABLE 7

Thin-layer chromatography of spectrophotometric assay mixtures

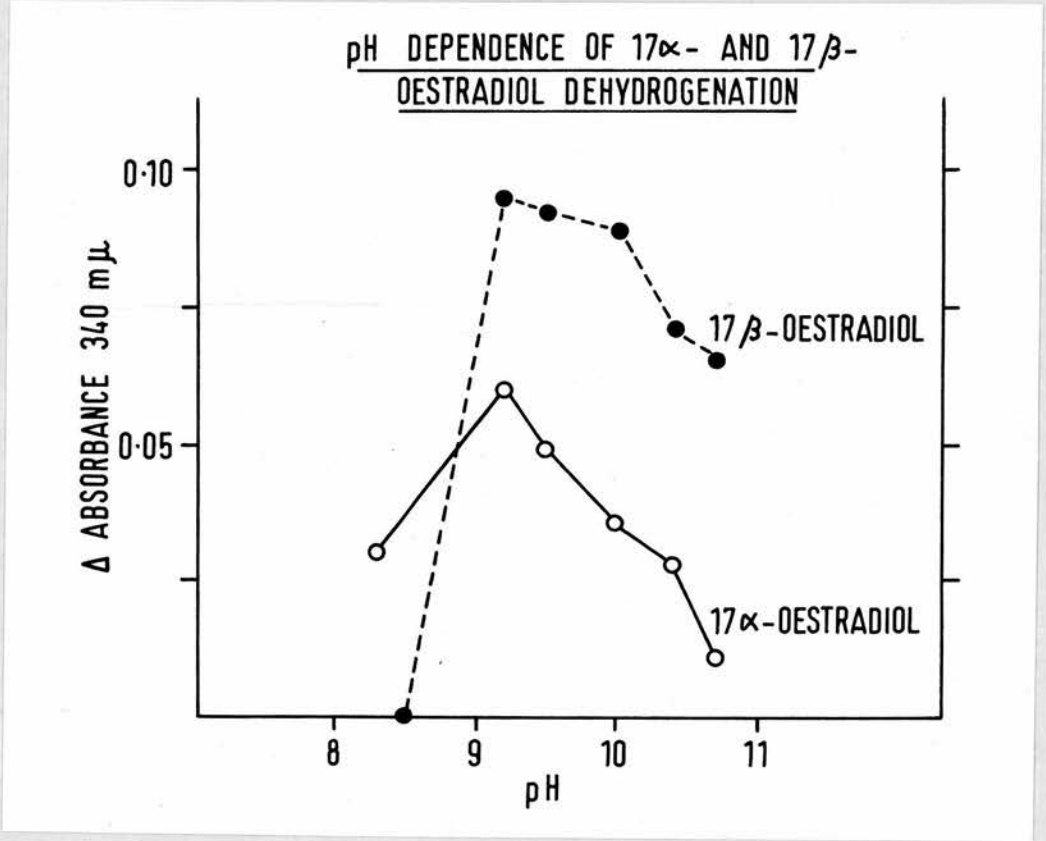
Assay buffer	pH	Pooled assay mixture (pH)	Oestrone formed
Sodium bicarbonate - carbonate	10.7	9.5	+++
Sodium bicarbonate - carbonate	10.4	9.3	+++
Sodium bicarbonate - carbonate	10.0	9.2	+++
Sodium bicarbonate - carbonate	9.5	9.0	+++
Sodium bicarbonate - carbonate	9.2	8.8	+++
tris-HCl	8.3	8.1	0
tris-HCl	7.9	7.1	0
tris-HCl	7.1	6.7	0
Sodium phosphate	6.4	5.8	0

+++ = very good conversion

0 = no conversion

These results were obtained when 17α- and 17β-oestradiol dehydrogenase activities were assayed at 37°.

Fig. 9



An ammonium sulphate precipitate taken between 0 - 50% saturation was stored for 6 days at -14° in glycerol-buffer and dialysed against 5 mM-sodium phosphate buffer (pH 7.2) containing EDTA (1 mM) and cysteine hydrochloride (1 mM).

The non-diffusible material was centrifuged at 34,600 x g for 30 min. and 0.5 ml. supernatant was assayed in duplicate for each substrate against a series of buffers. The rates of change of absorbance are plotted against pH. The assay mixtures for each substrate at each pH were pooled and extracted. The steroids were examined by thin-layer chromatography to confirm that the product was oestrone.

It was again found that an increase in absorbance occurred at 340 mμ as the pH of the assay mixtures was dropped from 8.1 to 5.8. This was present in blank and test cells and was caused by turbidity. The significance of this will be discussed later (p. 77).

Typical pH curves of both enzyme activities are shown in Fig. 9 .

CHARACTERISATION OF OESTRONE

The dehydrogenation product of both oestradiol epimers from incubations and from spectrophotometric assays was provisionally identified as oestrone by comparison of its R_F with that of the authentic steroid in the thin-layer system previously described. Further presumptive evidence of identification was obtained by comparison of mobility in the paper chromatographic system, ligroin-toluene-70% methanol (2:1:3, by volume) at 29°. The reduction products of oestrone from incubation experiments were also tentatively identified as 17α- and 17β-oestradiol by thin-layer chromatography with reference compounds.

When spectrophotometric assays were run with stored precipitates, or when new methods of purification were attempted, it was the practice to pool several assay mixtures in ethanol or methanol at 4°; precipitated protein was removed by centrifugation and extracted with fresh solvent. The steroid reactants were subjected to thin-layer chromatography and the product, oestrone, was thus provisionally identified.

The following experiments were designed for two purposes:-

1. to confirm that the product of dehydrogenation of 17α-oestradiol and of 17β-oestradiol was oestrone in each case.

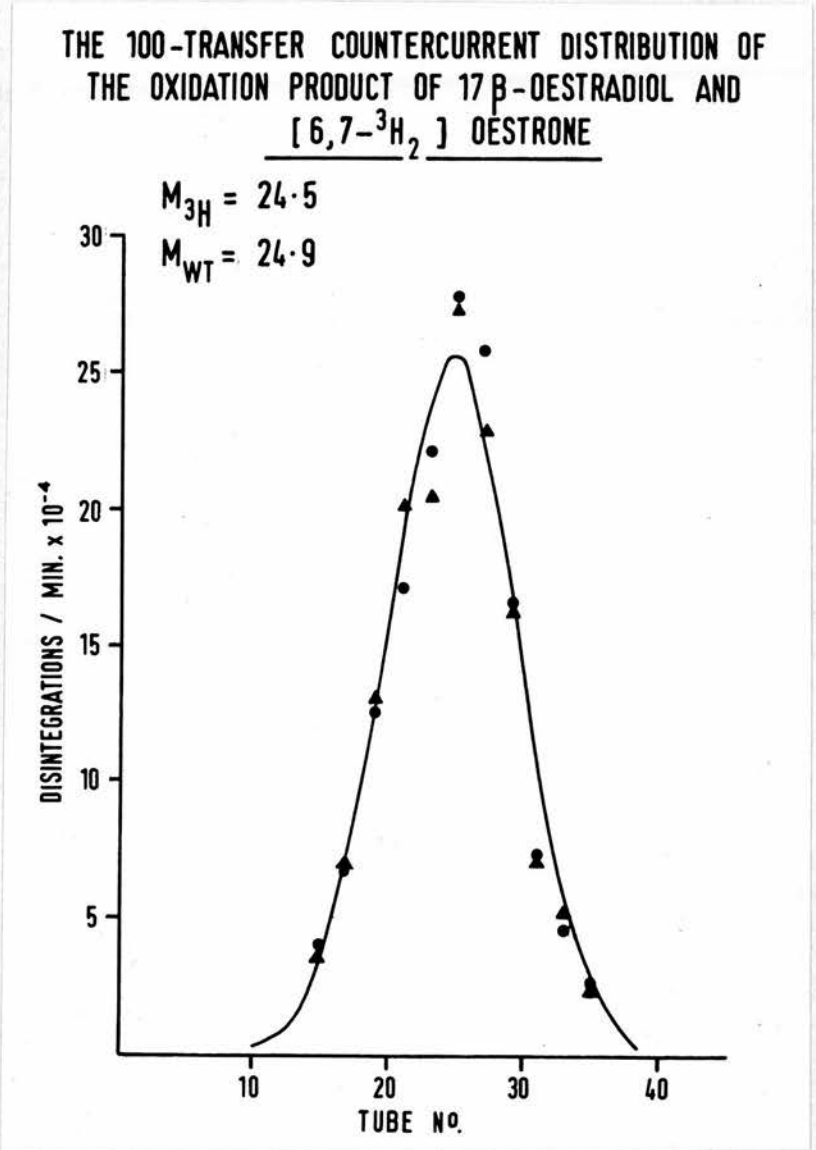
2. to determine the stoichiometry of these reactions which were assumed to be equimolar, i.e. for each mole of oestradiol oxidised, 1 mole of oestrone and 1 mole of NADPH were formed.

To test this hypothesis under standard assay conditions the amount of NADPH was determined spectrophotometrically and oestrone was measured by fluorescence.

Oestrone from 17β-oestradiol

Thirty-seven standard assays were run for 1 min. using a dialysed '0 - 50%' ammonium sulphate precipitate. The rate of change of absorbance was measured at 340 mμ and the contents of the reaction cuvettes were quickly transferred to ethanol at 4°. Preliminary experiments showed that the rate of formation of NADPH was constant for at least 2 min., thus it was possible to correct the absorbance reading for the time taken to raise the lid of the cuvette-holder, remove the cuvette, and discharge the reaction mixture.

Fig. 10



The solvent system is carbon tetrachloride-methanol-water (2:1:1, by volume). The partition coefficient (K) for oestrone in this system is 0.33. The odd-numbered tubes from 15 - 35 (inclusive) were used in these calculations.

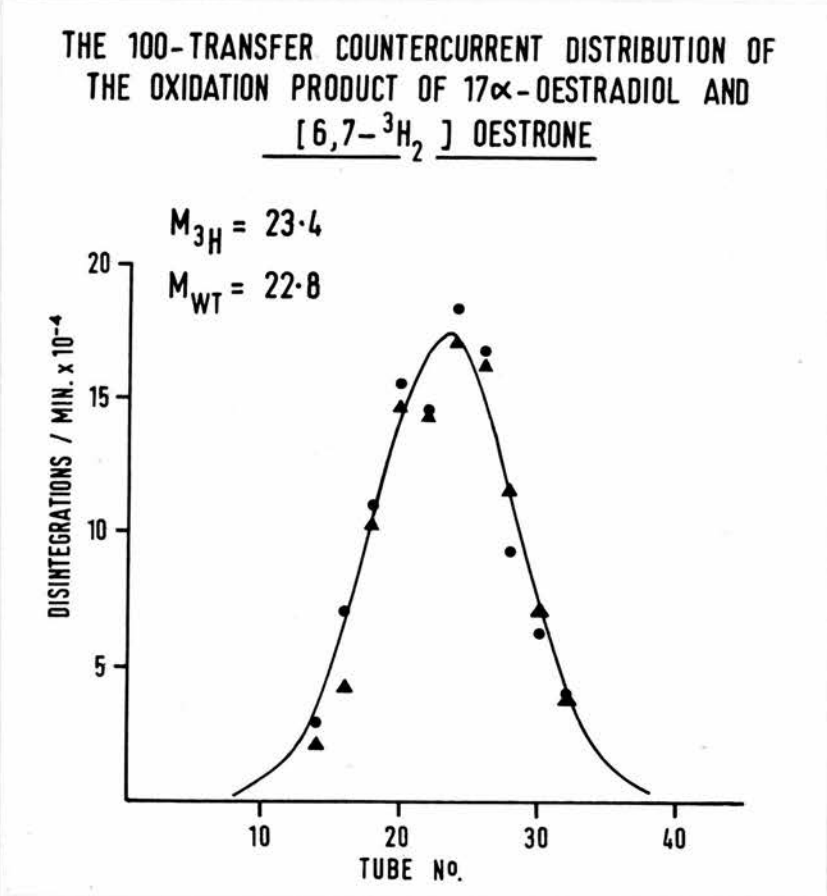
Weight in μg. x average specific activity (•) and radioactivity in disintegrations per minute (▲) are plotted as ordinates. The curve is that calculated by the computer from the experimental data.

M_{3H} and M_{wt} represent the peak tube number calculated by the computer for radioactivity and weight respectively. The standard error of the difference between M_{3H} and M_{wt} is ± 0.29 indicating agreement between the curves for radioactivity and weight ($p > 0.2$).

Each cuvette was rinsed twice with 0.5 ml. ethanol and the resultant pool was centrifuged to remove precipitated protein, which was resuspended and washed three times in ethanol. The combined washings were taken to dryness under reduced pressure. 318 x 10^4 disintegrations/min. of chromatographically pure [6,7-$^3H_2$] oestrone (Appendix D) were added to the dry residue which was dried again. It was then subjected to a four-tube countercurrent distribution with stripping, using 150 ml. of ethylacetate as upper phase and 30 ml. distilled water as lower phase. The aqueous extracts were discarded.

The organic extracts were pooled, dried under reduced pressure, then partitioned between hexane/90% methanol (v/v) in an eight-tube countercurrent distribution with stripping (Weliky and Engel, 1962). The aqueous methanol extracts were combined and dried under reduced pressure. The residue was transferred quantitatively to a Craig machine and a 100-transfer countercurrent distribution was run in carbon tetrachloride-methanol-water (2:1:1, by volume), (Appendix E). The computer analysis of the distribution data was carried out by Mr. Norman Goldman of the Boston University Computing Center, using the procedure described by Purdy, Goldman and Richardson (1965).

Fig. 11



The solvent system is carbon tetrachloride-methanol-water (2:1:1, by volume). The partition coefficient (K) for oestrone in this system is 0.33. The even-numbered tubes from 14 - 32 (inclusive) were used in these calculations.

Weight in μg. x average specific activity (•) and radioactivity in disintegrations per minute (▲) are plotted as ordinates. The curve is that calculated by the computer from the experimental data.

M_{3H} and M_{wt} represent the peak tube number calculated by the computer for radioactivity and weight respectively. The standard error of the difference between M_{3H} and M_{wt} is ± 0.23 indicating agreement between the curves for radioactivity and weight ($p > 0.05$).

Oestrone from 17α-oestradiol

The above procedure was modified in this experiment. Twenty-five standard assays were run and the reactions were terminated in methanol at 4° on this occasion. 307 x 10^4 disintegrations/min. of chromatographically pure [6,7-$^3H_2$] oestrone were added to the pooled reaction mixtures. The remainder of the experiment was carried out as previously described.

Results

The following values were used in the calculations of stoichiometry:-

Molar extinction coefficient of NADPH at 340 mμ	6.2×10^3 litre $mole^{-1}$ cm^{-1}.
Molecular weight of oestrone	270
Volume of reaction mixture	3 ml.
Light path of cuvette	1 cm

Oestrone from 17β-oestradiol

Total rate of change of absorbance per cuvette at 340 mμ 6.29

Concentration of NADPH = $\frac{6.29}{6.2}$ μmoles/ml. 1.014 μmoles/ml.

Stated purity of $NADP^+$ (Appendix G)	98%
Corrected concentration of NADPH	1.035 µmoles/ml.
Total amount of NADPH formed	3.105 µmoles

If the reaction is equimolar then the amount of oestrone formed is 3.105 x 0.27 mg. 0.8383 mg.

A theoretical distribution was calculated for a peak tube content of 0.0775 mg. and the values for the even-numbered tubes were derived from this curve (Table 8, p. 58). The total recoveries of oestrone from the countercurrent distributions are given in Appendix E.

Amount of oestrone recovered 0.7998 mg.

Amount of radioactive oestrone added to pooled assays 318 x 10^4 disintegrations/min.

Amount of radioactive oestrone recovered in countercurrent distribution 308 x 10^4 disintegrations/min.

Percentage recovery 96.8

Oestrone from 17α-oestradiol

Total rate of change of absorbance at 340 mμ	3.75
Concentration of NADPH = $\frac{3.75}{6.2}$ μmoles/ml.	0.65 μmoles/ml.
Stated purity of $NADP^+$ (Appendix G)	98%
Corrected concentration of NADPH	0.617 μmoles/ml.
Total amount of NADPH formed	1.851 μmoles
If the reaction is equimolar then the amount of oestrone formed is 1.851 x 0.27 mg.	0.4998 mg.
Amount of oestrone recovered by fluorescence	0.435 mg.
Percentage recovery	87

Amount of radioactive oestrone added to pooled assays 307 x 10^4 disintegrations/min.

Amount of radioactive oestrone recovered in countercurrent distribution 252 x 10^4 disintegrations/min.

Percentage recovery 82.1

TABLE 8

Theoretical distribution based on peak tube contents of 0.0775 mg.

Tube No.	Fraction of Θ	Ordinate	$\frac{\text{Ordinate}}{0.3989}$	Oestrone mg./tube
23/25	0.235	0.3885	0.975	0.075
22/26	0.470	0.3572	0.896	0.0694
21/27	0.705	0.3123	0.784	0.0608
20/28	0.94	0.2565	0.644	0.0499
19/29	1.175	0.2012	0.505	0.391
18/30	1.41	0.1476	0.369	0.0286
17/31	1.64	0.1040	0.261	0.0202
16/32	1.88	0.0681	0.171	0.0132
15/33	2.11	0.0431	0.108	0.0084
14/34	2.35	0.0252	0.063	0.0048
13/35	2.58	0.0143	0.036	0.0028
12/36	2.82	0.0075	0.019	0.0015
11/37	3.05	0.0038	0.0095	0.00074

ENZYME PURIFICATION

First procedure

Chicken liver was obtained within one hour of slaughter, transported in ice, dissected free of fatty tissue, minced in a chilled meat-grinder, and suspended in a 0.1M-sodium phosphate buffer, which contained nicotinamide (10 mM) and EDTA (1 mM). Three ml. of buffer mixture (pH 7.8) was used for each gram (wet weight) of tissue.

A homogenate was prepared in a Waring 'Blendor' at 80 volts for 1 min. at 5°. Phase-contrast microscopy of the homogenates and centrifuged debris showed that practically all the cells were ruptured under these conditions. The final pH of the homogenate was 7.7, it was centrifuged at 14,600 x g for 45 min. and the residue was discarded.

Ammonium sulphate (Mann's Special Enzyme Grade, 231 g./l.) was slowly added to the supernatant with constant stirring. The pH was maintained at 6.8 with 3M-ammonium hydroxide. After mixing for 30 min. at 4°, the precipitate was brought down at 14,600 x g for 30 min. then discarded.

This usually contained about 4 milli-units/ml. of 17β-oestradiol dehydrogenase activity after dialysis. No 17α-oestradiol dehydrogenase was ever detected in this fraction.

A second ammonium sulphate precipitate was made using 451 g./l. The precipitate was brought down at 14,600 x g and it was frequently necessary to centrifuge for 1 hr. to ensure adequate packing. This fraction was suspended in a minimal volume of 5 mM-sodium phosphate buffer (pH 7.2) which contained glycerol (50%, by volume). This was the stock preparation which usually measured 700 ml. from 1.5 Kg. fresh tissue. It was stable for several months when stored at -10 to -14°.

The ammonium sulphate fractions corresponded to 0 - 30% and 30 - 80% saturation calculated for 0°.

Chromatography on DEAE-cellulose

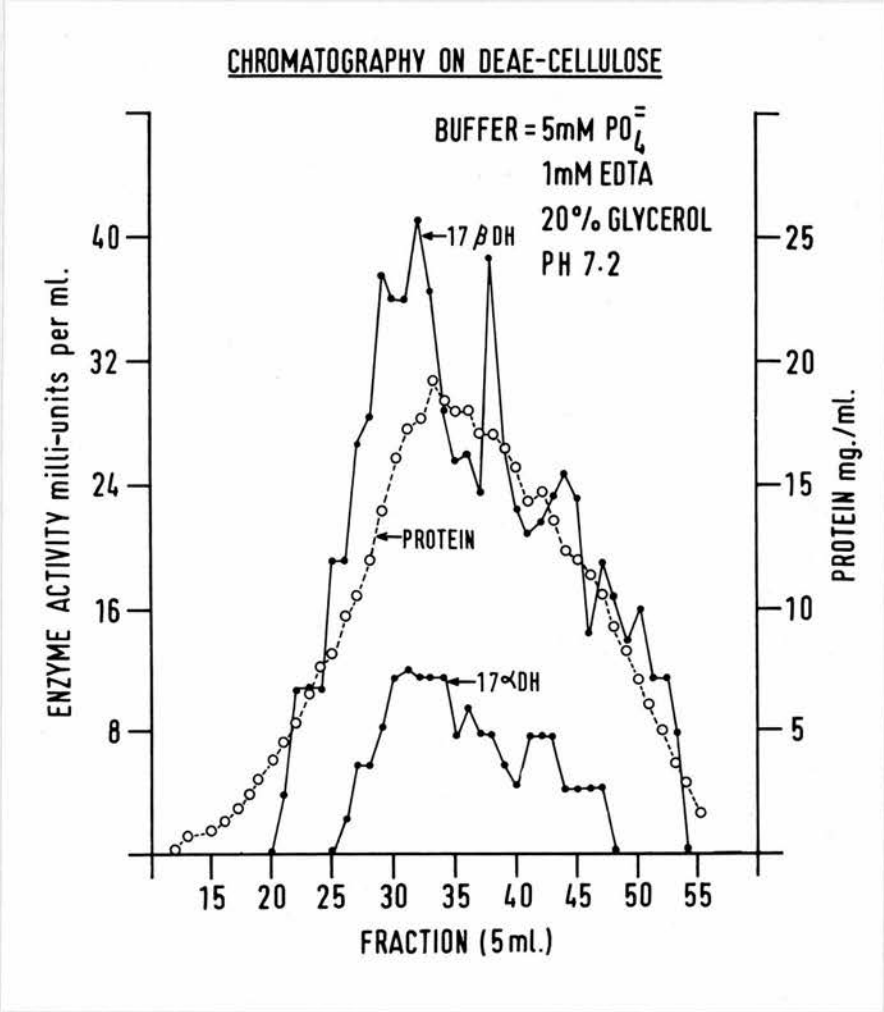
Further purification was effected on DEAE-cellulose (Appendix F). A jacketed glass column (diameter 5.0 cm.) was packed at room temperature using sodium phosphate buffer (5 mM) which contained EDTA (1 mM) and glycerol (20%, by volume). Preliminary experiments with a range of buffers from pH 7.0 - 8.6 and a final concentration of 1 - 10 mM showed that this mixture gave the best overall purification. The addition of glycerol was found to preserve the enzyme activities of column eluates for at least a week when stored at 5°. The cellulose column which measured 5 cm. x 40 cm. was gradually cooled to 5° by circulating refrigerant from a

Buchler refrigerated fraction collector and was held at that temperature for a minimum period of 24 hr. before use. Immediately before application of the sample, the column was washed with at least 1500 ml. of the same buffer mixture at 5°.

Approximately 40 ml. of the stored 30 - 80% ammonium sulphate precipitate was dialysed against four, 4-litre volumes of the equilibrating buffer at 5°, for 45 min. each change. The ammonium sulphate content of the dialysate was not checked, but longer periods of dialysis of up to 18 hr. duration showed no change in enzyme activity. The non-diffusible material which usually measured about 60 ml. was centrifuged at 34,800 x g for 20 min. The small amount of precipitate was discarded and the enzyme activity of the supernatant was assayed before chromatography.

Thirty ml. of this supernatant was applied to the column and eluted directly with the equilibrating buffer, using an L.K.B. 'Minipump' to ensure a constant flow-rate of 4 ml./cm^2/hr. Several experiments were carried out where the sample was held for 30 min. in the upper column bed prior to chromatography in the hope of achieving good equilibration, but no difference in elution pattern was observed. The eluate was monitored at 253 mμ on an L.K.B. fixed wavelength recorder and 80 drop fractions

Fig. 12



Approximately 40 ml. of the stock ammonium sulphate precipitate was dialysed against 5 mM-sodium phosphate buffer containing EDTA (1 mM) and glycerol (20%, by volume). The pH of this mixture was 7.2.

The non-diffusible material was centrifuged and 30 ml. of supernatant applied to a 5 cm. x 40 cm. column of DEAE-cellulose. 510 ml. of effluent was collected before the elution of protein. Enzyme assays and protein determinations were performed in duplicate on each fraction.

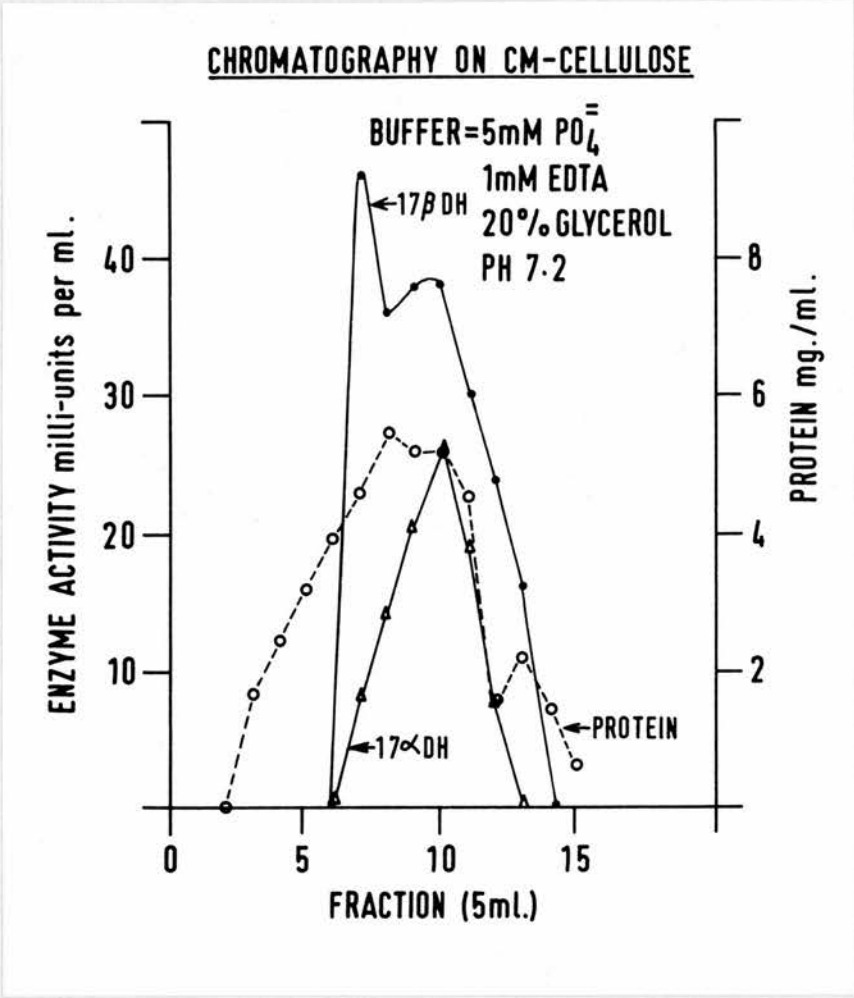
(4 - 5 ml.) were collected at 4°. A typical elution pattern is shown in Fig. 12. Enzyme assays and protein determinations were performed in duplicate on each fraction.

Chromatography on CM-cellulose

Those fractions which contained 17α-oestradiol dehydrogenase activity were pooled, and 30 ml. was chromatographed on a 5 cm. x 20 cm. column of CM-cellulose (Appendix F). The buffer mixture and method were those used for chromatography on DEAE-cellulose. A characteristic elution pattern is depicted in Fig. 13 where measurements of enzyme activities and protein concentration were carried out in duplicate on each fraction.

Representative tables of overall purification for both enzyme activities are given on pages 63 and 64 where the total recoveries after ammonium sulphate precipitation are estimates based on volumes used in chromatography. The yields of both activities were always greater after purification on DEAE-cellulose and this may have been due to removal of an 'inhibitor' present in crude homogenates and in the ammonium sulphate precipitate after dialysis.

Fig. 13



Those fractions which contained the most 17α-oestradiol dehydrogenase activity after chromatography on DEAE-cellulose were pooled and 30 ml. was applied to a 5 cm. x 20 cm. column of CM-cellulose, equilibrated and eluted with the phosphate-EDTA-glycerol mixture. A typical elution pattern is depicted where 240 ml. of effluent was collected before elution of protein. Enzyme assays and protein determinations were carried out in duplicate on each fraction.

TABLE 9

The purification of 17α-oestradiol dehydrogenase activity from 1.5 kilograms of fresh chicken liver

Procedure	Volume (ml.)	Total milli-units	Protein (mg./ml.)	Specific activity	Yield (%)	Purification
Homogenisation Tissue:Buffer = 1:3	7800	-	32	-	-	-
Centrifugation 14,600 x g	5860	23,440	31	0.1	100	1
'30-80%' $(NH_4)_2SO_4$ precipitation and dialysis	1000*	27,000*	67	0.4	115	4
DEAE-cellulose chromatography	3000*	54,000*	7.0	2.6	230	26
CM-cellulose chromatography	3000*	45,000*	3.0	5.0	191	50

*Estimated values based on volumes used in chromatography.

CHART II

Partial Purification of 17α- and 17β-Oestradiol Dehydrogenases from Chicken Liver (First Procedure)

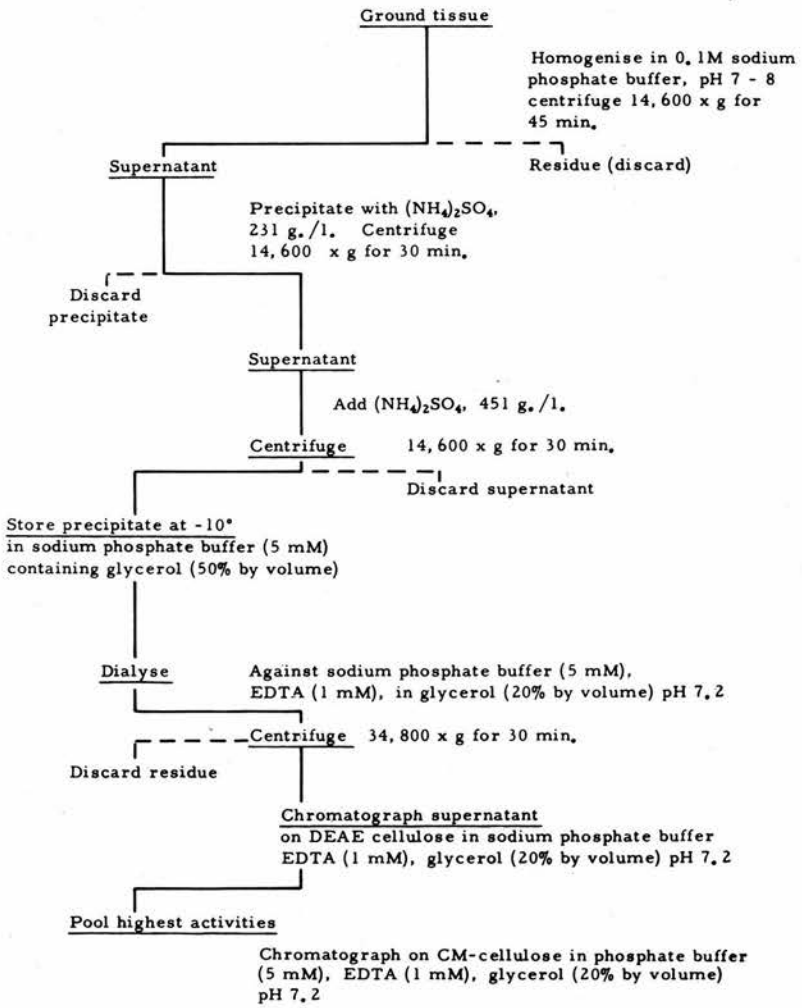


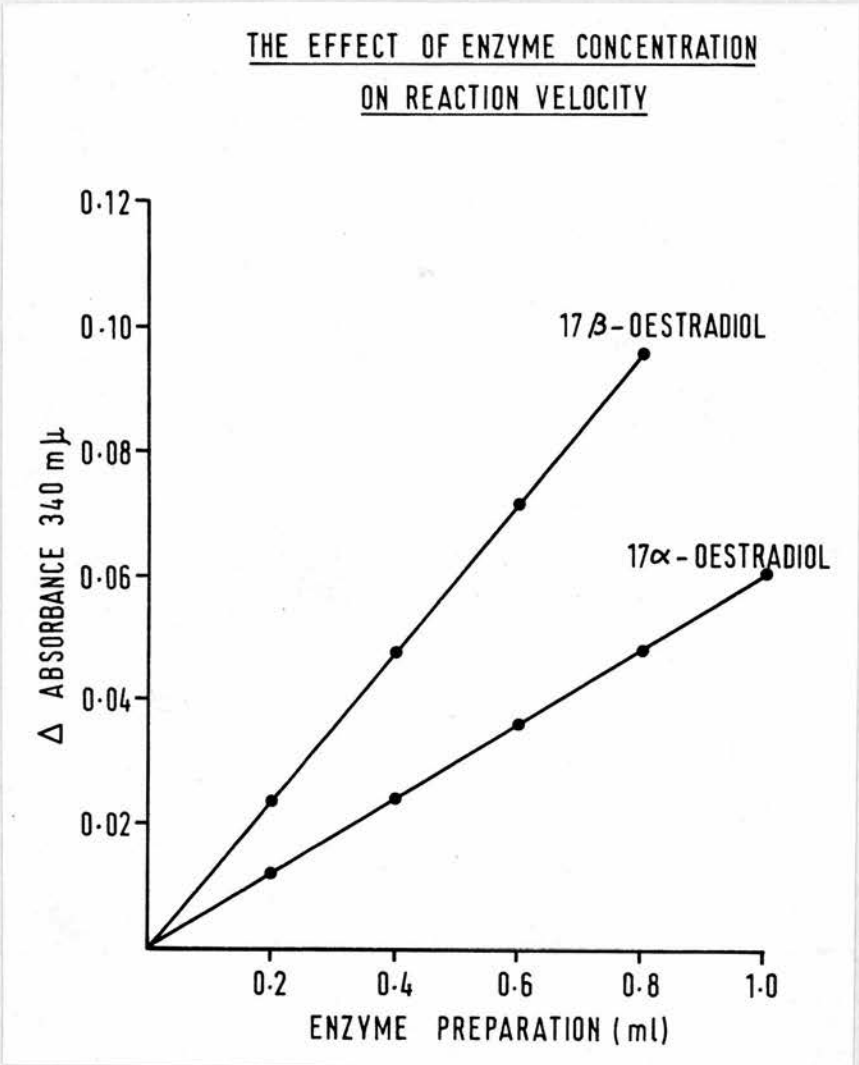
TABLE 10

The purification of 17β-oestradiol dehydrogenase activity from 1.5 kilograms of fresh chicken liver

Procedure	Volume (ml.)	Total Milli-units	Protein (mg./ml.)	Specific activity	Yield (%)	Purification
Homogenisation Tissue:Buffer 1:3	7800	-	32	-	-	-
Centrifugation 14,600 x g	5860	70,000	31	0.4	100	1
'30-80%' $(NH_4)_2SO_4$ precipitation and dialysis	1000*	62,000*	67	0.9	88	2
DEAE-cellulose chromatography	3000*	120,000*	7.0	5.7	171	8
CM-cellulose chromatography	3000*	105,000*	3.0	12.0	150	30

* Estimated values based on volumes used in chromatography.

Fig. 14



Ten ml. of an ammonium sulphate precipitate taken between 30 and 80% saturation and stored for 3 weeks at -10° was dialysed for 4 hr. against four changes of 5 mM-sodium phosphate buffer (pH 7.2) containing EDTA (1 mM). Four litres of buffer were used for each change. The non-diffusible material was centrifuged and 20 ml. of supernatant applied to a 2.3 cm. x 20 cm. column of DEAE-cellulose, equilibrated and eluted with the phosphate-EDTA buffer used for dialysis. The most active fractions were pooled and the rates of change of absorbance at 340 mμ were measured in duplicate for each volume of enzyme preparation using 17α- and 17β-oestradiol as substrates.

KINETIC PARAMETERS

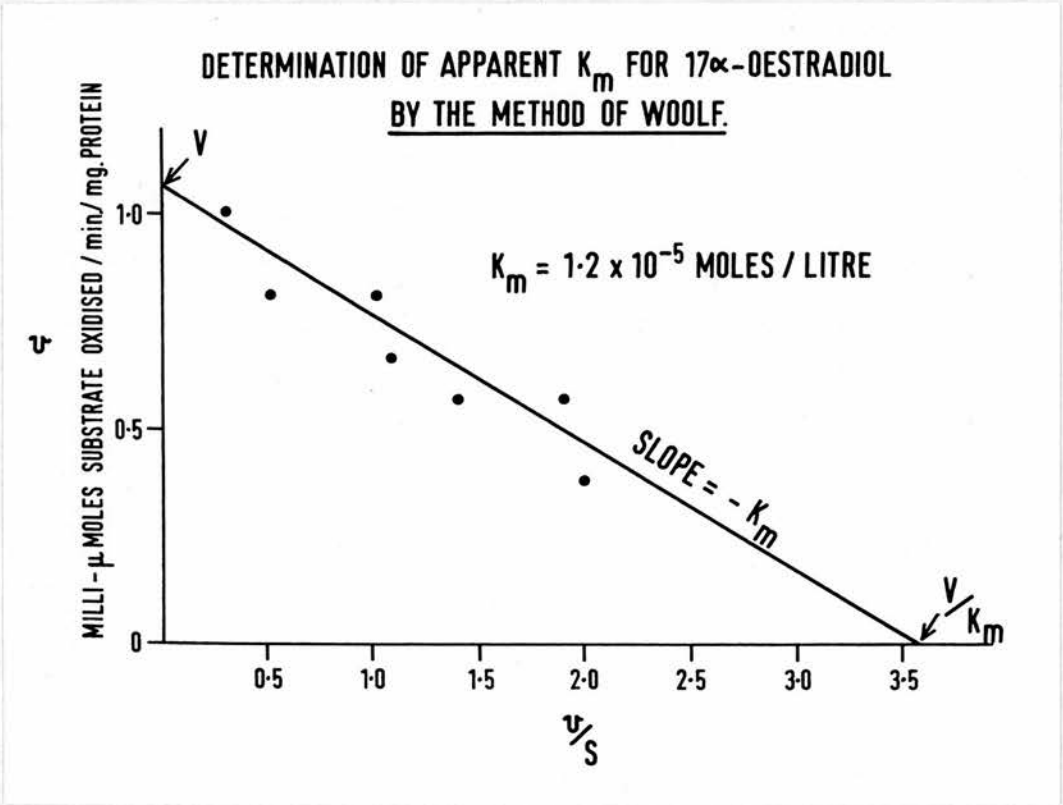
It was possible to determine certain kinetic parameters for both oestradiol dehydrogenase activities using eluates of DEAE-cellulose columns. The effect of enzyme concentration on reaction velocity is shown in Fig. 14.

The maximum initial velocity (V) which is theoretically obtained when the enzyme is 'saturated' with substrate, and the Michaelis constant (K_m), the value of (S) which is numerically equal to the concentration of substrate at half maximal velocity, were determined for 17α-oestradiol, 17β-oestradiol and $NADP^+$ (Appendix G).

Values of V and K_m were obtained by the linear method of Woolf (1932) advocated by Hofstee (1952). The regressions were obtained by the method of least squares without weighting (Figs. 15, 16, 17). I am indebted to Mr. I.A. Nimmo of the Department of Biochemistry, University of Edinburgh, for verification of these results by computer.

The reactions catalysed by 17α- and 17β-oestradiol dehydrogenase activities apparently followed Michaelis-Menten kinetics with respect to the steroid substrates 17α- and 17β-oestradiol. The determination of K_m for $NADP^+$ at 25° for both 'enzymes' gave non-linear plots (e.g. Fig. 17), although activities were low and rate differences between substrate concentrations were not marked. When measurements of K_m for $NADP^+$ were attempted at 37° for both 'enzymes', the plots were again non-linear and suggestive of inhibition of the reactions at high substrate concentrations (Tables 11, 12).

Fig. 15



A homogenate of fresh liver was prepared, heated to 50° in a 65° water bath, cooled rapidly and centrifuged. The supernatant was fractionated with ammonium sulphate, and the precipitate obtained between 40 - 60% saturation was stored in glycerol-buffer at -10° for 5 weeks before dialysis and chromatography on DEAE-cellulose. The most active fractions were pooled and used as the enzyme preparation in these experiments.

Assays were carried out in triplicate at 25° for each concentration of 17α-oestradiol in the presence of 'saturating' amounts of cofactor. Mean values of initial velocity were used for determination of K_m. The assay mixtures at each substrate concentration were pooled in redistilled ethanol and the reaction product, oestrone, was identified by thin-layer chromatography.

TABLE 11

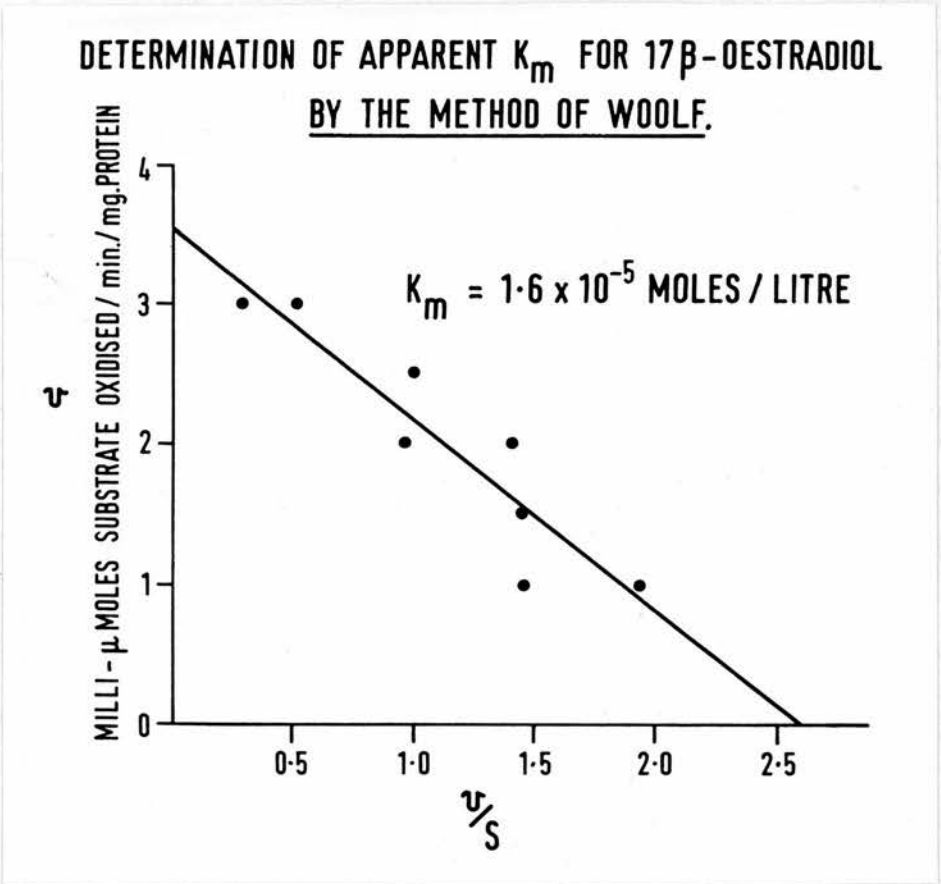
Typical values obtained in experiments to determine K_m for $NADP^+$ in respect of '17α-oestradiol dehydrogenase'.

Substrate Concentration ($\times 10^{-4}$M)	Initial velocity (v)	$\frac{\text{Initial velocity}}{\text{Substrate concentration}}$ $\left(\frac{v}{s}\right)$
1.30	64	49
1.00	62	62
0.60	60	100
0.50	60	120
0.30	50	167
0.25	40	160
0.17	24	141

An ammonium sulphate precipitate taken between 30 and 80% saturation was dialysed and chromatographed on DEAE-cellulose in 5 mM-sodium phosphate buffer (pH 7.2) containing glycerol (20%, by volume). The most active fractions were pooled and 0.5 ml. of this preparation was used in each assay carried out at 37° in duplicate at each substrate concentration.

The initial velocity (v) is the mean net rate of change of absorbance at 340 mμ expressed in optical density units to illustrate the small changes in net rate at each substrate concentration ($NADP^+$ is considered as substrate in this experiment).

Fig. 16



A homogenate of fresh liver was prepared, heated to 50° in a 65° water bath, cooled rapidly and centrifuged. The supernatant was fractionated with ammonium sulphate, and the precipitate obtained between 40 - 60% saturation was stored in glycerol-buffer at -10° for 5 weeks before dialysis and chromatography on DEAE-cellulose. The most active fractions were pooled and used as the enzyme preparation in these experiments.

Assays were carried out in triplicate at 25° for each concentration of 17β-oestradiol in the presence of 'saturating' amounts of cofactor. Mean values of initial velocity were used for determination of K_m. The assay mixtures at each substrate concentration were pooled in redistilled ethanol and the reaction product, oestrone, was identified by thin-layer chromatography.

TABLE 12

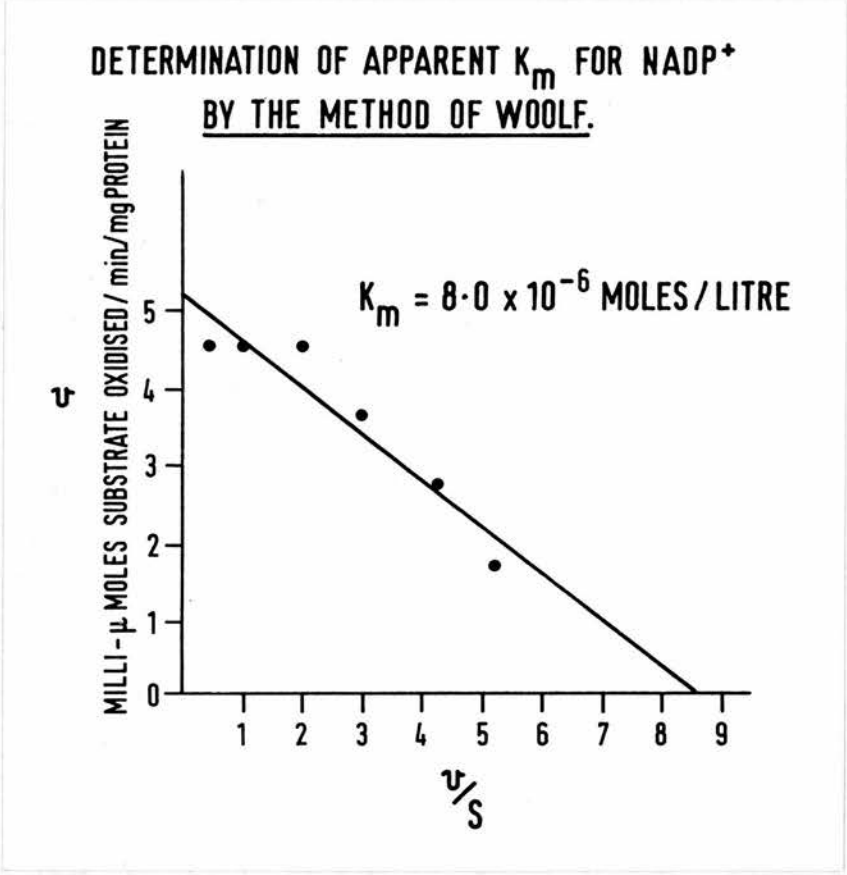
Typical values obtained during experiments to determine K_m for $NADP^+$ in respect of '17β-oestradiol dehydrogenase'.

Substrate Concentration ($\times 10^{-4}$M)	Initial velocity (v)	$\frac{\text{Initial velocity}}{\text{Substrate concentration}} \left(\frac{v}{s}\right)$
1.30	202	155
1.00	200	200
0.60	180	300
0.50	162	324
0.30	80	267
0.25	25	100

An ammonium sulphate precipitate taken between 30 and 80% saturation was dialysed and chromatographed on DEAE-cellulose in 5 mM-sodium phosphate buffer (pH 7.2) containing glycerol (20%, by volume). The most active fractions were pooled and 0.5 ml. of this preparation was used in each assay carried out at 37° in duplicate at each substrate concentration.

The initial velocity (v) is the mean net rate of change of absorbance at 340 mμ expressed in optical density units to illustrate the small changes in net rate at high substrate concentration ($NADP^+$ is considered as substrate in this experiment).

Fig. 17



A homogenate of fresh liver was prepared, heated to 50° in a 65° water bath, cooled rapidly and centrifuged. The supernatant was fractionated with ammonium sulphate and the precipitate obtained between 40 - 60% saturation was stored in glycerol-buffer at -10° for 6 weeks before dialysis and chromatography on DEAE-cellulose. The most active fractions were pooled and used as the enzyme preparation in these experiments.

Assays were carried out in triplicate at 25° for each concentration of $NADP^+$ in the presence of 'saturating' amounts of 17β-oestradiol. Mean values of initial velocity were used for determination of K_m. The assay mixtures at each concentration of $NADP^+$ were pooled in redistilled ethanol and the reaction product, oestrone, was identified by thin-layer chromatography.

Attempts to measure Michaelis constants for NAD^+ for both oestradiol dehydrogenase activities were unsuccessful. When this nucleotide was used in the routine oxidative assay in a final concentration of 1.3×10^{-4}M (i.e. equivalent to a 'saturating' concentration of $NADP^+$) the initial velocity was about one quarter of that obtained with $NADP^+$. No oestradiol dehydrogenase activity was found when the concentration of NAD^+ was reduced. On increasing the concentration of NAD^+ activity in the ethanol blank increased and it was impossible to measure a difference in the rate of change of absorbance between test and blank. These results suggested the presence of an 'NAD^+-specific alcohol dehydrogenase' in crude enzyme preparations and that the values of K_m for both oestradiol dehydrogenase activities may be considerably higher than those for $NADP^+$.

TABLE 13

Apparent Michaelis constants and maximal velocities for '17α-oestradiol dehydrogenase' at 25°.

Substrate	K_m (moles/litre)	Maximal velocity
17α-oestradiol	1.2×10^{-5}	1.03
	1.3×10^{-5}	1.1
$NADP^+$	3.8×10^{-6}	2.6
	3.7×10^{-6}	2.2

In column 1 'substrate' refers to the compound under test.

*Maximal velocity is expressed in milli-μmoles substrate oxidised/min./mg. protein.

Ammonium sulphate precipitates taken between 30 and 80% or 40 and 60% saturation were dialysed and chromatographed on DEAE-cellulose in 5 mM-sodium phosphate buffer (pH 7.2) containing glycerol (20%, by volume). Assays were carried out in duplicate or triplicate at each substrate concentration.

TABLE 14

Apparent Michaelis constants and maximal velocities for '17β-oestradiol dehydrogenase' at 25°.

Substrate	K_m (moles/litre)	Maximal velocity*
17β-oestradiol	1.6×10^{-5}	3.5
	1.6×10^{-5}	3.5
$NADP^+$	8×10^{-6}	5.2

In column 1 'substrate' refers to the compound under test.

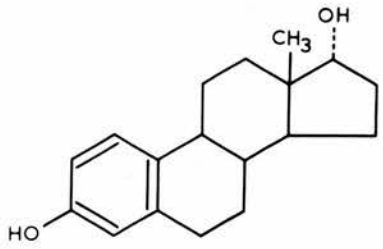
*Maximal velocity is expressed in milli-μmoles substrate oxidised/min./mg. protein.

Ammonium sulphate precipitates taken between 30 and 80% or 40 and 60% saturation were dialysed and chromatographed on DEAE-cellulose in 5 mM-sodium phosphate buffer (pH 7.2) containing glycerol (20%, by volume).

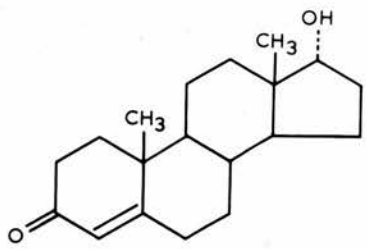
Assays were carried out in duplicate or triplicate at each substrate concentration.

Fig. 18

THE STRUCTURES OF STEROIDS MENTIONED IN THE TEXT.



17∝-OESTRADIOL



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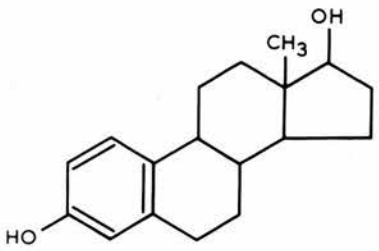

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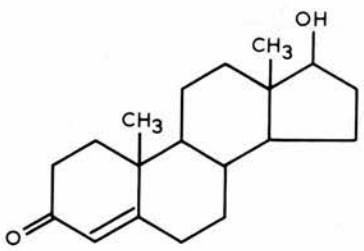
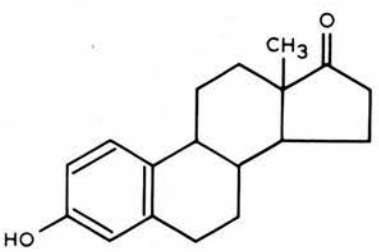
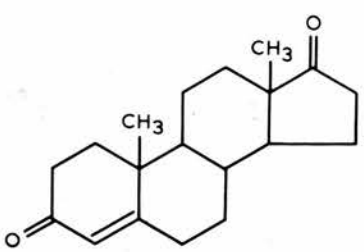


TESTOSTERONE



OESTRONE



ANDROSTENEDIONE

REACTIVITY TOWARDS OTHER STEROID SUBSTRATES

Eluates of DEAE-cellulose columns which oxidised 17α- and 17β-oestradiol to oestrone, also oxidised epi-testosterone and testosterone to androstenedione (Fig.18). This reaction product was provisionally identified by comparison of its mobility with that of the authentic compound in the thin-layer system described in Appendix A.

The initial velocities obtained with the C_{19}-substrates varied from 50 - 75% of those determined with the oestradiol epimers. Although the concentration of the C_{19}-steroids was 0.2 μmoles per cuvette (as in the routine oxidative assay of oestradiol dehydrogenase activities) maximal initial velocities and Michaelis constants were not determined for those compounds. It follows that no firm conclusions could be drawn from these experiments.

THE STABILITY OF 17α- AND 17β-OESTRADIOL DEHYDROGENASE ACTIVITIES

The yields of 17α- and 17β-oestradiol dehydrogenase activities were of the same order of magnitude whether the tissue was fresh or had been stored for 3 days at 4°. The addition of 1 mM-cysteine hydrochloride or 10 - 100 mM nicotinamide to the buffer used for extraction neither increased the yield nor the stability of these preparations.

Centrifuged homogenates prepared at pH 7 - 8 showed no loss of activity when stored for 24 hr. at 4°. The activities of the stock ammonium sulphate preparation (taken between 30 and 80% saturation) were stable for at least 4 months when stored in 5 mM-phosphate buffer containing glycerol (50%, by volume). Whilst there was no loss of 17β-dehydrogenase activity during that time the recovery of the 17α-enzyme varied from 75 - 100%.

Eluates of DEAE-cellulose columns frequently lost one third - one half of both enzyme activities after 12 - 18 hr. at 4°; the 17β-oestradiol dehydrogenase was the more stable of the two. When glycerol (20%, by volume) was included in the buffers used for chromatography, there was no loss of activity in eluates stored for 7 days

at 4°. The 17β-oestradiol dehydrogenase activity in these preparations was also stable at 19 - 23° for at least 3 days; the 17α-oestradiol dehydrogenase was almost completely lost within 12 hr. under the same conditions.

Preparations which had been purified sequentially on DEAE- and CM-cellulose retained full activity for at least 3 days when stored at 4° in 5 mM-sodium phosphate buffer (pH 7.2) containing glycerol (20%, by volume).

TABLE 15

The stability of 17α- and 17β-oestradiol dehydrogenase activities

Preparation	Specific Activity			
	17αDH		17βDH	
	I	II	I	II
Centrifuged homogenate after 24 hr. at 5°	0.1	0.1	0.3	0.3
'30-80%' ammonium sulphate precipitate kept for 4 months at -10° in glycerol (50%, by volume) and dialysed	0.4	0.4	0.9	0.9
Eluate of DEAE-cellulose column after 24 hr. at 5° (without glycerol)	0.4	0.2	3.0	3.0
Eluate of DEAE-cellulose column containing glycerol (20%, by volume) after 7 days at 5°.	3.0	3.0	6.0	6.0
Eluate of DEAE-cellulose containing glycerol (20%, by volume) after 3 days at 19-23°	3.0	0	6.0	6.0
Preparation obtained by chromatography on DEAE- and CM-cellulose stored at 5° for 3 days in 5 mM-sodium phosphate buffer containing glycerol (20%, by volume)	5.0	5.0	12.0	12.0

Column I lists enzyme activities assayed within 4 hr. of preparation.

Column II lists enzyme activities assayed after storage.

OTHER METHODS OF PURIFICATION

Alterations in tissue:buffer ratio

Attempts to extract more than 1.5 kilograms of fresh tissue using a tissue:buffer ratio of 1:3 were unsuccessful because of the difficulty of centrifuging more than 8 l. of homogenate in the 'Servall' instrument. Large-capacity ($\simeq$ 6L) centrifuges were unsuitable by reason of their low maximum 'g' values ($\simeq$2000). The 'Sharples' steam-driven centrifuge also proved inadequate, on account of the large amounts of cell debris which rapidly filled the collection chamber of that machine. Homogenates prepared between pH 7 - 8 with a tissue: buffer ratio of 1:2 could not be fractionated cleanly with ammonium sulphate.

The composition of the extraction buffer

The yields of 17α- and 17β-oestradiol dehydrogenase activities were not increased by the addition of glycerol (10 or 20%, by volume) to the buffer used for homogenisation. Salt fractionation in this mixture was also unsatisfactory.

Further chromatography on CM-cellulose

The use of two large columns in the first method of purification (p. 59) proved cumbersome and time-consuming. More important, the capacity of the ion-exchangers, particularly that of CM-cellulose, was not used to advantage, therefore experiments were carried out to define the optimal conditions for chromatography on that medium. It was hoped that sufficient purification could be obtained on CM-cellulose to eliminate the need for further large-scale chromatography.

Fifty ml. of an ammonium sulphate precipitate which had been taken between 40 and 60% saturation was dialysed against five 2-litre changes of a solution containing sodium bicarbonate (50 mM) and glycerol (20%, by volume). The final pH was 6.8. Dialysis was continued against five 2-litre changes of sodium phosphate buffer (1 mM) which contained glycerol (20%, by volume). The pH of this mixture was 6.4 at 5°. The total time of dialysis was 6 hr. The non-diffusible material was centrifuged at 34,800 x g for 30 min. and the residue discarded.

The supernatant was assayed for both 17α- and 17β-oestradiol dehydrogenase activities and 18 ml. was applied to a 2.3 cm. x 19 cm. column of CM-cellulose, equilibrated and eluted with 1.0 mM-sodium phosphate buffer (pH 6.4) which contained glycerol (20%, by volume).

A flow-rate of 4 ml./cm^2/hr. was maintained by L.K.B. 'Minipump'. Four discrete pigmented bands were formed. The most dense remained in the upper column bed. Sixty-five ml. of effluent was measured before 'ultraviolet-absorbing' material was eluted; this was collected in 5 ml. fractions at 5°.

During the routine oxidative assay of these fractions a precipitate formed in the cuvette on the addition of sodium bicarbonate-carbonate buffer. The precipitate came down irrespective of the order in which the assay constituents were added; however, it dissolved quickly on stirring. No 17α-oestradiol dehydrogenase activity was detected in the first seven fractions. Because of the possibility of loss of enzyme activity when precipitation occurred in the assay system, it was decided to incubate the fractions (which were pooled sequentially in batches of eight) and to examine the steroid reaction products by thin-layer chromatography.

TABLE 16

Thin-layer chromatography of CM-cellulose eluate

Fraction	Conversion to Oestrone	
	Substrate 17α-oestradiol	Substrate 17β-oestradiol
1 - 8	0	0
9 - 16	0	++
17 - 24	±	++
25 - 32	±	++
'Column bed'	0	++
Washed CM-cellulose	0	0

++ = good conversion
± = trace
0 = no conversion

Five ml. of eluate and 10 ml. of each 'slurry' were incubated at 37° for 30 min. in air, at pH 8.5 with 5 μ-moles substrate and 25 μmoles $NADP^+$.

Chromatography was repeated on a 1.5 cm. x 18 cm. column of CM-cellulose, equilibrated and eluted with 1 mM-sodium phosphate buffer containing glycerol (20%, by volume). As soon as 'ultraviolet-absorbing' material was eluted, a precipitate formed in the column outlet and in the 'Teflon' connecting tube which led to the fraction-collector. Incubations of pooled fractions showed that 17β-oestradiol dehydrogenase activity was eluted in the first fractions. No 17α-oestradiol dehydrogenase

activity was found in spite of increasing the molarity of the eluting buffer to 0.1M. The column bed was removed and 'slurries' were incubated at pH 8.5 - 9.0; oestrone was formed from both 17α- and 17β-oestradiol. Washed CM-cellulose which had not been used for chromatography did not oxidise the steroid substrates under the same conditions.

The diameter of the columns was increased to 5.0 cm. and a white 'waxy' material appeared in the upper column bed. The maximum amount of dialysate which could be chromatographed on a 5 cm. x 20 cm. column without disruption of the elution pattern was 30 ml. It seemed possible that this material was a protein which was precipitated below pH 7.0. This may also have been the explanation for the turbidity which occurred in the spectrophotometric assay when attempts were made to determine the pH dependence of the enzyme activities.

Prolonged dialysis against buffers of low pH

In order to increase the load on a 5 cm. x 20 cm. CM-cellulose column without disruption of the elution pattern, it was decided to dialyse samples of the stock ammonium sulphate preparation for 18 hr. against 5 mM-sodium phosphate buffer (pH 6.5) containing glycerol (20%, by volume). No 17α-oestradiol dehydrogenase

activity was found in the non-diffusible material although this had been present after 4 hr. dialysis against the same buffer mixture. The specific activity of the 17β-oestradiol dehydrogenase had risen from 0.5 to 1 milli-unit/mg. of protein.

Extraction of enzyme activities at low pH

The first method of purification (p. 59) was then modified to include precipitation at low pH. Fresh chicken liver stored at 4° for 48 hr. was hand-minced and 1.5 Kg. (wet weight) of tissue was homogenised with 3 l. 0.1M-ammonium citrate (pH 5.5) at 80 volts for 1 min. The pH was adjusted to 6.8 with 1.0M tribasic sodium phosphate and ammonium sulphate precipitates were taken at 30 and 80% saturation. The second precipitate was stored at -10° in 5 mM-phosphate buffer containing glycerol (50%, by volume). After dialysis of 20 ml. of this preparation against four, 4-litre volumes of 5 mM-sodium phosphate buffer (pH 7.2) for 1 hr. each change, the non-diffusible material was centrifuged at 34,800 x g for 20 min. The supernatant contained 0.6 - 0.8 milli-units 17α-oestradiol dehydrogenase and 0.8 - 1.0 milli-units 17β-oestradiol dehydrogenase per mg. of protein.

The effect of heat on 17α- and 17β-oestradiol dehydrogenase activities at various pH values

The effect of heat on 17α- and 17β-oestradiol dehydrogenase activities at various pH values indicated that the effect of heat should be studied over a wide range of pH. The following experiments were carried out before the spectrophotometric assay was established and thin-layer chromatography was used to determine the steroid reaction products from incubation mixtures.

Fresh chicken liver stored at -14° for 24 hr. was homogenised with 0.1M buffer (Table 17) in a 'Virtis' at reduced speed. The tissue:buffer ratio was 1:2. The homogenates were centrifuged at 5020 x g for 30 min. and the residues discarded. One ml. of 0.1M-calcium chloride in aqueous solution was added to each 10 ml. of supernatant which was stirred at 5° for 1 hr. The preparations were centrifuged at 14,600 x g for 30 min. and 100 ml. of each supernatant was heated in a stainless steel beaker to 55° in a 70° water-bath, and held at that temperature for 10 min. The preparations were cooled rapidly to 4° and centrifuged at 24,000 x g for 30 min.

Five ml. volumes of the second supernatant were incubated for 30 min. in air at 37° in a Dubnoff incubator. The pH of each incubation was adjusted where necessary to pH 7.4 - 8.0 with a few drops of N-NaOH or 0.1N-HCl. The steroids were extracted with 3 x 5 ml. volumes of redistilled methylene chloride, taken to dryness on a rotary evaporator, and examined by thin-layer chromatography.

TABLE 17

The effect of heat on 17α- and 17β-oestradiol dehydrogenase activities at various pH values

Homogenising buffer	pH at incubation	Substrate (3 μmoles)	Cofactor (0.1 μmole)	Result (oestrone formed)
Sodium carbonate-bicarbonate, pH 9.9	8.9	17α-oestradiol	NAD^+	+
		17α-oestradiol	$NADP^+$	+
		17β-oestradiol	NAD^+	+++
		17β-oestradiol	$NADP^+$	+++
Sodium carbonate-bicarbonate, pH 9.0	8.4	17α-oestradiol	NAD^+	++
		17α-oestradiol	$NADP^+$	++
		17β-oestradiol	NAD^+	+++
		17β-oestradiol	$NADP^+$	+++
Sodium phosphate-sodium dihydrogen orthophosphate, pH 8.0	7.3	17α-oestradiol	NAD^+	+
		17α-oestradiol	$NADP^+$	+
		17β-oestradiol	NAD^+	++
		17β-oestradiol	$NADP^+$	++
Sodium phosphate-sodium dihydrogen orthophosphate, pH 6.9	6.8	17α-oestradiol	NAD^+	±
		17α-oestradiol	$NADP^+$	±
		17β-oestradiol	NAD^+	+
		17β-oestradiol	$NADP^+$	+

(Continued on next page)

TABLE 17 (contd.)

Homogenising buffer	pH at incubation	Substrate (3 µmoles)	Cofactor (0.1 µmole)	Result (oestrone formed)
Sodium phosphate-sodium dihydrogen orthophosphate, pH 6.1	6.5	17α-oestradiol	NAD^+	0
		17α-oestradiol	$NADP^+$	0
		17β-oestradiol	NAD^+	0
		17β-oestradiol	$NADP^+$	+
Citric acid - sodium citrate, pH 5.0	4.8	17α-oestradiol	NAD^+	0
		17α-oestradiol	$NADP^+$	0
		17β-oestradiol	NAD^+	0
		17β-oestradiol	$NADP^+$	±
Citric acid - sodium citrate, pH 4.0	4.3	17α-oestradiol	NAD^+	±
		17α-oestradiol	$NADP^+$	±
		17β-oestradiol	NAD^+	±
		17β-oestradiol	$NADP^+$	0

+++ = very good conversion
++ = good conversion
+ = fairly good conversion
± = trace
0 = no conversion

Homogenates were heated to 55° for 10 min. then centrifuged. Five ml. of supernatant was used for each incubation at 37° for 30 min. in air.

The previous experiments were extended. Homogenates were prepared in the same way from the same batch of liver which had been stored at -14° for 3 days. Aliquots were heated to 45°, 50°, 55° and 60° for 10 min., cooled rapidly to 4° and centrifuged at 24,000 x g for 30 min. The pH of each incubation mixture was adjusted to 8.5, before heating to 37° for 30 min. in a Dubnoff water-bath. Protocols and results are summarised in Tables 18, 19, 20, 21.

TABLE 18

The effect of heat on 17α- and 17β-oestradiol dehydrogenase activities in a homogenate prepared with sodium dihydrogen orthophosphate-sodium phosphate buffer (0.1M) at pH 6.1

Heat treatment	Substrate (3 μmoles)	Cofactor (0.1 μmole)	Result (oestrone formed)
None	17α-oestradiol	NAD^+	+++
	17α-oestradiol	$NADP^+$	+++
	17β-oestradiol	NAD^+	+++
	17β-oestradiol	$NADP^+$	+++
45°	17α-oestradiol	NAD^+	++
	17α-oestradiol	$NADP^+$	+
	17β-oestradiol	NAD^+	+++
	17β-oestradiol	$NADP^+$	+++
50°	17α-oestradiol	NAD^+	+
	17α-oestradiol	$NADP^+$	+
	17β-oestradiol	NAD^+	+++
	17β-oestradiol	$NADP^+$	+++
55°	17α-oestradiol	NAD^+	++
	17α-oestradiol	$NADP^+$	++
	17β-oestradiol	NAD^+	+++
	17β-oestradiol	$NADP^+$	+++
60°	17α-oestradiol	NAD^+	++
	17α-oestradiol	$NADP^+$	++
	17β-oestradiol	NAD^+	+++
	17β-oestradiol	$NADP^+$	+++

+++ = very good conversion
++ = good conversion
+ = fairly good conversion

Homogenates were heated at the stated temperature for 10 min. then centrifuged. Five ml. of supernatant was used for each incubation at 37° in air for 30 min. at pH 8.5.

TABLE 19

The effect of heat on 17α- and 17β-oestradiol dehydrogenase activities in a homogenate prepared with sodium dihydrogen orthophosphate-sodium phosphate buffer (0.1M) at pH 6.9

Heat treatment	Substrate (3 μmoles)	Cofactor (0.1 μmole)	Result (oestrone formed)
None	17α-oestradiol	NAD^+	+++
	17α-oestradiol	$NADP^+$	+++
	17β-oestradiol	NAD^+	+++
	17β-oestradiol	$NADP^+$	+++
45°	17α-oestradiol	NAD^+	+++
	17α-oestradiol	$NADP^+$	+++
	17β-oestradiol	NAD^+	+++
	17β-oestradiol	$NADP^+$	+++
50°	17α-oestradiol	NAD^+	+++
	17α-oestradiol	$NADP^+$	+++
	17β-oestradiol	NAD^+	+++
	17β-oestradiol	$NADP^+$	+++
55°	17α-oestradiol	NAD^+	++
	17α-oestradiol	$NADP^+$	++
	17β-oestradiol	NAD^+	+++
	17β-oestradiol	$NADP^+$	+++
60°	17α-oestradiol	NAD^+	++
	17α-oestradiol	$NADP^+$	++
	17β-oestradiol	NAD^+	+++
	17β-oestradiol	$NADP^+$	+++

+++ = very good conversion
++ = good conversion

Homogenates were heated at the stated temperature for 10 min. then centrifuged. Five ml. of supernatant was used for each incubation at 37° for 30 min. at pH 8.5, in air.

TABLE 20

The effect of heat on 17α- and 17β-oestradiol dehydrogenase activities in a homogenate prepared with sodium dihydrogen orthophosphate-sodium phosphate buffer (0.1M) at pH 8.0

Heat treatment	Substrate (3 μmoles)	Cofactor (0.1 μmole)	Result (oestrone formed)
None	17α-oestradiol	NAD^+	+++
	17α-oestradiol	$NADP^+$	+++
	17β-oestradiol	NAD^+	+++
	17β-oestradiol	$NADP^+$	+++
45°	17α-oestradiol	NAD^+	+++
	17α-oestradiol	$NADP^+$	+++
	17β-oestradiol	NAD^+	+++
	17β-oestradiol	$NADP^+$	+++
50°	17α-oestradiol	NAD^+	+++
	17α-oestradiol	$NADP^+$	+++
	17β-oestradiol	NAD^+	+++
	17β-oestradiol	$NADP^+$	+++
55°	17α-oestradiol	NAD^+	++
	17α-oestradiol	$NADP^+$	++
	17β-oestradiol	NAD^+	+++
	17β-oestradiol	$NADP^+$	+++
60°	17α-oestradiol	NAD^+	++
	17α-oestradiol	$NADP^+$	++
	17β-oestradiol	NAD^+	+++
	17β-oestradiol	$NADP^+$	+++

+++ = very good conversion
++ = good conversion

Homogenates were heated at the stated temperature for 10 min. then centrifuged. Five ml. of supernatant was used for each incubation at 37° for 30 min. at pH 8.5, in air.

TABLE 21

The effect of heat on 17α- and 17β-oestradiol dehydrogenase activities in a homogenate prepared with sodium bicarbonate-sodium carbonate buffer (0.1M) at pH 9.0

Heat treatment	Substrate (3 μmoles)	Cofactor (0.1 μmole)	Result (oestrone formed)
None	17α-oestradiol	NAD^+	++
	17α-oestradiol	$NADP^+$	+++
	17β-oestradiol	NAD^+	+++
	17β-oestradiol	$NADP^+$	+++
45°	17α-oestradiol	NAD^+	+
	17α-oestradiol	$NADP^+$	++
	17β-oestradiol	NAD^+	+++
	17β-oestradiol	$NADP^+$	+++
50°	17α-oestradiol	NAD^+	±
	17α-oestradiol	$NADP^+$	±
	17β-oestradiol	NAD^+	++
	17β-oestradiol	$NADP^+$	+++
55°	17α-oestradiol	NAD^+	±
	17α-oestradiol	$NADP^+$	±
	17β-oestradiol	NAD^+	++
	17β-oestradiol	$NADP^+$	++
60°	17α-oestradiol	NAD^+	±
	17α-oestradiol	$NADP^+$	±
	17β-oestradiol	NAD^+	±
	17β-oestradiol	$NADP^+$	±

+++ = very good conversion
++ = good conversion
+ = fairly good conversion
± = trace

Homogenates were heated at the stated temperature for 10 min. then centrifuged. Five ml. of supernatant was used for each incubation at 37° for 30 min. at pH 8.5, in air.

The effects of heat on 17α- and 17β-oestradiol dehydrogenase activities were reinvestigated when the spectrophotometric assay was established. At that time assays were carried out at 25°, and it was rarely possible to detect any 17α- or 17β-oestradiol dehydrogenase activity in crude homogenates at that temperature. Heating for 10 min. at 50° followed by centrifugation, resulted in removal of some 12 mg. protein/ml. About 50% of both activities was lost; this estimate was based on subsequent purifications and allowance was made for increased reactivity at the higher assay temperature (37°).

Fresh preparations were heated to 50° and cooled quickly to 4° but losses remained high. Volumes greater than 1 l. proved difficult to cool quickly, and heat treatment of the crude homogenate from 1.5 Kg. of liver was impracticable in small volumes. Attempts to concentrate the enzyme activities in centrifuged, heat-treated homogenates by ammonium sulphate fractionation resulted in further losses. Because of such losses in the first stages of purification and the risk of permanent structural alterations to the remaining enzyme molecules, purification by heat treatment was discontinued.

Freeze-drying

An attempt was made to prepare a concentrate of 17α- and 17β-oestradiol dehydrogenase activities by freeze-drying.

Chicken liver stored at -7° for two weeks was homogenised in a 'Virtis' at 60 volts for 1 min. Two ml. of 10 mM-sodium phosphate buffer (pH 7.2) which contained EDTA (1 mM) was used for each gram (wet weight) of tissue. The preparation was centrifuged at 16,300 x g for 30 min. and the residue discarded. The supernatant was centrifuged for a further 30 min. at 24,000 x g and 30 ml. of the second supernatant was applied to a column of DEAE-cellulose (1.9 cm. x 39 cm.). This had been previously equilibrated with the homogenising buffer at 5°. Elution was carried out with the same buffer mixture.

Five ml. fractions were collected at 5° and assayed spectrophotometrically. The most active were pooled, and 20 ml. of the pooled eluate was shell-frozen in a 500 ml. round bottom flask and lyophilised. The dry residue was suspended in 2 ml. 10 mM-sodium phosphate buffer (pH 7.2) prior to spectrophotometric assay at 25°. The results are shown in Table 22.

TABLE 22

The effect of freeze-drying on 17α- and 17β-oestradiol dehydrogenase activities

Preparation	Specific Activity (milli-units/mg. protein) 17α-oestradiol dehydrogenase	17β-oestradiol dehydrogenase
Pooled eluate	0.6	1.2
Reconstituted after freeze-drying	0.3	0.5

Preparation of acetone powder

One hundred grams (wet weight) of fresh chicken liver was homogenised in a 'Virtis' for 1 min. at 60 volts with 200 ml. 0.1M-sodium phosphate buffer pH 8.0 at 4°. The homogenate was centrifuged at 4,920 x g for 30 min. and the residue discarded.

The supernatant was heated to 50° in a 65° water-bath, held at that temperature for 10 min., cooled rapidly to 4° and centrifuged for 30 min. at 14,600 x g. The residue was discarded and 90 ml. of supernatant was stirred into 1 l. of acetone at -5°. A pale pink flocculating precipitate was formed, stirring was continued for 5 min. then the acetone suspension was centrifuged at 14,600 x g for 30 min. at -10°.

The supernatant was discarded and the powder resuspended and washed twice with fresh acetone at -10°. The precipitate from 250 ml. of suspension was immediately dissolved in 20 ml. 0.1M-sodium phosphate buffer, pH 8.0. Five ml. volumes were incubated at 37° for 30 min. with steroid substrates in the presence of added cofactors. The steroid reaction products were examined by thin-layer chromatography. Incubations were repeated with acetone powders stored at -14° for 24 hr. and for 18 days. The results are shown in Table 23.

TABLE 23

Incubation of acetone powder of chicken liver homogenate

How stored	Substrate (3 μmoles)	Cofactor (0.1 μmole)	Result (oestrone formed)
Incubated	17α-oestradiol	NAD^+	++
immediately	17α-oestradiol	$NADP^+$	++
after	17β-oestradiol	NAD^+	+++
preparation	17β-oestradiol	$NADP^+$	+++
	17α-oestradiol	NAD^+	+
24 hr.	17α-oestradiol	$NADP^+$	+
at	17β-oestradiol	NAD^+	+++
-14°	17β-oestradiol	$NADP^+$	+++
	17α-oestradiol	NAD^+	±
18 days	17α-oestradiol	$NADP^+$	±
at	17β-oestradiol	NAD^+	++
-14°	17β-oestradiol	$NADP^+$	++

+++ = very good conversion
++ = good conversion
+ = fairly good conversion
± = trace

The acetone powder from 250 ml. of suspension was dissolved in 20 ml. 0.1M-sodium phosphate buffer. Five ml. was used for each incubation in air at 37° for 30 min.

Calcium phosphate gel adsorption

Preliminary experiments with calcium phosphate gel prepared one year previously (Appendix H) gave a two-fold increase in specific activity of 17α-oestradiol dehydrogenase (17α DH) and a six-fold purification of 17β-oestradiol dehydrogenase (17β DH).

TABLE 24

The attempted adsorption of 17α- and 17β-oestradiol dehydrogenase activities on calcium phosphate gel

Fraction	Volume of gel 4 mg. (dry wt.) per ml.	Volume after fractionation	Protein mg./ml.	Specific activity milli-units/g. protein	
				17αDH	17βDH
Dialysate	-	160	99	0.1	0.2
1	50	198	38.1	0.2	0.5
2	50	235	27.6	0.2	0.7
3	100	304	16.5	0.2	1.2

Because of an insufficiency of aged gel, the investigation could not be continued until adsorption of the enzyme protein occurred. It was thought that it would be profitable to repeat these experiments as the procedure seemed useful for the purification of large batches of enzyme.

Four hundred ml. of an ammonium sulphate precipitate taken between 30 - 80% saturation and stored in glycerol-buffer at -10° was dialysed against 5 mM-sodium phosphate buffer containing EDTA (1 mM), and cysteine hydrochloride (1 mM). The final pH was 6.0 at 4°, and three 7-litre volumes were used for dialysis, one hour each change.

The non-diffusible material was centrifuged at 14,600 x g for 30 min. and the supernatant (650 ml.) fractionated. Calcium phosphate gel (250 ml., 4 mg. dry weight/ml. prepared 6 weeks previously) was used for each fractionation step. On the addition of gel the mixture was stirred for 15 min. at 5°, then centrifuged at 4,920 x g for 15 min. The supernatant was immediately assayed (Table 25).

TABLE 25

The attempted purification of 17α- and 17β-oestradiol dehydrogenase activities by calcium phosphate gel adsorption

Fraction	Volume after fractionation	Protein mg./ml.	Specific activity milli-units/mg.protein	
			17α DH	17β DH
Dialysate	650	58	0.3	0.6
1	860	33.3	0.3	1.0
2	1060	26.4	0.4	1.2
3	1270	19.5	0.4	1.0
4	1660	13.8	0.6	1.7
5	2030	9.6	0.6	1.4

There was little or no adsorption of enzyme protein. Therefore it was decided to concentrate the activities by salt fractionation. Crystalline $(NH_4)_2SO_4$ (Mann's Special Enzyme Grade) was added to the enzyme solution and two precipitates were made; the first, at pH 6.9, was equivalent to 50% saturation at 0°, the second at pH 6.7 was equivalent to 75% saturation at 0°. These fractions were stored in 5 mM-phosphate buffer containing glycerol (50%, by volume) for 48 hr. and 5 ml. of each was dialysed against three 4-litre changes of 5 mM-sodium phosphate buffer containing EDTA (1 mM), cysteine hydrochloride (1 mM) and glycerol (20%, by volume). The total time of dialysis was 3 hr. The results are summarised in Table 26.

TABLE 26

The enzyme activities of ammonium sulphate precipitates taken after calcium phosphate gel adsorption

Fraction	Protein mg./ml.	Specific activity milli-units/mg.protein	
		17α DH	17β DH
0-50% precipitate after dialysis	127.0	0.1	0.3
50-75% precipitate after dialysis	17.4	-	0.8

Year-old gel adsorbed more protein per mg. than six-week old material. Because of this variation and the shortage of aged gel, the method was not pursued further.

Chromatography on hydroxylapatite

Attempts at adsorption fractionation of dialysed ammonium sulphate precipitates were continued using hydroxylapatite (Anacker and Stoy, 1958; Appendix I). This material was packed and eluted at room temperature (19 - 23°) with 5 mM-sodium phosphate buffer which contained glycerol (20%, by volume). The column measured 8 cm. in width by 2 cm. in depth. No purification of 17α- or 17β-oestradiol dehydrogenase activities occurred at pH 7.0 or 7.2.

DISCUSSION

When this work was undertaken the assumption was made that tissues of those animals (e.g. sheep, calf and rabbit) known to metabolise or to excrete free or conjugated 17α-oestradiol probably contained a specific 17α-oestradiol dehydrogenase. The results of incubations with sheep, calf and rabbit liver were therefore disappointing. Although sheep and rabbit liver homogenates did oxidise 17α-oestradiol to oestrone, the activities were low, but the ovine tissue may have lost activity in the 36 - 48 hr. before incubation. It was also possible that the experimental findings reflected a sex difference in enzyme activities, however this aspect could not be investigated because of difficulty in obtaining tissues. Sex differences are known to occur in the distribution of enzymes concerned in steroid metabolism, e.g. the 3β-hydroxysteroid dehydrogenases of rat liver (Rubin and Strecker, 1961), nevertheless these observations may not pertain to chicken liver. In the present study homogenates of fresh liver from laboratory-reared cockerels were shown to interconvert 17α-oestradiol, 17β-oestradiol and oestrone. When it is considered how modifications of steroid hormone metabolism may be evoked by hypo- or hyperfunction of other endocrine tissues the possibility of seasonal or cyclic fluctuations in enzyme activity is not remote. Although this facet could not

be studied, its discussion is relevant, and a good illustration of the effect of one endocrine secretion upon steroid metabolism has been provided by Gallagher, Hellman, Bradlow, Zumoff and Fukushima (1960) who found that the ratio of 5α:5β metabolites of C_{19}-steroids ranges from very low in myxoedematous patients to greater than 1 in those with hyperthyroidism.

The choice of chicken liver as starting-material for the purification of 17α- and 17β-oestradiol dehydrogenase activities was based on the finding that crude homogenates of this tissue, without added cofactor, could catalyse the reversible oxidation of 17α- and 17β-oestradiol to oestrone. The yields of the 17-ketone were high and it appeared that the optimum pH for both reactions was above 8.4. It was noted that incubation of 17α-oestradiol gave the 17β-epimer in addition to oestrone; similarly, the 17α-epimer (as well as oestrone) was formed from 17β-oestradiol.

The simplest explanation for this observation was that chicken liver contained at least two oestradiol dehydrogenases each specific for the 17α- or 17β-epimer. As these activities were located in the non-particulate sub-cellular fraction, extensive purification was feasible. It was therefore resolved to attempt the isolation and separation of both 'enzymes' because the system offered

unique potential as a model for kinetic and mechanistic studies of the hydroxysteroid dehydrogenases.

An alternate reason for the production of oestrone and the substrate epimer was that these transformations may have been catalysed by a specific epimerase, i.e. an enzyme which effects inversion of the 17α-hydroxyl to the 17β-configuration without formation of the free 17-keto intermediate. An analogous chemical mechanism cannot be postulated.

Biological epimerisation of the steroid molecule has not been established although an enzymic basis for epimerisation of cardiotonic steroids at C-3 has been proposed by Repke and Samuels (1964) using subcellular fractions of rat liver homogenates. The 3β-hydroxyl group of the cardiotonic steroids is known to undergo inversion to the 3α-configuration in the animal body (Repke and Lauterbach, 1959), but to consider 'inversion' as synonymous with biological epimerisation is inaccurate. Although instances are well-known where a common symmetrical product can result from the action of pairs of enzymes, respectively specific for two isomers, there is evidence that single proteins are concerned with the epimerisation of sugars or the racemisation of amino-acids.

In a recent publication Ozon and Breuer (1965) claimed to have demonstrated 16α-, 16β-, 17α-, and 17β-hydroxysteroid oxidoreductases as well as 16α- and 16β-hydroxylases in incubations of chicken liver slices and subcellular fractions. These authors also claimed the partial separation of NAD(P)-dependent 17α-hydroxysteroid dehydrogenases from NAD(P)-dependent 17β-hydroxysteroid dehydrogenases. The results of Ozon and Breuer's experiments with the 17α- and 17β-oestradiol dehydrogenase activities are in broad agreement with those reported in this thesis but their conclusions must be interpreted with some scepticism. One important difference in experimental method concerns the duration of incubation which in Ozon and Breuer's publication was reported as 60 min. at 38°, whereas in the present study all incubations were carried out for 30 min. at 37°.

Only a limited assessment of enzyme activity can be made from qualitative examination of incubation products of a steroid and crude tissue homogenates but no attempt was made to devise an assay based on incubation of substrate followed by thin-layer or gas-liquid chromatography of the reaction products. Such methods are time-consuming but they may be the only procedures available for the study of particulate enzymes. The most suitable procedure for the assay of a

pyridine-nucleotide-linked dehydrogenase is the measurement of the rate of formation or disappearance of the quinonoid bond structure of the dihydronicotinamide ring of NADH or NADPH. This chromophore has a molar extinction coefficient of 6.2×10^3 litre $mole^{-1}$, cm^{-1}, at 340 mμ.

The pH optima for both oestradiol dehydrogenase activities (determined spectrophotometrically) were found to lie between pH 9 - 9.5, a range similar to that reported by Langer and Engel (1958) for the 17β-oestradiol dehydrogenase of human placenta. In the latter case the proximity of the optimum pH to 10 suggested a possible influence of the ionisation of the phenolic hydroxyl of 17β-oestradiol (pK of oestrone = 9.36). Langer and Engel therefore studied the relationship of the initial rate of oxidation of the 3-methyl ether of 17β-oestradiol under similar experimental conditions. The relationship of reaction rate to pH coincided roughly with that for 17β-oestradiol from pH 7.5 - 10.2 and it was concluded that the peak in reaction rate near pH 10 reflected ionisation of groups on the enzyme rather than that of the phenolic hydroxyl.

The results of incubations with chicken liver homogenates indicated that the pH optima were not lower than 8.4 but such results must be interpreted with caution.

The values quoted for the avian liver activities are not in agreement with those reported by Ozon and Breuer (1965) who used a different experimental method.

The determination of the stoichiometry of the reactions catalysed by the 17α- and 17β-oestradiol dehydrogenase activities was based on the measurement of product at the end of the first minute of the reaction, i.e. when the kinetics were zero-order in respect of substrate and cofactor. Thin-layer chromatography of the reaction products at the end of the first or second minute always showed oestrone and never the corresponding substrate epimer.

Comparison of mobility of the product with that of the reference compound in thin-layer and paper chromatographic systems was considered inadequate for characterisation, hence resort to countercurrent distribution. Statistical analysis of the data by means of the computer programme devised by Purdy, Goldman and Richardson (1965) showed that there was significant agreement between the curves for radioactivity and weight. Furthermore the weights of oestrone produced enzymically from both oestradiol epimers proved that these were equimolar reactions.

The computer programme was based on the statistical procedure described by Sheps, Purdy, Engel and Oncley (1960a; 1960b) which superceded the method for

statistical analysis of countercurrent distribution data published by Baggett and Engel (1957).

Purdy *et al.* (1965) recommended that radioactive samples be randomised before counting in order to reduce the effect of any time dependence of the counting efficiency which may occur over prolonged periods of counting. Since colorimetric or spectrofluorimetric methods used for the determination of mass are often dependent on time or temperature or both, Purdy and his colleagues also advocated the random arrangement of samples and standards before measurement. This procedure was designed to obviate a source of serial correlation in sequential determinations of weight in adjacent tubes.

Apparent Michaelis constants indicate high affinity of both 'enzymes' for their substrates. The values are of a similar order to those reported for other steroid dehydrogenases, e.g. the K_m for oestrone and 17β-oestradiol for the placental enzyme was given as 2.2×10^{-8} moles/litre (Langer *et al.* 1959).

The graphical method of determining K_m and V described by Woolf (1932) was preferred to that published by Lineweaver and Burk (1934). A recent penetrating analysis of graphical methods showed the double reciprocal plot to be the least satisfactory (Dowd and Riggs, 1965).

The results of incubation experiments showed that yields of oestrone and the corresponding substrate epimer were enhanced by the addition of $NADP^+$ and to a much lesser extent by NAD^+. This preference was confirmed in the spectrophotometric assay by which it was possible to determine an apparent Michaelis constant for $NADP^+$ in respect of both enzyme activities. Plots of initial velocity (v) against the ratio of initial velocity: substrate concentration (v/s) suggested that both dehydrogenase reactions were inhibited by high concentrations of cofactor. Apparent Michaelis constants for NAD^+ could not be measured. These results are at variance with those of Ozon and Breuer (1965) who claimed that the 17α- and 17β-hydroxysteroid oxidoreductases of chicken liver reacted equally well with NAD^+ or $NADP^+$ but their experimental methods differed from those used in the present study.

There are at least two dehydrogenases which prefer $NADP^+$ as cofactor and which catalyse the reversible oxidation of 17α- and 17β-oestradiol to oestrone. Final proof must await complete separation of the activities, however, there is evidence to support this view. It was found, as Ozon and Breuer (1965) claimed, that a partial separation of the 17α- and 17β-oestradiol dehydrogenase activities could be achieved by salt fractionation.

An accurate comparison cannot be made between those findings and the results reported in this thesis, but they agree in that the 17β-oestradiol dehydrogenase activity is widely distributed throughout the ammonium sulphate fractions whereas the 17α-dehydrogenase activity is largely confined to the fraction obtained between 30 - 40% saturation (Ozon and Breuer) and the 30 - 50% precipitate (p. 42).

Further support for the existence of at least two $NADP^+$-linked dehydrogenases stems from the observation that the ratios of both activities were not constant during ion-exchange chromatography. Prolonged dialysis gave only 17β-oestradiol dehydrogenase activity, however this is not proof of the existence of two distinct enzymes; it may be that some small molecule necessary for the stability of 17α-oestradiol dehydrogenase activity was lost during dialysis. Conclusive evidence must await further purification, separation, and substrate specificity studies.

The correct nomenclature of the activities studied in this thesis cannot be assigned until the completion of detailed investigations of substrate specificity with highly purified enzyme preparations. Although eluates of DEAE-cellulose columns catalysed the oxidation of the testosterone epimers, these transformations may not have

been effected by the same enzymes which attacked 17α- and 17β-oestradiol. The presence or absence of other hydroxysteroid dehydrogenases (e.g. those specific for C_{19}-steroids) has yet to be proved.

Precise investigations of steroid-enzyme interactions demand extensive purification of the activities concerned. Ideally the ultimate goal would be crystallisation of the catalytic proteins but there are difficulties inherent in the purification of enzymes involved in steroid metabolism. Not the least of these is the small yield of activity particularly where endocrine tissues are used as starting-material. This problem was not encountered with the enzymes discussed in this thesis.

The major restriction was imposed by the limited capacity of the centrifuge and the resultant inability to extract large quantities of tissue with minimum delay. A second obstacle was the low specific activity of the enzymes used for column chromatography. Although the first method of purification gave a satisfactory yield the procedure required improvement for the reasons given (p. 76).

The finding that considerable enzyme purification occurred on CM-cellulose at pH 6.0 - 6.5 was encouraging and the most profitable method of extraction would seem

to be at pH 5.5 followed by ammonium sulphate fractionation. The stability of ammonium sulphate precipitates and column eluates in glycerol-buffer could permit 'stockpiling' of activities by successive or recycling chromatography on CM-cellulose. Evidence was obtained that the 17β-dehydrogenase activity began to be eluted before the 17α-activity and it may be possible to exploit a difference in charge to separate the 'enzymes' by gradient elution with buffers of increasing molarity or pH.

If such methods were successful the purified material could be pooled and concentrated by means of a high molecular weight disperse polymer such as 'Ficoll' (Pharmacia). Alternatively further attempts at lyophilisation may be profitable.

The choice of chromatographic materials is wide and stronger cation-exchangers such as phosphonic acid- or sulphoethylcellulose deserve consideration. The possibility of further purification on the combined ion-exchange:cross-linked dextrans, such as CM-Sephadex is not remote. Should attempts to separate the activities fail before this stage then gel-filtration on a cross-linked dextran (e.g. Sephadex G-100 or G-200) may fulfil the purpose. Such procedures have been most effectively employed in the purification and separation of the

bacterial hydroxysteroid dehydrogenases (Delin, Squire and Porath, 1964). However this would appear to be a late step for use with preparations of high specific activity. Column electrophoresis or electrophoresis on starch or acrylamide gel are also procedures of greatest potential in the final stages of purification. It is clear that the most interesting aspects of this study must await separation and extensive purification of the 17α- and 17β-oestradiol dehydrogenases, therefore the remainder of the discussion will be largely speculative.

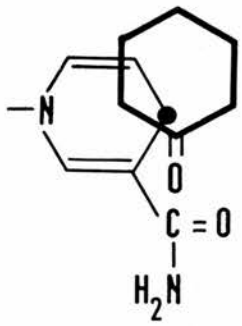
Theorell and Chance (1951) proposed a kinetic, compulsory order mechanism for horse liver alcohol dehydrogenase whereby the enzyme-coenzyme complex must form before substrate is bound. There is now considerable evidence to support this concept in the case of the liver enzyme and McKinley-McKee (1964) has stated that the Theorell-Chance mechanism is probably applicable to all dehydrogenases. It is possible that the enzyme-coenzyme complex induces profound conformational changes in the protein which in turn cause rearrangement of the polypeptide chains to form the active site which binds substrate. Such conformational changes are amenable to study by optical rotatory dispersion but separation and rigorous purification of the 17α- and 17β-oestradiol dehydrogenases would be obligatory before these potential models could be investigated by such sophisticated techniques.

It is probable that the 17α- and 17β-oestradiol dehydrogenases of chicken liver each reversibly catalyses the 'direct' transfer of hydrogen from C-17 to position 4 of the nicotinamide ring in position B (Fig. 2). All hydroxysteroid dehydrogenases so far investigated use the B side of the nicotinamide ring of the pyridine nucleotides and it would be of particular interest to investigate the stereochemistry of hydrogen transfer in model reactions in which there were two enzymes, each specific for the 17α- and 17β-configuration. The choice of isotope lies between deuterium and tritium; the former is preferred where high accuracy is sought, but tritium has the greater ease of estimation.

It is now known from the absolute configuration at C-4 of the dihydronicotinamide ring (Fig. 2) that yeast and liver alcohol dehydrogenases transfer H_A, and the a- and e-ketone reductases of C. falcata transfer H_B. Kinetic measurements with cyclohexane and decalin derivatives led Prelog (1963) to propose models of the steric relationship of enzyme, coenzyme and substrate in the neighbourhood of the active site. The possible importance of the interaction of substrate with the carboxamide group of the coenzyme was emphasised as a factor in the determination of product specificity.

Fig. 19

THE POSITION OF NADH RELATIVE TO SUBSTRATE



The black dot represents the A hydrogen of NADH and is understood to be beneath the carbonyl group. The planes of the dihydropyridine and cyclohexanone rings need not be parallel to each other.

(After Graves et al., 1965)

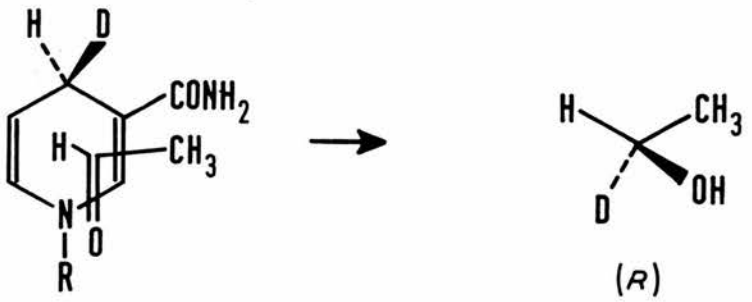
Graves, Clark and Ringold (1965) investigated the reduction of 3- and 4-alkyl cyclohexanones and the four 10-methyl-2-decalones by horse liver alcohol dehydrogenase. By correlating reaction rates and steric specificity they were able to define (1) areas common to reactive substrates and (2) areas that gave reduced rates of reaction. Graves and his colleagues also suggested preliminary mapping of enzyme, coenzyme and substrate in the vicinity of the active site.

The position of NAD and NADH relative to the substrate was considered by Prelog (1963, see Fig. 3). He held that the cofactor was located directly in front of the substrate so that H_A which was transferred lay directly under the substrate carbonyl and the carboxamide group lay to the right. Graves, Clark and Ringold prefer another position for NADH in the reaction with liver alcohol dehydrogenase, i.e. with a portion of NADH under the substrate but at a right angle to the cyclohexanone ring (Fig. 19).

They indicated that if the 1,4-dihydronicotinamide ring is planar as suggested by the nuclear magnetic resonance studies reported by Meyer, Mahler and Baker (1962), H_A and H_B of NADH will be staggered relative to the nicotinamide ring and not in true axial-equatorial positions. Graves et al. (1965) maintained that hydride

Fig. 20

THE SPATIAL RELATIONSHIP BETWEEN NADH AND ACETALDEHYDE IN REACTIONS CATALYSED BY YEAST AND LIVER ALCOHOL DEHYDROGENASES



(AFTER KARABATSOS ET AL.,1966)

transfer could occur with this conformation but if there was a strict requirement for axial transfer in the transition state then the two rings under discussion must be essentially parallel.

Karabatsos, Fleming, Hsi and Abeles (1966) have argued against Prelog's concept of the importance of the carboxamide group in the control of product stereospecificity. They described the spatial relationship between NADH and acetaldehyde in the yeast and liver alcohol dehydrogenation reactions (Fig. 20) by correlating the absolute configuration of H_A and H_B with the following evidence:-

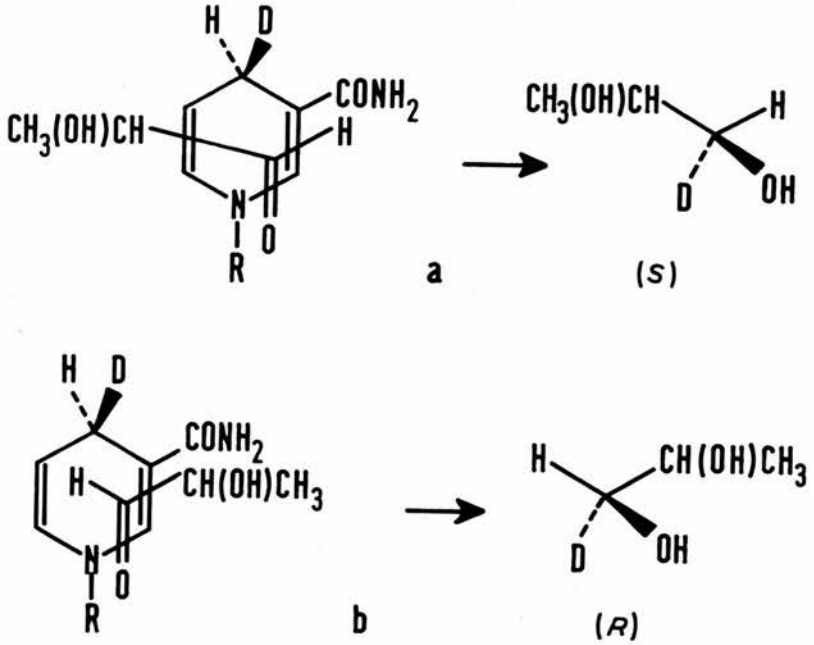
"NADH and 1-deuterioacetaldehyde give (-)-ethanol-1-d. The absolute configuration of (+)-ethanol-1-d is (R)".

Karabatsos et al. claimed that these facts, together with the finding that yeast alcohol dehydrogenase and NAD^+ react with (S)-2-octanol but not (R)-2-octanol, indicate that Prelog's concept is followed with enzymes associated with the transfer of H_A as well as with H_B.

In order to evaluate the importance of hydrophilic and hydrophobic regions in these reactions these workers determined the absolute configuration at C-1 of 1,2-propanediol-1-d obtained from the reduction of D- and L-lactaldehyde with deuterated NAD and horse liver alcohol dehydrogenase. They suggested that the polar

Fig. 21

THE REDUCTION OF D- AND L- LACTALDEHYDE WITH DEUTERATED NAD AND HORSE LIVER ALCOHOL DEHYDROGENASE



(AFTER KARABATSOS ET AL., 1966)

group that is capable of hydrogen bonding might conceivably 'force deuterated NAD' in a rather than b (Fig. 21). It was also claimed on the basis of nuclear magnetic resonance studies that as far as product stereospecificity is concerned D- or L-lactaldehyde and acetaldehyde have the same substrate-coenzyme relationship (Figs. 19 and 21b).

Steroids possess unusual advantages for investigations of enzyme-substrate interactions. They are chemically well-defined, rigid molecules, amenable to many subtle variations in structure and they can be readily measured in minute amounts. Substrate specificity studies of the type described by Langer et al. (1959) for the 17β-oestradiol dehydrogenase of human placenta would provide a basis for the construction of models according to Prelog's 'diamond-lattice' theory. Thus it should be possible to picture the relationships between enzyme, coenzyme and substrate in the vicinity of the active sites and to 'map' these areas by the use of two enzymes each specific for the 17α- and 17β-configuration. Should further investigation of reaction mechanisms be suggested from these projected kinetic experiments then the deuterium or tritium isotope effect could be used to advantage. According to Westheimer (1961) "The deuterium effect has become one of the most important tools which physical organic chemists employ in the elucidation of the

mechanisms of chemical reactions". Thus when a bond to a hydrogen or deuterium atom is broken in the rate-determining step of a reaction, the rate constant (K_H) for the reaction of the hydrogen compound exceeds the constant (K_D) for the same reaction with the corresponding deuterium compound.

Engel and his colleagues (1964) have compared the relative rates of oxidation of testosterone derivatives by chromic acid and by the soluble $NADP^+$-linked testosterone dehydrogenase of guinea-pig liver. In this way they attempted to contrast the effects of variation in steroid structure which influence chemical oxidation at carbon-17 with the results obtained by enzymic oxidation. Although the studies were incomplete, distinctions could be made between the rates of oxidation of certain C_{19}-compounds with chromic acid and those with the dehydrogenase.

The physiological importance of such <u>in vitro</u> studies may seem remote, but steroid hormones are potent modifiers of biochemical reactions <u>in vivo</u>, and metabolic control mechanisms cannot be described without detailed knowledge of the reactions concerned. Three concepts are of particular relevance in this respect. The first is that of the 'allosteric effect' (Monod, Changeux and Jacob, 1963) cited by Engel (1964) as a useful model for the study of hormone action. By exerting this effect a

hormone could alter the conformation of an enzyme protein and thereby influence binding at the catalytic site. This hypothesis implies that the active site of an enzyme is not rigid and the argument has been upheld by Koshland (1964) in his theory of 'induced fit' of substrate and enzyme.

It is not known whether steroid hormones can function as 'allosteric ligands' but Monod (1965) has stated that their physico-chemical properties make these compounds 'rather unfit' to serve this purpose. He indicated that virtually all the metabolites known to function as 'allosteric ligands' are highly polar, mostly ionic compounds in contrast to steroids.

Although the mechanism(s) of steroid hormone action are obscure the assumption is widely held that these compounds exert their effects by interaction with a macromolecule and therein lies the basis of Engel's concept which was restated in 1964. He believes "that if a steroid hormone interacts with enzymes concerned in its metabolism, with proteins involved in its transport and with receptor sites concerned in its physiological action, then these substances must all share structurally related sites complementary to the steroid hormone".

In kinetic studies with the 17β-oestradiol dehydrogenase of human placenta, Langer et al. (1959) showed that the steroid substrate must possess a highly planar ring A

or B or both for significant reactivity, and the high affinity for substrates, the steric specificity, and the frequent pronounced effects of distant modifications upon substrate reactivity indicated multiple sites of interaction with the enzyme. Further investigations of the effect of nitro-groups in ring A were made with the same enzyme (Engel, Stoffyn and Scott, 1964) and it was suggested that the phenolic ring A of the oestrogen might interact with a tyrosyl group at the binding site of the enzyme.

The experiments reported in this thesis were directed towards the isolation and purification of the oestradiol dehydrogenases of avian liver in order to provide a model for the investigation of steroid-enzyme interactions. Such investigations constitute an approach to the understanding of the mechanism(s) of hormone action, a fundamental biological problem which must ultimately be resolved in chemical terms.

SUMMARY

This work was undertaken to find a source of 17α-oestradiol dehydrogenase, to purify and characterise this enzyme and to study the effects of substrate structure upon its reaction kinetics in an *in vitro* model system.

The literature was reviewed concerning the *in vivo* metabolism of 17α-oestradiol, the interconversion of 17α-oestradiol and oestrone by animal tissues *in vitro*, and the histochemical demonstration of 17α-hydroxysteroid dehydrogenase activity. Publications dealing with the 17β-hydroxysteroid dehydrogenases and topics related to the mechanism of action of the hydroxysteroid dehydrogenases were also reviewed.

Chicken liver was chosen as the best source of '17α-oestradiol dehydrogenase' on the basis of incubation experiments with sheep, calf and rabbit liver, calf kidney and avian liver. On subcellular fractionation when $NADP^+$ was used as cofactor, most activity was found in the 'soluble' preparation. Because this fraction also contained 17β-oestradiol dehydrogenase activity the purification of both 'enzymes' was attempted.

A spectrophotometric assay (at 340 mμ) based on the formation of NADPH was devised for these studies. The pH optima were in the range 9 - 9.5 and the product of both reactions was identified by comparison of its mobility with that of authentic oestrone in thin-layer and paper chromatographic systems and by countercurrent

distribution of the enzymic oxidation product of both epimers in the presence of tritiated oestrone. Statistical analysis of the experimental data by computer revealed significant agreement between the curves for radioactivity and weight, and the fluorescence measurement of oestrone showed that both dehydrogenations were equimolar reactions.

Extraction of the oestradiol dehydrogenase activities was best carried out between pH 7 - 8 in 0.1M sodium phosphate buffer using a tissue:buffer ratio of 1:3. All the 17α-dehydrogenase activity and almost all of the 17β-dehydrogenase was precipitated by ammonium sulphate between 30 and 80% saturation. Further purification was effected on DEAE- and CM-cellulose in 5 mM-sodium phosphate buffer (pH 7.2) containing glycerol (20%, by volume). This procedure gave a 50-fold purification of the 17α-dehydrogenase and a 30-fold purification of the β-enzyme. The existence of at least two $NADP^+$-linked oestradiol dehydrogenases each specific for the 17α- and 17β-configuration was claimed on the grounds of partial separation of those activities by salt fractionation and the inconstant ratios of both activities during ion-exchange chromatography.

The reactions appeared to follow Michaelis-Menten kinetics at 25° in respect of both steroid substrates, and apparent Michaelis constants were of the order of 1.2×10^{-5} moles/litre for 17α-oestradiol and 1.6×10^{-5} moles/litre for 17β-oestradiol. Plots of initial velocity against the ratio of initial velocity:substrate concentration were not linear when attempts were made to determine the apparent K_m for $NADP^+$ in respect of both enzymes. The results were suggestive of inhibition at high cofactor concentrations. Apparent Michaelis constants for NAD^+ could not be determined because of high activity in the 'blank' cuvette when this nucleotide was used as cofactor.

The oestradiol dehydrogenases were stable for at least 4 months when stored at -10° in dilute sodium phosphate buffer containing glycerol (50%, by volume), and the enzymic activities of eluates of DEAE- and CM-cellulose columns were also preserved by the addition of glycerol (20%, by volume).

Other methods of purification, e.g. chromatography on CM-cellulose at pH 6 - 6.5 and adsorption on calcium phosphate gel were described and suggestions for further purification were advanced. The potential use of two separate, highly purified 17α- and 17β-oestradiol dehydrogenases in model reactions was discussed in

conjunction with current concepts of steroid/enzyme interactions and the mechanism of action of steroid hormones.

A preliminary account of this work was given at the Forty-Seventh Meeting of the Endocrine Society in New York City, New York, June, 1965.

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APPENDICES

APPENDIX A

Thin-layer chromatography

The following thin-layer system was used for the examination of the steroid reaction products from incubation mixtures and spectrophotometric assays:-

35 g. Silica gel G (Merck, according to Stahl)
73 ml. Distilled water
3 ml. Fluorescein solution (12.5 mg. fluorescein made up to 500 ml. with 0.1N-NaOH)

The micrometer on the Desaga-Brinkmann Model S spreader was set at about 350 microns. Plates were activated for 13 - 14 hr. at 60 - 70° before use.

The mobilities of the steroid reaction products were compared with those of reference compounds in the system ether-benzene (2:1, by volume). The plate was examined under ultraviolet light and sprayed with 95% ethanol/ concentrated sulphuric acid (v/v). 17α-Oestradiol appeared as a bright yellow spot immediately after spraying with fresh reagent. Colours developed after heating at 110° for 5 - 10 min. Oestrone, 17α- and 17β-oestradiol showed as bright orange spots on heating. The colours gradually dulled on exposure to air.

APPENDIX C

Dialysis

Dialysis was carried out against buffer at 4 - 5°. Dialysis tubing (flat width 1 3/16") was obtained from Arthur H. Thomas Co., Philadelphia, Pa. It was found that this tubing produced a yellow discolouration on immersion in aqueous buffers. It was therefore soaked in an aqueous solution of EDTA (5 g./100 ml.) for at least 48 hr. before use. This was followed by thorough washing in de-ionised water and in the dialysis buffer.

APPENDIX D

Purification of [6,7-$^3H_2$] oestrone

Two hundred and fifty μc. of compound NET-51, [6,7-$^3H_2$] oestrone, lot No. 134-21-77 was obtained from New England Nuclear Corporation. This had a stated specific activity of 40.6 c/m-mole. The weight was given as '0.00167 mg. in 0.5 ml. 10% methanol/benzene'. The compound was sealed under nitrogen and dated 12.22.64. Two hundred and fifty μc. was transferred quantitatively to a 250 ml. volumetric flask with 'Spectrograde' benzene. Fifty μl. was placed in a vial, dried under nitrogen, and 10 ml. scintillation fluid added. This gave a total count per min. of 2.7×10^4. A second sample of 50 μl. was put in a vial, then applied to a silica gel plate which had been subdivided into tracks approximately 1.5 cm. wide. The vial was rinsed twice with one drop of redistilled ethanol, and the washings were applied to the same origin. The mobility of the labelled oestrone was compared to that of authentic oestrone in the ether-benzene system. A 17β-oestradiol reference was also used. The plate was examined under ultraviolet light and the position of the authentic compounds was marked. One cm. lengths of silica gel were scraped from the radio-active track into Pasteur pipettes loosely plugged with cotton wool. The steroids were eluted with ethanol into

a counting vial and dried under nitrogen.

A contaminant, which corresponded in mobility to 17β-oestradiol, was present in the stock solution. Two ml. of stock (1 μc./ml.) was dried under nitrogen and taken up in one drop of redistilled ethyl acetate; this sample was applied to a 'curtain' of Whatman No. 1 paper cut in multiple strips. The capillary-applicator was rinsed with one drop of solvent and this was applied to the same origin. Twenty μg. oestrone and 20 μg. 17β-oestradiol were included for reference on separate strips. A 'blank', which consisted of two drops of ethyl acetate, was applied to a fourth strip. The position of the front was marked with 'Oil Red O'.

The paper was pre-equilibrated by the rapid method of Bush and Crowshaw (1965) in which the steroids were washed to the start line with ethyl acetate-methanol-chloroform (1:1:1, by volume). The paper was dried in air and the procedure was repeated twice. The paper was then soaked in a mixture of ether-75% methanol (1:1, by volume) and placed in the system ligroin-toluene-70% methanol (2:1:3, by volume) at 29°.

The reference strips were cut out and stained with 1% $FeCl_3$-1% $K_3 Fe(CN)_6$ (v/v) for 5 min., then washed in

cold running tap-water. Sections which corresponded to oestrone and 17β-oestradiol in the blank and radioactive strips were cut out and counted after elution in ethanol overnight.

The procedure was repeated using compound NET-51, [6,7-$^{3}H_{2}$] oestrone, Lot No. 134-21-75. This had a stated specific activity of '40.6 c/m mole and a weight of 0.00167 mg. in 0.5 ml. 10% methanol/benzene'.

The radioactive oestrone was found to contain approximately 1% of a compound of similar mobility to authentic 17β-oestradiol in the petroleum ether-toluene-methanol system. The radioactive compound which corresponded in mobility to the oestrone reference was used for countercurrent distribution.

APPENDIX E

Countercurrent distribution

The carbon tetrachloride-methanol-water system was prepared by mixing 1 l. redistilled methanol with 1 l. deionised, glass-distilled water; this mixture was allowed to cool to room temperature and 2 l. redistilled carbon tetrachloride was added. A 100-tube all-glass Craig apparatus (H.O. Post Scientific Company, New York) calibrated for 10 ml. lower phase was used for the countercurrent distributions. The tubes were capped except for every fourth one which was filled with 40 ml. lower phase. This was transferred by hand until all the tubes were filled. The uncapped tubes were covered, 10 ml. of upper phase was introduced into tube 0 and the machine was tilted to mix ten times. The phases were allowed to separate and the upper layer was transferred to tube 1. Another 10 ml. of upper phase was added to tube 0 and the procedure was repeated until eight upper layers were introduced. The contents of tube 0 were withdrawn by syringe and 10 ml. of each phase was added to the dried sample in 2 ml. aliquots which were then transferred to tube 0. The machine was tilted by hand to mix, the phases were allowed to settle and the first transfer was made to tube 1. Ten ml. of upper phase was added to tube 0, the reservoir was primed and the robot mechanism set to give a total of 100 transfers.

In order to locate the peak of radioactivity every fourth tube was dried down under reduced pressure and 5 ml. of pure ethyl alcohol (United States National Formulary) was added to each flask.

Ten µl. volumes were removed at the same time for fluorescence assay and for counting in a Packard 'Tri-Carb' liquid scintillation spectrometer (Model 314 Ex). The efficiency was about 23%. Sufficient counts were accumulated to ensure an accuracy of about 1%. Complete analysis of the peak was carried out in this manner. Oestrone was measured spectrofluorimetrically (Slaunwhite, Engel, Scott and Ham, 1953). Oestrogen assays and ^{3}H-counting were performed on randomised samples in accordance with the recommendations of Purdy, Goldman and Richardson (1965).

Experimental data are shown in Tables 27, 28, 29, 30.

TABLE 27

Oestrone from 17β-oestradiol
Recovery of [6,7$^{3}H_{2}$]oestrone

Tube	Disintegrations per min. x 10^{-4}
12	0.6
13	0.9
14	0.9
15	3.5
16	10.9
17	6.8
18	11.4
19	13.7
20	11.8
21	20.0
22	23.0
23	20.5
24	28.0
25	27.3
26	26.9
27	22.7
28	18.6
29	16.1
30	15.1
31	7.0
32	9.0
33	5.3
34	4.2
35	2.4
36	1.5

Total 308 x 10^{-4}

TABLE 28

Oestrone from 17α-oestradiol
Recovery of [6,7$^{3}H_{2}$]oestrone

Tube	Disintegrations per min. x 10^{-4}
12	0.3
13	0.9
14	2.1
15	3.5
16	4.3
17	6.8
18	10.3
19	13.7
20	14.9
21	20.0
22	14.5
23	20.5
24	17.7
25	27.3
26	16.2
27	22.7
28	11.6
29	16.1
30	7.1
31	7.0
32	3.6
33	5.3
34	2.0
35	2.4
36	1.0

Total 252 x 10^{-4}

TABLE 29

The measurement of oestrone from 17β-oestradiol by fluorescence

Tube	µg. Oestrone
12	1.5
13	3.5
14	4.8
15	10.5
16	13.2
17	17.5
18	28.6
19	32.5
20	49.9
21	44.5
22	69.4
23	57.5
24	77.5
25	72.5
26	69.4
27	67.5
28	49.9
29	43.5
30	28.6
31	19.0
32	13.2
33	12.0
34	4.8
35	7.0
36	1.5

The content of the even-numbered tubes was derived from the theoretical curve (Table 8, p. 58). The content of the odd-numbered tubes was that determined by fluorescence assay.

Total oestrone = 0.7998 mg.

TABLE 30

The measurement of oestrone from 17α-oestradiol by fluorescence

Tube	µg. Oestrone
12	3.5
13	5.0
14	6.5
15	8.0
16	15.5
17	15.5
18	24.0
19	18.0
20	34.0
21	25.5
22	31.5
23	30.0
24	39.5
25	30.0
26	36.5
27	27.5
28	20.0
29	16.5
30	13.5
31	10.5
32	8.5
33	6.5
34	5.5
35	1.0
36	2.5

Total oestrone recovered (i.e. the sum of the amounts found in each tube by fluorescence) = 0.435 mg.

APPENDIX F

Ion-exchange celluloses

The diethylaminoethyl (DEAE)-cellulose and carboxymethyl (CM)-cellulose used throughout this work were obtained from Brown Company, Berlin, New Hampshire. The capacity of the DEAE-cellulose was 0.7 m-equiv./g. and the value for CM-cellulose was 0.6 m-equiv./g. Both ion-exchangers were subjected to the same washing procedure in the hope that any heavy metals would be removed as their salts.

Two hundred g. DEAE- or CM-cellulose was allowed to sink into 5 l. distilled water. The suspension was gently stirred, then made up to 10 l. with distilled water. Stirring was continued for 3 - 4 min. The heavier particles of cellulose were allowed to settle and 5 l. of 'fines' was siphoned off. The volume was again made up to 10 l., a second 5 l. volume of 'fines' was removed and the cellulose was allowed to settle; this occupied a volume of about four and a half litres.

Five l. 0.1N-HCl (reagent grade) was added to the cellulose suspension which was stirred for 30 min. The supernatant was decanted after 30 min. and 5 l. 0.1N-NaOH was added. The suspension was stirred for 30 min. allowed to settle, then 6 l. of supernatant was decanted.

The suspension was washed at least ten times with 6 l. distilled water before storage in water at 5°.

The capacities of the ion-exchange celluloses were determined before and after treatment with acid and alkali and were found to be unchanged.

One gram each of DEAE- and CM-cellulose was spread on watch-glasses, dried for 12 hr. at 60° then reweighed. The capacities of each ion-exchanger were determined as follows. The DEAE-cellulose was converted to the hydroxide form on a Buchner funnel by washing with 50 ml. 0.5 N-NaOH and then with freshly-distilled water until the washings were neutral. The cellulose was transferred quantitatively to a 100 ml. beaker, 25 ml. of 1.0N-NaCl was added and this mixture was titrated with 0.1N-HCl to pH 3.0 using a glass electrode.

The CM-cellulose was converted to the hydrogen form on a Buchner funnel with 50 ml. of 0.5N-HCl. The cellulose was washed with freshly-distilled water until the washings were neutral; the ion-exchanger was then transferred quantitatively to a 100 ml. beaker, 25 ml. of 1.0N-NaCl was added and the mixture titrated with 0.1N-NaOH to pH 10.0 using a glass electrode.

APPENDIX G

Cofactors

The β forms of NAD^+, $NADP^+$ and the reduced forms were obtained from Sigma Chemical Company, St. Louis, Mo. The stated purity was 98% (corrected for 3 moles H_2O). A 1.0 mM solution of nucleotide was made up in distilled water and 10 µl. were applied to a poly (ethyleneimine) cellulose thin-layer sheet. This was chromatographed in 0.2M aqueous lithium chloride for 30 min. at 23° (K. Randerath, personal communication). On examination in ultraviolet light no contaminants were seen at this concentration. The less polar NAD^+ migrated from the origin, $NADP^+$ remained as a discrete spot.

Purity of the oxidised forms was also confirmed by enzymatic reduction with glucose-6-phosphate dehydrogenase (Sigma) using an assay system based on that described by Horecker and Kornberg (1957).

APPENDIX H

Preparation of calcium phosphate gel

The method was essentially that of Kunitz, described by Dixon and Webb (1964). One hundred and fifty ml. calcium chloride solution (132 g. $CaCl_2$, $6H_2O$/1.) was diluted to 1,600 ml. with deionised water. This was shaken with 150 ml. trisodium phosphate solution (152 g. $Na_3PO_4.12H_2O$/1.) and the pH adjusted to 7.4 by the addition of 0.5M acetic acid. The gel was washed eight times by decantation, using 10 l. deionised water for each change. It was then stored in a dark bottle at 4 - 5° for at least a month before use.

APPENDIX I

Preparation of hydroxylapatite

25.2 g. $CaH_4(PO_4)_2.H_2O$ was dissolved in 5 l. deionised water. The following solutions were added dropwise with constant stirring over a two-hour period:-

1 l. 1.5M-$K_2H\ PO_4$

1 l. 1.4M-$CaCl_2.H_2O$

The precipitate was allowed to settle at room temperature for 40 min. The supernatant was siphoned off and the precipitate transferred to a 24 cm. Buchner funnel fitted with Whatman paper (No. 5). The precipitate was washed with 2 l. deionised water and gently mixed with 2.5 l. 50 mM-NaOH for 3 hr. at 37°. The supernatant was removed and 1.5 l. 50 mM-NaOH added; this mixture was stirred for 12 hr. at 37°. Two further changes each for 2 hr. were made with 1.5 l. 50 mM-NaOH. The precipitate was washed with 50 mM-$NaH_2PO_4.H_2O$ until the pH was 6 - 7. The precipitate was then washed with 4 l. deionised water and stored in deionised water at room temperature.

APPENDIX J

Steroids

All steroid substrates were supplied by courtesy of Dr. Lewis Engel.

Stock solutions of steroids (2×10^{-3}M) stored at 4 - 5° in redistilled ethanol were used in enzyme assays. Homogeneity of oestrone, 17α- and 17β-oestradiol (recrystallised from ethanol) was confirmed by thin-layer chromatography in ether-benzene and by paper chromatography in the ligroin-toluene-70% methanol system.

No contaminants were found by thin-layer chromatography of recrystallised epitestosterone, testosterone and androstenedione in the ether-benzene system.

ABBREVIATIONS AND DEFINITIONS

AcPyAD; AcPyADH	Oxidised and reduced forms of the 3-acetylpyridine analogue of nicotinamide-adenine-dinucleotide.
CM-cellulose	Carboxymethylcellulose
DEAE-cellulose	Diethylaminoethylcellulose
EDTA	Ethylenediaminetetra-acetate (disodium salt)
Ligroin	A saturated volatile fraction of petroleum B.P. 20 - 135°
NAD^+; NADH	Oxidised and reduced forms of nicotinamide-adenine-dinucleotide.
$NADP^+$; NADPH	Oxidised and reduced forms of nicotinamide-adenine-dinucleotide phosphate.
NAD(P)	The cofactor specificity of an enzyme which uses either pyridine nucleotide.
$(NH_4)_2SO_4$	Ammonium sulphate
R_F	The ratio of the velocity of the substance under consideration to the velocity of the mobile phase in a chromatographic system.
v/v	Volume for volume.

All molarities reported in the text are final concentrations.

Systematic name	Trivial name
Androst-4-ene-3,17-dione	Androstenedione
3α-hydroxy-5α-androstan-17-one	Androsterone
3β-hydroxy-5α-androstan-17-one	Epiandrosterone
17α-hydroxyandrost-4-en-3-one	Epitestosterone
17β-hydroxyandrost-4-en-3-one	Testosterone
Oestra-1,3,5(10)-triene-3,17α-diol	17α-oestradiol
Oestra-1,3,5(10)-triene-3,17β-diol	17β-oestradiol
Oestra-1,3,5(10),11-tetraene-3,17α-diol	11-dehydro-17α-oestradiol
Oestra-1,3,5(10)-triene-3,16α,17β-triol	Oestriol
Oestra-1,3,5(10)-triene-3,16β,17β-triol	16-epioestriol
Oestra-1,3,5(10)-triene-3,16α,17α-triol	17-epioestriol
Oestra-1,3,5(10)-triene-3,16β,17α-triol	16,17-epioestriol
3-hydroxyoestra-1,3,5(10)-trien-17-one	Oestrone
3,16α-dihydroxyoestra-1,3,5(10)-trien-17-one	16α-hydroxyoestrone
3,16β-dihydroxyoestra-1,3,5(10)-trien-17-one	16β-hydroxyoestrone
Pregn-4-ene-3,20-dione	Progesterone

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