

3 $\alpha$ -HYDROXYSTEROID DEHYDROGENASE ACTIVITY  
IN THE CYTOSOLIC FRACTIONS OF HUMAN  
PLACENTA, KIDNEY AND LIVER

CENTRE FOR NEWFOUNDLAND STUDIES

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TONY EMMANUEL GYAPONG









**3 $\alpha$ -HYDROXYSTEROID DEHYDROGENASE ACTIVITY**

**IN THE CYTOSOLIC FRACTIONS**

**OF HUMAN PLACENTA, KIDNEY AND LIVER.**

by



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A thesis submitted to the School of Graduate Studies  
in partial fulfilment of the requirements  
for the degree of Master of Science.

Faculty of Medicine  
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## ABSTRACT

Although the major metabolites of 3-ketosteroids voided in human urine are 3 $\alpha$ -hydroxysteroids or their derivatives, there has been very little published work on the purification and characterization of the human 3 $\alpha$ -hydroxysteroid dehydrogenases which must be involved in their genesis.

The presence of the 3 $\alpha$ -hydroxysteroid dehydrogenases in human liver, kidney and placenta has been demonstrated by incubation of extracts of these tissues with 17 $\alpha$ -methyl-5 $\alpha$ -androstane-3 $\alpha$ ,17 $\beta$ -diol which was converted to the 3-ketone. This was identified by thin layer chromatography and by high performance liquid chromatography and by further characterization after sodium borohydride reduction of the 3-ketone to the 3 $\beta$ -alcohol.

The NAD-dependent liver cytosol enzyme activity found in the 40-60% ammonium sulphate precipitate has been partially purified using ion-exchange and gel filtration chromatography. The 3 $\alpha$ -HSD activity appears to reside in a number of isozymes separable by carboxymethyl-cellulose chromatography; one is tentatively identified as being a human liver ADH,  $\beta$ 1 $\beta$ 1 which was shown by iso-electric focussing to have a pI between 9 and 10. 4-Methyl pyrazole was found to inhibit the ethanol dehydrogenase activity but not the 3 $\alpha$ -HSD activity of these isozymes.

A two-fold ratio of 3 $\alpha$  : 3 $\beta$ -HSD activity was observed in the human kidney cytosol but the activity in the microsomal fraction was entirely due to 3 $\beta$ -HSD with only a trace of 3 $\alpha$ -HSD activity. The 3 $\alpha$ -HSD activity found in the cytosolic fraction of the human placenta was very unstable.

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# Glossary

## Trivial and Systematic Names of Steroids

Androstenedione	5 $\alpha$ -Androstane-3,17-dione
Androstenedione	4-Androstene-3,17-dione
Androsterone	3 $\alpha$ -Hydroxy-5 $\alpha$ -androstane-17-one
Cortisol	11 $\beta$ ,17,21-Trihydroxy-4-pregnene-3,20-dione
Cortisone	17,21-Dihydroxy-4-pregnene-3,11,20-trione
Cholesterol	5-Cholesten-3 $\beta$ -ol
3 $\alpha$ -Androstanediol	5 $\alpha$ -Androstane-3 $\alpha$ ,17 $\beta$ -diol
5 $\alpha$ -Dihydrotestosterone	5 $\alpha$ -Androstane-17 $\beta$ -ol-3-one
Pregnenolone	3-Hydroxy-5-pregnen-20-one
Progesterone	4-Pregnene-3,20-dione
Testosterone	17 $\beta$ -Hydroxy-4-androsten-3-one
Tetrahydrocortisone	3 $\alpha$ ,11 $\beta$ ,17 $\alpha$ ,21-Tetrahydroxy-5 $\alpha$ -pregnan-20-one
Tetrahydrocortisol	3,11,17,21-Tetrahydroxy-5-pregnan-20-one

## Abbreviations

DHT	5 $\alpha$ -Dihydrotestosterone
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NADH	Nicotinamide adenine dinucleotide (reduced)

NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
TLC	Thin layer chromatography
HPLC	High pressure liquid chromatography
DEAE-Sephacel	Diethylaminoethyl-Sephacel
CM	Carboxymethyl-cellulose
HSD	Hydroxysteroid dehydrogenase
ME	2-Mercaptoethanol
UV	Ultraviolet
2-Mercaptoethanol	2-Hydroxyethylmercaptan
NBT	Nitro blue tetrazolium
PMS	Phenazine methosulphate
TEMED	N,N,N',N'-Tetramethylethylenediamine
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
Tris	2-Amino-2-hydroxymethylpropane-1,3-diol
K' (HPLC)	Retention time of the sample relative to solvent peak
R <sub>f</sub> (TLC)	Relative distance of migration with respect to the solvent front
SM	Starting material
pI	Isoelectric point
ADH	Alcohol dehydrogenase

## CHAPTER I

### INTRODUCTION

With the exception of the estrogens, the major biologically active steroid hormones of man all contain a 3-ketone group. The major steroid excretory products in adult human urine are the 3 $\alpha$ -hydroxy and 3 $\beta$ -hydroxy androstanes and pregnanes of the 5 $\alpha$ - and 5 $\beta$ -series and their derivatives. The 3 $\alpha$ -hydroxysteroid dehydrogenases (E.C.1.1.1.50) of human liver are thus on a major route of steroid inactivation.

This thesis reports a study of the 3 $\alpha$ -hydroxysteroid dehydrogenase activity of human liver and, to a lesser extent, that of placenta, and kidney cytosol. The importance of the 3 $\alpha$ -hydroxysteroid dehydrogenases in the metabolism of androgens, progestins and in the synthesis of the bile acids, and the fact that, in man, the enzymes involved have not been characterized previously prompted this work. Although, as will be seen below, 3 $\alpha$ -HSD activity has been incidentally found in human liver, there has as yet been no publication in which this enzyme activity of human liver cytosol has been followed through several steps of purification. The substrate used in this study for detection and assay of 3 $\alpha$ -HSD activity is a 5 $\alpha$ -androstane-3 $\alpha$ ,17 $\beta$ -diol blocked at the 17-position by insertion of a 17 $\alpha$ -methyl group so that it is capable only of metabolism at the 3-position.

This thesis reports a partial purification and characterization of one of the 3 $\alpha$ -HSD enzymes, and its tentative identification as human liver alcohol dehydrogenase isozyme  $\beta$ 1 $\beta$ 1.

## CHAPTER II

### LITERATURE REVIEW

#### 1. The Steroid Hormones

##### i) Physiological Activities of the Hormones

It has long been known that the biologically active steroid hormones containing a conjugated 4-en-3-one structure in ring A are extensively inactivated by  $5\beta$ -reduction of the double bond, followed by 3-ketone reduction to the  $3\alpha$ -alcohols. The two most abundant C(19) steroids in human urine are the two  $3\alpha$ -hydroxysteroids shown at the bottom of Fig. 1. The  $3\alpha$ -HSD activity is thus of considerable importance in the regulation of the concentrations of steroid hormones and may therefore play a role in such hormonal effects as sex differentiation, maintenance of pregnancy, the regulation of energy utilization and electrolyte balance, muscle and hair growth, maintenance of cell integrity, suppression of the immune reaction and of inflammation generally. Steroid hormones also play an important role in modifying the rate of growth of certain tumours, particularly those of the breast and prostate gland.

##### ii) Catabolism of Steroid Hormones

The principal metabolic changes undergone by steroids are oxidation and/or reduction, conjugation and hydrolysis. Conjugation usually involves conversion of steroid hydroxyl groups to either the glucuronide or the sulphate ester. Although it is often claimed that these processes render the steroids water-soluble, it is likely that conjugation is part of a more complex control process, as the hormonally active

FIG 1 Representative Pathways of Testosterone Metabolism in Man



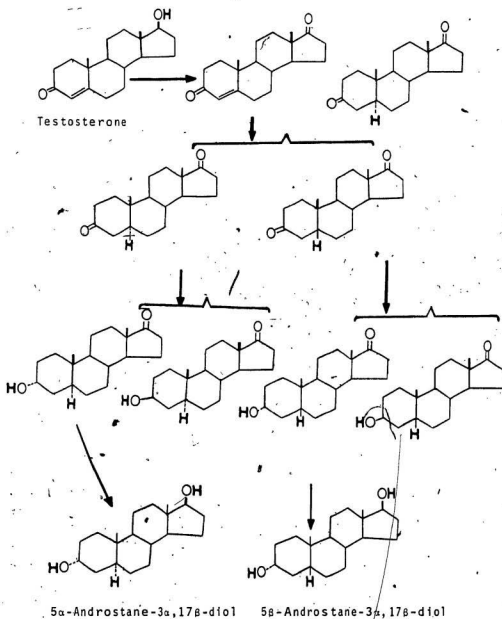


FIG 1

steroids are already at concentrations well below their solubility limit.

The processes of oxidation and reduction include hydroxy-ketone interconversion, reduction of double bonds, hydroxylations or oxidative scissions of the side chain. The liver and kidney are major steroid-metabolizing organs in the body, the liver being the more important. Both in animals and in man, it has been demonstrated that steroids are normally metabolized so rapidly in the liver that only poorly metabolizable synthetic substrates or very high doses of natural estrogens can bypass the enzymatic machinery present in the liver.

### iii) Androgens and their Metabolism

Male sex hormone effects in the body have been considered to be due to the presence of testosterone. In many of its effects, however, testosterone appears to act only after conversion to  $5\alpha$ -DHT. A third considerably weaker androgen is  $3\beta$ -hydroxyandrost-5-en-17-one (DHA). All three are to a large extent converted to  $3\alpha$ -hydroxysteroids before excretion.

Androgen effects are diverse; most apparent is that they control early development in utero, and later development at puberty to the male habitus.  $3\alpha$ -HSD activity is important in the metabolism and regulation of the levels of testosterone and of  $5\alpha$ -DHT. Testosterone has been implicated in the organization of neuronal pathways in the neonate brain (McEwen, 1982) and may account for differences between male and female behaviour (Rainbow and McEwen, 1984). In addition, male sexual behaviour in females and castrates, for example, clasping in the frog, song in the bird and mating in the rat can be stimulated by testosterone and its metabolites (Erulkar et al., 1981; Nottenbohm, 1980; Goy and McEwen, 1980). Testosterone is also involved in the mediation of the release of luteinizing hormone-releasing hormone in the hypothalamus (Martini, 1982; Schally et al., 1973 a,b).

In many androgen target tissues, testosterone is reduced by enzymes of the cell nucleus to an active metabolite, 17 $\beta$ -hydroxy-5 $\alpha$ -androstane-3-one (5 $\alpha$ -DHT) (Wilson and Gloyna, 1970). Extensive evidence has been accumulated that this metabolite is an important mediator of androgen action both in utero and in adult life (Bruchovsky and Wilson, 1968; Gloyna and Wilson, 1969).

In nearly all tissues, 5 $\alpha$ -DHT can further be reduced to 5 $\alpha$ -androstane-3 $\alpha$ ,17 $\beta$ -diol (Fig 1) (Gore and Baron, 1965). The latter metabolite has generally been regarded as a less active degradation product (Bruchovsky and Wilson, 1968), however in several organs such as the rat kidney (Verhoeven, 1976), rat exorbital lacrimal gland (Cavellero and Offner, 1967), rat prostate and seminal vesicles (Dorfman and Dorfman, 1963) and mouse kidney (Kochakian, 1944; Ohno et al., 1971; Bardin et al., 1973), the activity of 5 $\alpha$ -androstane-3 $\alpha$ ,17 $\beta$ -diol equals or surpasses that of testosterone (Cekan and Pelc, 1966; Moore and Wilson, 1972). Furthermore, it has been shown that, in vivo, 3 $\alpha$ -androstenediol can be converted to 5 $\alpha$ -DHT (Bardin et al., 1973; Bruchovsky, 1971; Becker et al., 1973). The site of this conversion, however and its significance in the overall scheme of androgen metabolism and action remain obscure. It has been suggested that the conversion of 5 $\alpha$ -dihydrotestosterone (5 $\alpha$ -DHT) into 5 $\alpha$ -androstane-3 $\alpha$ ,17 $\beta$ -diol may regulate androgen action in androgen-dependent tissues (Walsh and Wilson, 1976; DeKlerk et al., 1979). 3 $\alpha$ -HSD-mediated conversion of 5 $\alpha$ -DHT to 5 $\alpha$ -androstane-3 $\alpha$ ,17 $\beta$ -diol has been reported to be localized in the cytosol of human breast cancer cells (MacIndoe and Woods, 1981) and ovaries of immature rats (Suzuki et al., 1978).

#### iv) Pregnanes and Corticosteroids and their Metabolism

In the metabolism of the corticosteroids, 3 $\alpha$ -HSDs predominate over 3 $\beta$ -HSD in man; in most animals, however, 3 $\beta$ -HSDs appear to predominate (Gower and Honour,

1984). The first stage in the reduction of the A-ring activities involves a 4-ene-5 $\beta$ -reductase or a 4-ene-5 $\alpha$ -reductase. The second stage utilizes the 3 $\alpha$ - and 3 $\beta$ -HSD enzyme activities. Thus for progesterone, the major metabolite is, 5 $\beta$ -pregnane-3 $\alpha$ ,20 $\alpha$ -diol, and for cortisol (11 $\beta$ ,17,21-trihydroxy-4-pregnene-3,20-dione) and cortisone (17,21-dihydroxy-4-pregnene-3,11,20-trione), the major products are tetrahydrocortisol (3 $\alpha$ ,11 $\beta$ ,17 $\alpha$ ,21-tetrahydroxy-5 $\alpha$ -pregnan-20-one) and tetrahydrocortisone (3 $\alpha$ ,17 $\alpha$ ,21-tetrahydroxy-5 $\alpha$ -pregnan-11,20-dione).

#### v) Bile Acids and their Metabolism

In the conversion of cholesterol to the primary bile acids chenodeoxycholic acid and cholic acid, the 5-ene-3 $\beta$ -hydroxy configuration of cholesterol is transformed in liver by several steps to the saturated 3-ketone, then by a 3 $\alpha$ -HSD to the 3 $\alpha$ -hydroxy-5 $\beta$  bile acids (Fig 2).

The crude 3 $\alpha$ -HSD of rat liver cytosol functions as well with NADPH as with NADH as cofactor (Tomkins, 1956). It is believed that, in vivo, in animals, the reversible dehydrogenation of 3 $\alpha$ -hydroxy bile acids is not catalyzed by liver enzymes but by intestinal microorganisms (Hatanaka et al., 1972). Ogura (1959) and Berseus (1967) have reported, however, that rat liver enzymes can reduce 3-oxo bile acids to 3-hydroxy bile acids.

In common with other dual nucleotide-dependent hydroxysteroid dehydrogenases, 3 $\alpha$ -hydroxysteroid dehydrogenases promote transhydrogenation between NADPH and NAD in the presence of catalytic concentrations of steroids and may therefore also be involved in the regulation of nicotinamide nucleotide concentrations (Hurlock and Talalay, 1958; Talalay and Williams-Ashman, 1958 a,b).

## 2. Dehydrogenases and their Properties

### i) Alcohol dehydrogenases

Hydroxysteroid dehydrogenases are members of the larger class of alcohol dehydrogenases about which much is known, both with respect to the physical and chemical properties of the enzymes, and to the mechanism and stereochemistry of the reactions they catalyze. Some dehydrogenases, named the A-type, cause transfer of the 4-proR hydrogen of the NAD(P)H cofactor. The remainder (the B-type) transfer only the 4-proS hydrogen. Rat liver microsomal 3 $\alpha$ -HSD is described as being a B-type of hydroxysteroid dehydrogenase (Bjorkhem et al., 1973; see Fig 3).

The soluble enzyme from the rat liver cytosol was however found to be the opposite, being a A-type of hydroxysteroid dehydrogenase (Bjorkhem and Danielsson, 1970).

The NAD(P)-linked alcohol dehydrogenases constitute a group of enzymes that is both widespread and important in phyla from plant to man. Once obtained in preparations of defined characteristics, these enzymes are especially amenable to detailed mechanistic and stereochemical study. The reaction is in general readily reversible; both substrate and coenzyme reactions are stereospecific; the transferred hydride ion does not usually exchange with hydrogen of water and there are two experimentally available heavier isotopes differing so greatly in mass from the common isotope that the three have appreciably different rates of reaction in many cases. Hydrogen transfer from nicotinamide coenzymes in alcohol dehydrogenases is hydride-like and does not involve radical intermediates (Nonhebel et al., 1982). Our knowledge of the mechanism, stereochemistry and other characteristics of alcohol dehydrogenase reaction derives principally from early work on the enzymic oxidation of ethanol.

Ethanol dehydrogenases have been identified in widely varied life forms including bacteria, yeast, insects, plants, fish and mammals (Branden et al., 1975) but the enzymes have been purified in only relatively few instances.

FIG. 2 Role of 3 $\alpha$ -Hydroxysteroid Dehydrogenase in Bile Acid Synthesis.

R = (CH<sub>2</sub>)<sub>2</sub>.SO<sub>2</sub>OH (taurocholic acid)

R = CH<sub>2</sub>.COOH (glycocholic acid)

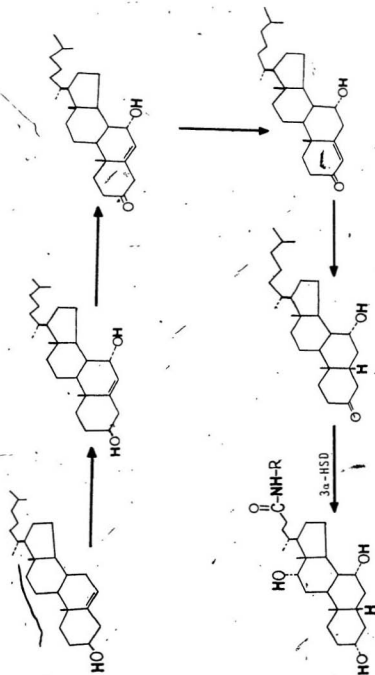


FIG 2

FIG. 3 Stereochemistry of NAD Reduction Catalyzed by Rat Liver  
3 $\alpha$ -Hydroxysteroid Dehydrogenase, a Class "B" Dehydrogenase  
(Bjorkhem et al. 1973).



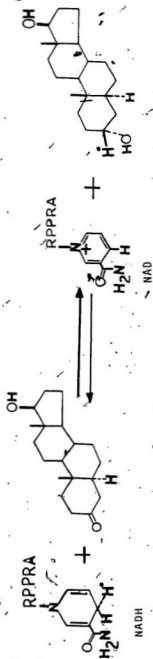


FIG 3

Horse liver and yeast alcohol dehydrogenases have been purified and crystallized and have been used in many studies including structure-function relationships (Branden et al., 1975; Jornvall, 1973; Li and Vallee, 1965), enzyme kinetics, and of the role of zinc in biological systems (Drum et al., 1967).

Some genetic studies of *Drosophila* and maize alcohol dehydrogenases (Scandalios, 1969; Ursprung and Leone, 1965) and also of human liver alcohol dehydrogenase (Smith et al., 1973) have been performed utilizing activity measurements of partially purified enzymes. Ethanol oxidation has been accepted as the primary function of yeast alcohol dehydrogenase since the enzyme was first isolated and crystallized by Theorell and Bonnichsen (1951). This assumed role has been extended to the horse (Bonnichsen and Wassen, 1948) and human forms (von Wartburg et al., 1965) of the enzyme.

#### ii) Human liver alcohol dehydrogenase

Human liver alcohol dehydrogenase was first isolated by von Wartburg et al. (1964) and fractionated into chromatographically distinct forms by Blair and Vallee (1966). Subsequently, after three major molecular forms of horse liver alcohol dehydrogenase were found to be the dimers of dissimilar polypeptide chains, Schenker et al. (1971) and Pietruszko et al. (1972) concluded that similar considerations may also pertain to some of the molecular forms of human liver alcohol dehydrogenases then known. This has been confirmed by the detailed investigations of Vallee and his co-workers (1983).

Alcohol dehydrogenase is the primary enzyme responsible for the metabolism of ethanol in humans (Li, 1977). This enzyme represents 2-3% of the soluble protein in normal human liver, which suggests that aside from its action on exogenous ethanol, it may serve other important though presently unknown function(s) in intermediary

metabolism. Human liver alcohol dehydrogenase (ADH; E.C. 1.1.1.1) has a complex isozyme pattern with forms differing in electrophoretic mobility, kinetic and immunological properties. The human liver alcohol dehydrogenases have been divided into three classes (Vallee and Bazzone, 1983). Class I consists of pyrazole-sensitive, basic isozymes; class II consists of less pyrazole sensitive, less basic isozymes and class III is composed of forms with anodal electrophoretic mobility and low ethanol dehydrogenase activity (Strydom and Vallee, 1982).

Class I isozymes consist of homo- and heterodimers of  $\alpha$ ,  $\beta$  and  $\gamma$  subunits, which are coded for by three gene loci, ADH1, ADH2 and ADH3 (Smith et al., 1971). Genetic alleles are known to occur at the ADH2 locus coding for  $\beta 1$  and  $\beta 2$  subunits of known structures (Jornvall et al., 1984a; Buhler et al. 1984a) and for  $\beta$ -Indianapolis subunits (Bosron et al., 1980). Similarly  $\gamma 1$  and  $\gamma 2$  subunits have been ascribed to the ADH3 locus (Smith et al., 1971) while allelic variants have not been defined for the  $\alpha$ -subunit. The  $\alpha$ -polypeptide is predominant in early fetal liver (Smith et al., 1971), while in adult livers  $\beta$ -subunits and (apparently to a lesser extent)  $\gamma$ -subunits predominate. Differences in efficiency of ethanol oxidation and in other enzymatic properties are found for the homodimeric class I isozymes (Bosron et al., 1983). The primary structures of the  $\beta 1$   $\beta 2$  and  $\gamma 1$  subunits have been determined as well as nucleotide sequence of the cDNA corresponding to the  $\beta$ mRNA (Duester et al., 1984; Ikuta et al., 1985; Heden et al., 1986). Three different size classes of cDNA clones coding for the  $\beta$ -subunit of the human liver alcohol dehydrogenases have been characterized from a human liver cDNA library (Lars-Olof Heden et al., 1986), however, one is only a partial sequence (Duester et al., 1984). Another (Ikuta et al., 1985) contains a few differences in relation to the protein structure reported (Hempel et al., 1984), and results from a third suggest a cDNA with the presence of a deletion. Active site structures and sequence corresponding to a missing region in distantly related alcohol

dehydrogenases have also been studied for the  $\alpha$ -subunit, showing the overall relationship among the class I polypeptides (Hempel et al., 1985). The class I isozymes migrate as a group towards the cathode on starch gel electrophoresis at pH 7.7-8.6. The iso-electric points of the human liver isozymes is as follows: Class I, 9-11 (Li and Theorell, 1969); class II, 8-9 (Li et al., 1977); and class III, 6.4 (Wagner et al., 1984).

The class I isozymes have been isolated as a group by chromatography on DEAE-cellulose and on an affinity resin, Sepharose 4B activated with cyanogen bromide and coupled to 4(3-(N-6-aminocaproyl)aminocaproyl)pyrazole (CappGapp-Sepharose) which contains a pyrazole-like end group (Lange et al., 1976). An appreciable amount (10-30%) of enzymically active protein does not bind to the CappGapp-Sepharose immobilized ligand. This is associated with the  $\pi$  isozyme. There are four homodimers,  $\alpha\alpha$ ,  $\beta\beta$ ,  $\gamma\gamma$  and  $\gamma_2\gamma_2$  and six heterodimers,  $\alpha\beta$ ,  $\alpha\gamma$ ,  $\alpha\gamma_2$ ,  $\beta\gamma$ ,  $\beta\gamma_2$  and  $\gamma\gamma_2$  that would be formed by the random combination of the four subunits, and all have been detected.

The individual class I isozymes were separated from one another by CM-cellulose chromatography (Wagner et al., 1983). All class I isozymes are very stable and retain virtually full activity towards ethanol over a period of one month when stored in 5mM tris-Cl buffer with 0.5mM NAD and 0.1mM DTT, pH 7.3 (Deetz et al., 1984). The specific activity of the  $\beta\beta$  isozyme with ethanol as substrate is 0.2U/mg, while that for the other class I isozymes is 0.6-1.2U/mg. Class III has an activity of 0.4-0.6U/mg protein. Discontinuous polyacrylamide gel electrophoresis in the presence of 7M urea at pH 7.2 resolves the cathodic subunit chains of human liver alcohol dehydrogenase i.e.  $\pi$ ,  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\gamma_2$  (Keung et al., 1985). From the relative positions of the resulting subunit bands, individual alcohol dehydrogenases can be identified unequivocally (Keung et al., 1985). The tryptic peptide HPLC elution profiles of the class I isozymes are similar to one another (Strydom and Vallee., 1982) and antibody prepared to  $\beta\beta$

cross-reacts with all the isozymes within this group (Adinolfi et al., 1979) suggesting close structural homology.

The enzyme forms belonging to the class II ( $\pi$ -ADH) and III ( $\chi$ -ADH) differ substantially from the class I forms with respect to electrophoretic mobility, tryptic peptide maps, antibody cross-reactivity and kinetic properties. The eight predominant cathodic class I human liver isozymes  $\alpha\gamma 2$ ,  $\gamma 2\gamma 2$ ,  $\alpha\gamma 1$ ,  $\alpha\beta 1$ ,  $\beta 1\gamma 2$ ,  $\gamma 1\gamma 1$ ,  $\beta 1\gamma 1$  and  $\beta 1\beta 1$  all oxidize ethanol, ethylene glycol, methanol, benzyl alcohol, octanol, cyclohexanol and 16-hydroxyhexadecanoic acid at the pH optimum, 10.0. However as judged by kinetic criteria, in no case is ethanol the best substrate for any of the class I isozymes (Pietruszko, 1979; Wagner et al., 1983).

The class II ( $\pi$ -ADHs) are less pyrazole sensitive, cationic proteins which have been purified and characterized (Bosron et al., 1980; Wagner et al., 1983). The class II ADHs catalyze the oxidation of ethanol, pentanol and 3-pyridinyl-carbinol but do not oxidize methanol, glycerol or any of the cardiac sterols (Vallee and Bazzzone, 1983).

Screening liver extracts by means of starch gel electrophoresis followed by staining with an alcohol of longer chain length, i.e. pentanol, led to the identification of an isozyme which hardly oxidizes ethanol at all (Pares and Vallee, 1981). The substrate specificity and the kinetic and physicochemical behaviour of this enzyme proved to differ from those of class I and class II; it is anionic at neutral to slightly alkaline pH values and is not saturated by ethanol at concentrations up to 2M. Similar ADH isozymes have now been detected and isolated from liver extracts of species other than the human and have been named provisionally  $\chi$ -ADH (class III). (Pares and Vallee, 1981).

Class III ADH is the sole molecular form in human placenta (Wagner et al., 1984) and brain (Beisswenger et al., 1985) while class II may well be liver-specific but

coexist there with other ADH forms (Ditlow et al., 1984). In contrast the  $\beta$ -subunits of class I were detected in lung, skin, hair roots and adult kidney (Smith et al., 1971; Goedde et al., 1980) and  $\gamma$ -subunits were identified in stomach and infant kidney. In the testis, immunohistochemical localization of ADH is primarily in the seminiferous epithelium and Leydig cells (Buhler et al., 1984), the major molecular form of testicular ADH is class III ADH.  $\chi$ -ADH is genetically distinct from other isozyme classes. Antibodies prepared against  $\beta\gamma 2$ ,  $\beta\gamma 1$  and  $\beta\beta$  (class I) and  $\pi$ -ADH (class II) do not cross-react with human  $\chi$ -ADH. However antibodies towards both horse and human liver  $\chi$ -isozymes do cross-react with each other (Vallee and Bazzone, 1983). The optimum activity of ethanol oxidation for all three classes of human liver alcohol dehydrogenase occurs between pH 9.0 and 11.0 (Lutstorf et al., 1970; Wagner et al., 1984; Ditlow et al., 1984).

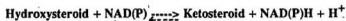
The remarkable polymorphism of human liver ADH and the accompanying differences in substrate specificities and kinetic parameters suggests participation in multiple metabolic pathways. Clearly the oxidation of ethanol is not the sole or perhaps even the primary function of all ADH molecular forms. All the human liver alcohol dehydrogenases characterized to date are dimers of molecular weight approximately 85,000. Those that have been tested contain 4g atoms of zinc/mol, bind two mols of NAD and readily oxidize long chain aliphatic and aromatic primary alcohols (Bosron et al., 1979; Lange and Vallee, 1976; Pares and Vallee, 1981). Most of the catalytic properties of the human protein are similar to those of the enzyme from horse liver, but they differ in regard to the magnitudes of the Michaelis constants for substrates and coenzymes and in substrate specificity. In particular, human liver alcohol dehydrogenase oxidizes ethylene glycol and methanol at high rates, whereas the horse enzyme exhibits little or no activity towards these alcohols. Unlike horse liver alcohol dehydrogenase isozymes, the isolated components of the human liver could not be

distinguished from each other by their steroid activity. Earlier work reported that all the isolated isozymes appeared to be active with steroids with a  $3\beta$ -HSD activity about 5% of that of ethanol.

While there was a two-fold difference seen between males and females in the amount of ADH contained in adult mouse and human kidney per gram of tissue, the same could not be said of human liver (Ohno et al., 1970). Kidney ADH activity of adult females is about one-fourth and that of adult males is about one-half of the liver ADH activity. In man, the ethanol dehydrogenase activity is not inducible. It has also been observed that neither ADH nor aldehyde dehydrogenase of liver was affected by ingestion of androgenic steroids (Ohno et al., 1970).

### iii) Hydroxysteroid dehydrogenases

Among the alcohol dehydrogenases, hydroxysteroid dehydrogenases are a particularly interesting group because of the importance of steroids in man. All steroid hormones have in their biosynthesis at least one reaction catalyzed by a hydroxysteroid dehydrogenase. Hydroxysteroid dehydrogenases (ketosteroid oxidoreductases; E.C. 1.1.1.n) are a family of enzymes catalyzing the interconversion of hydroxyl and carbonyl functions located on the steroid skeleton or the side chain (Talalay, 1963). These dehydrogenases are pyridine nucleotide-dependent enzymes. A general formulation of the oxidoreduction reaction is:



Dehydrogenases acting on 3 $\alpha$ -, 3 $\beta$ -, 6 $\alpha$ -, 6 $\beta$ -, 7 $\alpha$ -, 7 $\beta$ -, 11 $\alpha$ -, 11 $\beta$ -, 12 $\alpha$ -, 12 $\beta$ -, 15 $\alpha$ -, 15 $\beta$ -, 16 $\alpha$ -, 16 $\beta$ -, 17 $\alpha$ -, 17 $\beta$ -, 20 $\alpha$ -, 20 $\beta$ -, and 21-hydroxy-steroids are among those which have been demonstrated either histochemically or by incubation and isolation of metabolites, either with crude enzyme preparations or after purification of the enzymes (Talalay, 1963; Baillie et al., 1966). Estrogen oxidoreductases viz. 6 $\alpha$ -, 6 $\beta$ -, 7 $\alpha$ -, 7 $\beta$ -, 11 $\beta$ -, 15 $\alpha$ -, 15 $\beta$ -, 16 $\alpha$ -, 16 $\beta$ -, 17 $\alpha$ -, and 17 $\beta$ - have been reviewed by Breuer and Knuppen (1969).

In addition to their role in steroid metabolism, HSDs serve as models for the study of steroid receptor proteins. The highly specific interaction between steroids and receptor proteins, vital to hormonal activity, is perhaps more readily studied in hydroxysteroid dehydrogenases for which rapid and sensitive assays of the enzymatic activity are available.

Some of these enzymes, e.g placental estradiol 17 $\beta$ -dehydrogenase (Engel and Groman, 1974) have narrow specificity with only estradiol and a few closely similar estrogens acting as substrates. Others, for example those of *Pseudomonas testosteroni* 3(17) $\beta$ -HSD (Marcus and Talalay, 1956) and *Streptomyces hydrogenans* (3 $\alpha$ ,20 $\beta$ -



HSD) have wider specificities; both are capable of recognizing carbonyl groups at both ends of the steroid molecule as substrates. All of the above have been used for covalent steroid-protein linkage experiments which have improved our understanding of the requirements for forming a steroid receptor site on a protein.

Of the commercially available steroid dehydrogenases, only two are offered in crystalline form. The  $3\alpha,20\beta$ -HSD of *Streptomyces hydrogenans* has been obtained crystalline (Edwards and Orr, 1978), though the commercially available enzyme has deliberately been diluted with bovine serum albumin to give a product of reproducible specific activity. The only other hydroxysteroid dehydrogenase obtainable commercially in crystalline form is horse liver alcohol dehydrogenase, but the steroid activity of this enzyme is associated primarily with the minor (S; steroid-active) subunit, rather than the major (E; ethanol active) subunit (Pietruszko and Theorell, 1969).

The majority of the steroid alcohol dehydrogenases thus far examined (Bentley, 1970) catalyze the transfer of hydride from the steroids to the 4-pro-S-position of the reduced NAD(P) (Henbest and Jackson, 1962). Akhtar et al., (1972) have listed a series of nine NADPH steroid reductases of mammalian origin in which the hydride transferred to the  $\alpha$ -face of the steroid was the 4-pro-S hydrogen of the coenzyme and that transferred to the  $\beta$ -face was the 4-pro-R hydrogen. The avian enzymes also employ NADP as cofactor but do not follow the mechanism described above. In the case of the  $3\alpha$ - and the  $3\beta$ -hydroxysteroid dehydrogenases of *Pseudomonas testosteroni*, both utilize the 4-pro-S proton of NADPH.

#### iv) Human Liver $3\alpha$ -Hydroxysteroid Dehydrogenases

$5\alpha$ - and  $5\beta$ -DHT are reduced to a small extent to the corresponding  $3\alpha$ - and  $3\beta$ -hydroxysteroids by the microsomal fraction of human liver in the presence of NADPH and NADH; NADH being the preferred cofactor (Bjorkhem et al., 1972).  $3\alpha$ - and

$3\beta$ -hydroxy-androstane oxidation by the microsomal fraction of human liver has also been reported (Bjorkhem, 1976) although no attempts were made to purify or characterize the enzymes. The ratio of  $3\alpha$ - to  $3\beta$ -reduction was about 2:1 in male liver microsomes. No sex differences could be detected in the activity of  $3\alpha$ -hydroxysteroid dehydrogenase in human liver (Bjorkhem et al., 1972, 1976). Age-dependent changes in the activities of various human liver steroid-metabolizing enzymes, including the  $3\alpha$ -HSD activity, were reported by Reynolds (1966).

Using biopsy samples from 17 patients, Lang et al. (1986) detected soluble and microsomal  $3\alpha$ -HSD from human liver, and optimized assay systems for the determination of the enzymes were developed. The mean and standard deviations for the cytoplasmic  $3\alpha$ -HSD activity was  $1.03 \pm 0.34$  nmol/min/mg protein and that for the microsomal enzyme was  $2.07 \pm 1.16$  nmol/min/mg protein. Using radioactive  $5\alpha$ -DHT as substrate, pH optima for the cytosolic and microsomal enzyme activities were found to be 6.0 and 5.5 respectively. The  $V_{max}$  for the microsomal  $3\alpha$ -HSD was reached at a NADH concentration of about 1.0mM. No correlation with sex, age, or disease was detected.

#### v) Other Mammalian $3\alpha$ -Hydroxysteroid Dehydrogenases

The rat is the major animal used for  $3\alpha$ -HSD research and liver is the predominant tissue studied.  $3\alpha$ -HSD activity has, however, been found in homogenates of rat kidney (Verhoeven et al., 1977) and in several other tissues (Inano et al., 1977; Thien et al., 1977).

The presence of soluble  $3\alpha$ -HSD activity, capable of reducing androstanes and pregnanes, in unfractionated rat liver cytosol was first described by Tomkins in 1956, and has been reviewed by Talalay (1963). More recently, four isozymes of  $3\alpha$ -HSD, designated F1 to F-4, one NADH- and three NADPH-linked, have been found in rat

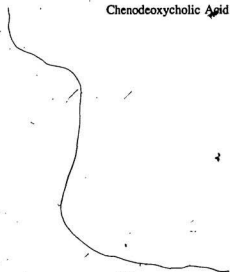
liver cytosol (Ikeda et al., 1981). The four fractions were separated by DEAE-cellulose. The molecular masses were estimated to be 32,000 Da. The main fraction, F-1, is NADH-dependent while F-3 was NADPH-dependent; these two isozymes have been characterized.

In the conversion of cholesterol to the main primary bile acids, cholic acid and chenodeoxycholic acid, both an NAD-dependent  $3\alpha$ -HSD and a  $3\alpha$ -HSD which can utilize either NAD or NADP are involved. At least six isozymes of  $3\alpha$ -HSD of rat liver cytosol have later been described (Ikeda et al., 1984). All the isozymes were able to catalyze the dehydrogenation of  $3\alpha$ -hydroxysteroids of differing carbon skeletons (C-19, C-22, C-23, C-24, C-25 and C-26). They also found that  $5\beta$ -cholestan- $3\alpha,7\alpha$ -diol which is believed to be an important intermediate in the formation of chenodeoxycholic acid from cholesterol, was the poorest substrate for all of their F-4 enzymes. Their findings suggest that the pathways of chenodeoxycholic acid formation presented by Mitropoulos and Myant (1967), (pathway B), and Ayaki and Yamasaki (1972), (pathway C), may function in rats (Fig 4).

This work supports results obtained by Koide (1969) who found that the  $3\alpha$ -HSD involved in the metabolism of steroid hormones also catalyzes reactions concerned with the biosynthesis of bile acids from cholesterol.

Sanyal et al., (1974) reported that in the study of  $5\beta$ -reduction of testosterone to  $5\beta$ -androstane- $3\alpha,17\beta$ -diol by unfractionated rat liver cytosol, production of the  $3\alpha$ -allylic steroid alcohol, androst-4-ene- $3\alpha,17\beta$ -diol, was apparently an early, but reversible event. They then established that dehydrogenases present in the cytosol were capable of reducing the 3-ketone of both the unconjugated 4-en-3-one and the saturated  $5\beta$ -DHT to the corresponding  $3\alpha$ -hydroxysteroid. By studying the metabolism of the  $3\beta$ -deuterio- $3\alpha$ -allylic alcohol they showed that deuterium loss was an obligatory step in its conversion to the saturated  $5\beta$ -androstane- $3\alpha,17\beta$ -diol and concluded that the

FIG. 4 Role of  $3\alpha$ -Hydroxysteroid Dehydrogenase in Cholic Acid and  
Chenodeoxycholic Acid Biosynthesis (Ikeda et al., 1984).



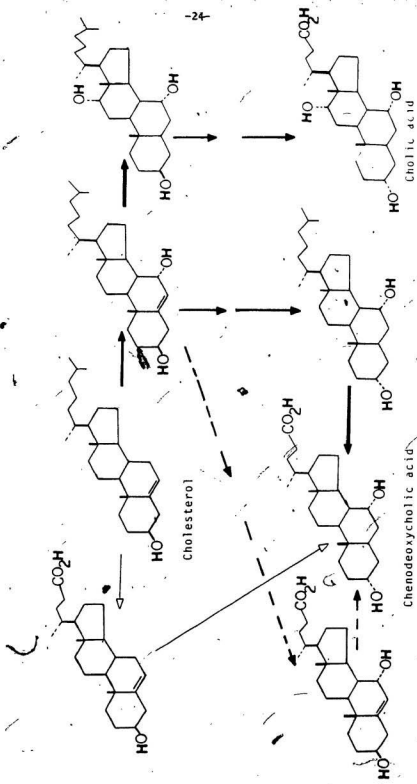


FIG. 4

reductase requires the conjugated 4-en-3-one structure.

The conversion of 5 $\alpha$ -DHT to 3 $\alpha$ -androstenediol appears to be the major route for the metabolism of testosterone and 5 $\alpha$ -DHT in brain and has been demonstrated *in vivo* and in brain tissue slices (Denef et al., 1973; Celotti et al., 1979; Martini, 1982). Krieger and Scott (1984), using <sup>14</sup>C-DHT and NADPH as cofactor described a single method for the assay of the soluble 3 $\alpha$ -HSD in homogenates of rat brain cytosol and mapped out the activity in fourteen brain regions. An NADPH-dependent 3 $\alpha$ -HSD from rat brain cytosol was also purified by Penning et al., (1985).

Three 3 $\alpha$ -HSD activities were found in the rat kidney (Verhoeven et al., 1977). One is microsomal, NADPH linked. The other two are soluble; one NADH-linked, one NADPH-linked. The microsomal NAD-dependent 3 $\alpha$ -HSD was found to convert 3 $\alpha$ -androstenediol to 5 $\alpha$ -DHT (Verhoeven et al., 1977). The activity of 3 $\alpha$ -HSDs in mammalian kidney cytosol has been reported (Verhoeven et al., 1976; Ghraf et al., 1978). An NADP-dependent 3 $\alpha$ -HSD was found in the rat kidney cytosol. The enzyme exhibits a higher affinity for 5 $\alpha$ -DHT than for 3 $\alpha$ -androstenediol (de Moor et al., 1975; Verhoeven et al., 1976). Because NADPH/NADP ratios are greater than 1 in the cytosol of mammalian tissues, de Moor et al., (1975) concluded that the cytoplasmic activity of 3 $\alpha$ -HSD plays a major role in the transformation of 5 $\alpha$ -DHT to the 3 $\alpha$ -androstenediol under *in vivo* as well as *in vitro* conditions.

Hastings et al., (1980), have also described the presence and kinetics of both NADPH and NADH-dependent 3 $\alpha$ -HSD activity in the rat testis. Taurog and his colleagues (1975) have shown that the reduction of 5 $\alpha$ -dihydrotestosterone to 3 $\alpha$ -androstenediol is the major metabolic route for the androgens in the rat prostate and have implicated the 3-hydroxysteroid dehydrogenases in the regulation of androgen action. The conversion of 5 $\alpha$ -DHT to 3 $\alpha$ -androstenediol is in general reversible in target organs (Taurog et al., 1975; Becker et al., 1973; Krieg et al., 1975; Dionne et al.,

1974). Furthermore, the metabolism of 5 $\alpha$ -DHT to 3 $\alpha$ -androstenediol in the muscles may be an important factor for the total body-androgen status, since the musculature contributes more than one-third of the body mass.

The presence of at least two isozymes of 3 $\alpha$ -HSD in rat testis, prostate and epididymis cytosol preparations has been demonstrated (Krause and Karavolas, 1980). The NADPH- and NADH-dependent enzymes could be separated from each other on Sephadex G-200 column chromatography. The one eluting in the void volume is primarily NADH-dependent whereas the other with a molecular weight of 34,000 is predominantly NADPH-dependent (Hastings et al., 1980).

In contrast to conventional wisdom that 3 $\alpha$ -HSDs are involved in the destruction of hormonal activity of steroids, Jacobi et al., (1978) reported induction of significant growth in rat prostate as a result of the presence of 5 $\alpha$ -androstane-3 $\alpha$ ,17 $\beta$ -diol.

The majority of the 3 $\alpha$ -HSD activity was found in the cytosolic fraction rather than the nuclear and microsomal fractions in rat heart, diaphragm, skeletal muscle and the bulbocavernosus/levator ani (BCLA), (Smith et al., 1980). Extensive studies on the characteristics of the C-19 steroid 3 $\alpha$ -HSD activities in rat liver (Koide, 1969; Hoff and Schriefers, 1973), kidney Verhoeven et al., 1976; Ghraf et al., 1977), prostate (Taurog et al., 1975; van Doorn et al., 1975; Inano et al., 1977) and anterior pituitary (Celotti et al., 1979; Thien et al., 1977) have been made. Both NADPH- and NADH-dependent 3 $\alpha$ -HSD activities were found in the hypothalamus, the former being a cytosolic enzyme, and the latter, a plasma membrane enzyme (Krause and Karavolas, 1980). Rat hypothalamic 5 $\alpha$ -dihydroprogesterone, NADH-linked 3 $\alpha$ -HSD is reported to be associated with plasma membranes with a broad pH optimum of 6-10 (Krause and Karavolas, 1981). Smithgall and Penning (1985) also demonstrated the presence of soluble 3 $\alpha$ -HSD activity in rat lung and testis.

A  $3\alpha$ -HSD has also been reported to have been isolated from rabbit liver cytosol and was named F-3. This enzyme was NADPH-dependent and had a M.W. of 29,000 (Sawada et al., 1980). An enzyme having dual  $3(17)\alpha$ -HSD activity in the rabbit kidney has been described by Lau et al., (1979).

It has been found that  $5\alpha$ -androstane-3,17-dione is converted to mixtures of  $3\alpha$ -hydroxy- $5\alpha$ -androstan-17-one and  $3\beta$ -hydroxy- $5\alpha$ -androstan-17-one by  $3\alpha$ - and  $3\beta$ -HSD in boar testis (Brophy and Gower, 1972) and submaxillary salivary glands (Kotkov et al., 1972) and gilt nasal epithelium. In boar testis,  $5\alpha$ -androst-16-en- $3\beta$ -ol predominates but in the immature animal, the  $3\alpha$ -hydroxy compound predominates, the change to  $3\beta$ -hydroxy compound occurring gradually as the age of the animal increases (Booth, 1975).

The baboon kidney was found to have  $3\alpha$ -HSD activity, converting  $3\alpha$ -androstanediol to  $5\alpha$ -DHT (Okumura et al., 1979).

vi) Other Enzyme Activities Associated with Mammalian  $3\alpha$ -HSD

It has been reported that human liver ethanol dehydrogenases are also  $3\beta$ -hydroxysteroid dehydrogenases, and that their corresponding ketones are all substrates for the human liver alcohol dehydrogenases. Although a  $3\alpha$ -hydroxysteroid is described as an inhibitor of this  $3\beta$ -HSD activity, no metabolism of the  $3\alpha$ -hydroxysteroid by human liver ethanol dehydrogenase has been reported (Reynier et al., 1969). These observations were made, however, long before the multiple human ethanol dehydrogenases were characterized.

$3\alpha$ -HSD activity has been implicated in the detoxification of carcinogens derived by oxidation in vivo of polycyclic aromatic hydrocarbons.  $3\alpha$ -Hydroxysteroids can also be oxidized by enzymes other than those primarily recognized as  $3\alpha$ -HSD. Multiple forms of mouse liver cytosolic benzene dihydrodiol (trans-1,2-dihydroxy-3,5-



cyclohexadiene) NADP-dependent dehydrogenase, (dihydrodiol dehydrogenase) designated DD1, DD2, DD3 and DD4 have been purified (Bolcsak and Nerland, 1983). The rat liver enzyme, also NADP-linked, has a M.W. of 35,000 (Penning and Talalay, 1983). These enzymes have been shown to catalyze the oxidation of  $3\alpha$ -hydroxysteroids as well as benzenediol. An NAD(P)-dependent  $3\alpha$ -HSD was purified to homogeneity from rat liver cytosol where it was responsible for most if not all of the capacity for the oxidation of androsterone, 1-acenaphthenol and benzenediol (Penning, Mukharji, Barrows and Talalay, 1984).

Vogel (1980) has reported that the dihydrodiol dehydrogenase activity of rat liver co-purifies with  $3\alpha$ -HSD and may play a vital role in the detoxification of ultimate carcinogens (Glatt et al., 1982). The enzyme also promoted the reduction of quinones and certain aromatic aldehydes and ketones and is inhibited by most of the major types of non-steroidal anti-inflammatory drugs. The dehydrogenase was found to bind arachidonic acid and was potently inhibited by certain prostaglandins (Penning and Talalay, 1983).

It has been shown (Hurlock and Talalay, 1958; Talalay and Williams-Ashman, 1958a,b) that the NAD(P)-linked rat liver cytosolic  $3\alpha$ -HSD promoted an efficient hydrogen flow (transhydrogenation) between NADPH and NAD in the presence of catalytic amounts of steroid substrates. Transhydrogenation may serve a regulatory role by controlling hydrogen flow between NADH- and NADPH-dependent reactions.

vii) The role of  $3\alpha$ -HSD in development and control

Both NADH- and NADPH-linked  $3\alpha$ -HSD activities are sex-linked in the rat (Verhoeven et al., 1977). The soluble NADPH-linked activity is more active in the female. The microsomal NADPH-linked enzyme activity was however 35 times more active in the male than in the female. The NADH-linked  $3\alpha$ -HSD activity is subject to

complex developmental changes (Verhoeven et al., 1977). Hoff and Schriefers (1973) have demonstrated sex differences in the rate of  $5\alpha$ -androstane-3,17-dione reduction catalyzed by rat liver cytosolic  $3\alpha$ -HSD, while Verhoeven et al., (1976) have shown that the reduction of  $5\alpha$ -DHT to  $3\alpha$ -androstenediol catalyzed by rat kidney cytosol is two-fold higher in females. The latter difference has also been attributed to regulation of the enzyme by estrogen (Ghrat et al., 1975).

Due to their potential importance in regulating  $5\alpha$ -DHT metabolism,  $3\alpha$ -HSD activities in rat heart, diaphragm, skeletal muscle and the BCLA have been studied. The role of the  $3\alpha$ -HSD in hormone metabolism has also been studied by Pietruszko and Chen (1976). They found that the acetaldehyde reductase from rat liver cytosol is also a  $3\alpha$ -hydroxysteroid dehydrogenase.

$3\alpha$ -Hydroxysteroids have been implicated as inhibitors of ethanol dehydrogenases and must therefore bind to them. In the conversion of steroid ketone to alcohol, compounds of the A/B cis configuration ( $5\beta$ -) were found to be metabolized at about ten times faster rates than steroids of A/B trans ( $5\alpha$ -) configuration. In the oxidation from alcohol to ketone, A/B trans steroidal  $3\beta$ -alcohols were better substrates than the A/B cis compounds. The  $K_m/V_{max}$  values determined for  $3\beta$ -hydroxysteroids and their ketones are generally low, suggesting that such steroids are good substrates (Cronholm et al., 1975).

#### viii) Bacterial $3\alpha$ -Hydroxysteroid Dehydrogenases

Several bacterial  $3$ -hydroxysteroid dehydrogenases have been studied in considerable detail. An advantage of bacterial steroid dehydrogenases is that the enzymes are inducible, offering the possibility of obtaining significant amounts of protein. They are also reasonably stable and can be purified to apparent homogeneity. These enzymes can be subjected to genetic manipulations, permitting amino acid substitutions to be

made, and allowing a novel approach to studying protein-steroid interactions. Purified bacterial  $\alpha$ - and  $\beta$ -hydroxysteroid dehydrogenases serve as models for the study of the interaction of steroids with enzymatically active protein surfaces. The affinity of these enzymes for their steroidal substrates is high and relatively specific.

Using testosterone as the sole source of carbon, Marcus and Talalay (1956) isolated a *Pseudomonad* which they named *Pseudomonas testosteroni*. This microorganism had  $\alpha$ - and  $\beta$ -HSD activities, catalyzing oxidation-reduction at the C-3 and C-17 positions of androstanes.

By selecting growth on testosterone or estradiol-17 $\beta$  as providing the only source of organic carbon, Payne and Talalay (1985), isolated a number of soil microorganisms which contain highly active and inducible, NAD-linked 3 $\alpha$ -, 3 $\beta$ -, and 17 $\beta$ -hydroxysteroid dehydrogenases. Such enzymes are suitable for the microanalysis of steroids and of steroid-transforming enzymes, as well as for performing stereoselective oxidations and reductions of steroids. Microbial 3 $\alpha$ - and 3 $\beta$ -HSDs are now available for analytical purposes (Shikita and Talalay, 1979). An observation that may be relevant to the work reported in this thesis is that the 3 $\alpha$ -HSD of *P. testosteroni* is inhibited by superoxide radicals ( $O_2^{\cdot -}$ ) and that the presence of NADH prevents this inhibition (Kim et al., 1986).

The inducible 3 $\alpha$ ,20 $\beta$ -hydroxysteroid dehydrogenase from *Streptomyces hydrogenans* has been the subject of a large number of studies. Much is now known about its physical properties and the range of acceptable steroid substrates. The enzyme is tetrameric, each identical subunit has a M.W. of approximately 24,000. For 5 $\alpha$ -DHT, the 3 $\alpha$ -HSD activity has a  $V_{max}$  of 19.6  $\mu$ mol/min mg of protein and a  $K_m$  of 289  $\mu$ M. The properties of this enzyme have been reviewed (Edwards and Orr, 1978). Preliminary X-ray crystallographic data has now been obtained by Fitzgerald, Duax, Punzi and Orr (1984) on the *Streptomyces hydrogenans* 3 $\alpha$ ,20 $\beta$ -HSD. More recently, single

crystals of the  $3\alpha,20\beta$ -hydroxysteroid dehydrogenase of square bipyramid shape have been grown at room temperature in the presence of excess NADH (Ghosh et al., 1986). This enzyme may soon become the first HSD of known three-dimensional geometry.

## CHAPTER III

### MATERIALS AND METHODS

#### 1. MATERIALS

##### i) Steroids

17 $\beta$ -hydroxy-17 $\alpha$ -methyl-5 $\alpha$ -androstane-3-one, 17 $\alpha$ -methyl-5 $\alpha$ -androstane-3 $\alpha$ ,17 $\beta$ -diol, 17 $\alpha$ -methyl-5 $\alpha$ -androstane-3 $\beta$ ,17 $\beta$ -diol, 17 $\alpha$ -methyl-5 $\beta$ -androstane-17 $\beta$ -ol-3-one, 5 $\beta$ -androstane-3,17-dione, and 5 $\alpha$ -androstane-3,17-dione were purchased from Steraloids Inc. Wilton, NH. 5 $\alpha$ -androstane-17 $\beta$ -ol-3-one, 5 $\alpha$ -androstane-3 $\alpha$ ,17 $\beta$ -diol, 5 $\alpha$ -androstane-3 $\beta$ ,17 $\beta$ -diol, 5 $\beta$ -androstane-17 $\beta$ -ol-3-one, 5 $\beta$ -androstane-3 $\alpha$ ,17 $\beta$ -diol, 5 $\alpha$ -androstane-3 $\beta$ ,17 $\beta$ -diol, 5 $\beta$ -androstane-3 $\beta$ -ol-17-one, 5 $\beta$ -androstane-3 $\alpha$ -ol-17-one, 5 $\beta$ -pregnan-3 $\alpha$ -ol-20-one, and 5 $\alpha$ -pregnan-3 $\alpha$ -ol-20-one were purchased from Sigma Chemical Co. St Louis, Missouri. 17 $\alpha$ -methyltestosterone and testosterone were purchased from Searle Chemicals, Inc., Chicago, Ill. 5 $\alpha$ -androstane-3 $\alpha$ -ol-17-one was purchased from Schwarz/Mann, Division of Becton, Dickinson and Co. N.Y.

##### ii) Coenzymes

NAD, (Grade III from yeast), NADP (monosodium salt), NADP, (Monosodium salt, Sigma grade, from yeast), NADPH (tetrasodium salt) were from Sigma Chemical Co., St Louis, Missouri.

iii) Chromatographic and Electrophoretic Chemicals

Sephadex G-25, G-200, DEAE-Sephacel were from Pharmacia Fine Chemicals, Uppsala, Sweden. CM52 (CM-cellulose, preswollen) was from Whatman, England. I.C.I. Procion Blue MX3G dye bound to Sepharose 4B-200 was prepared by Mr. Gordon Murphy in our laboratory. Starch-Hydrolysed was from Connaught Laboratories Ltd., Willowdale, Ont. Acrylamide, bisacrylamide, TEMED, ammonium persulphate, Pyronin Y(G) were from Bio-Rad Laboratories, Richmond, Calif. Glutathione (reduced) was from Aldrich-Fine Chemicals, Milwaukee, Wis. Amido Black 10B was from The British Drug Houses (Canada) Ltd., Toronto, Ont. Redi-Plate Silica Gel GF were from Fisher Scientific Company, Pittsburgh, Pa. Ampholine pH ranges 9-11 and 3.5-10 were from LKB, Bromma, Sweden. Spectrapor membrane tubing was from Spectrum Medical Industries, Inc. Los Angeles, Ca.

Negative pressure protein dialysis membrane,  $\alpha$ -ProDiMem, was purchased from Pierce Chemical Co., Rockford, Ill. Enzyme assays were done on the Gilford U.V. Spectrophotometer, Model 240 with a Model 6050 sample changer.

iv) Protein Standards, Enzymes and Related Compounds

Bovine serum albumin, 2-mercaptoethanol, NBT, PMS, 3 $\alpha$ -HSD (Pseudomonas testosterone) were from Sigma Chemical Co., St Louis, Missouri. Coomassie Brilliant Blue R-250 was from Eastman Kodak Co., Rochester, N.Y. Human-placenta was obtained immediately after delivery from St Clare's Hospital, St John's, Nfld. Human kidney and liver, free from disease, were obtained immediately after autopsy from the Health Sciences Centre, St John's, Nfld. with the appropriate approval of the Human Investigations Committee.

v) Buffer salts and solvents

All salts and solvents were the highest purity preparation available, of reagent grade or better. They were purchased from Fisher Scientific Co., Ottawa, Ont., from Canadian Lab. Supplies, Ottawa, Ont., from Aldrich Chemical Co., Inc. Milwaukee, Wis., from Sigma Chemical Co., St Louis, Missouri. or from BDH Chemicals, Toronto, Ont. HPLC solvents were purchased from the suppliers listed above or from Burdick and Jackson, Inc., Muskegon, Michigan, USA. The water was de-ionized and further purified by a reverse-osmosis system (Milli-Q, Millipore Corporation).

## 2. METHODS

Melting points were measured on a Kofler apparatus and are uncorrected. NMR spectra were determined with a Bruker WP 80 for solutions in deuteriochloroform with tetramethylsilane as internal standard. Products were analysed or separated by HPLC using a Waters 244 liquid chromatograph with a R401 differential refractometer and a Model 450 variable wavelength detector with a Whatman Partisil silica 10/2 column. The solvent system was hexane/isopropanol 95:5 by volume run at 1 mL/min, at about 1000 psi. Retention times of the steroids varied somewhat from day to day, being critically dependent on the exact composition of the solvent mixture, but were satisfactorily consistent for a given solvent batch. Standards were run each time; phenanthrene and cholesterol were also run as additional standards. Retention times relative to cholesterol were constant. The tlc Rf values reported are on silica GF, Analtech Inc., Newark, Delaware, with solvent system ether/ toluene 2:1 in which androst-4-ene-3,17-dione standard had a Rf of 0.51 and testosterone 0.37.

### a) Enzyme Assays

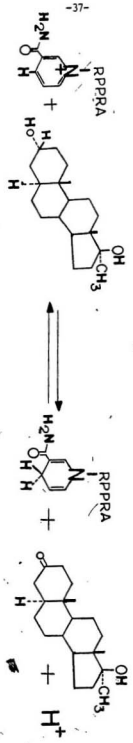
Enzyme assays are based on the generation or loss of NADH, readily detected by its absorption at 340 nm, (Fig. 5).



FIG. 5 Reaction used in Assay of 3 $\alpha$ -Hydroxysteroid Dehydrogenase Activity.

(The forward reaction proceeds optimally at pH 9.0 while the reverse is optimum at pH 7.0.  $\lambda_{\text{max}}$  340 nm;  $\epsilon$  6,200).

FIG 5



i) Alcohol oxidation with NAD(P)

The incubation mixture consisted of steroid alcohol substrate (10mM) in freshly distilled tert-butanol, 100 $\mu$ L; 4.4mL of 50mM sodium pyrophosphate buffer containing 2.3mM NAD(P), pH 9.0 and generally 0.5mL of the enzyme solution. The blank contained all of the above except the steroid substrate. Instead, 100 $\mu$ L tert-butanol was added. For kinetic assays, the change in NAD(P)H concentration was measured at 340 nm on the Gilford spectrophotometer. A typical assay medium consisted of a 50 mM sodium pyrophosphate buffer containing 2.3 mM NAD(P), 2.9 mL, 100 $\mu$ L of the enzyme solution and 10 $\mu$ L of the 5 mM steroid substrate. The control cuvette contained 10 $\mu$ L of t-butanol instead of the steroid substrate. Where identification of metabolites was desired, incubation was carried out in the warm room at 37°C overnight.

For the ethanol dehydrogenase assay, 0.5 mL of 2.0 M ethanol (6.06 mL of ethanol in 50 mL deionized water) was used as substrate with pyrophosphate buffer (1.5 mL, pH 9) and NAD (1.0mL of 25 mM in pyrophosphate buffer).

ii) Carbonyl group reduction with NAD(P)H

The conditions and concentrations were as above except that the ketosteroid, 0.176 mM NAD(P)H, and 50mM potassium phosphate buffer of pH 7.0 were used. The control experiment contained no steroid.

b) Isolation of Metabolites after Incubation

Incubations were terminated by the extraction of the steroid substrate and product with 5 mL of methylene chloride. The mixture was then spun in an IEC Clinical Centrifuge to separate the organic layer from the water. The methylene chloride layer was then passed through a small plug of cotton wool in a Pasteur pipette to remove water droplets and then evaporated to dryness under nitrogen. A few drops of methylene

chloride were added to the dried samples and the mixture was then spotted on thin layer plates coated with silica gel GF and subsequently separated by chromatography in the solvent system ether: toluene (2:1). Appropriate authentic reference samples were also spotted on the same plate.

In the case of samples which were later studied by high performance liquid chromatography (HPLC), the methylene chloride mixture was refiltered through another plug of cotton wool before injection. For further characterization in one case where the  $3\alpha$ -hydroxysteroid had been converted to  $17\alpha$ -methyl- $5\alpha$ -dihydrotestosterone, the 3-ketosteroid was further characterized by dissolving in ethanol and reduced to the  $3\beta$ -hydroxysteroid; the product isolated as before and characterized by HPLC (see results).

#### c) Thin layer chromatography (TLC)

The thin layer chromatography was carried out on  $20 \times 10$  cm plates coated with 0.25mm of silica gel GF. Samples were spotted 3 cm from the lower edge of the plate and 1.5 cm apart. Chromatography was done at room temperature. After development, the plates were allowed to air dry. The plates were then visualized under ultraviolet light (UV) at 375 nm to determine which spots were UV absorbent. The steroids were then detected by spraying the plates with a 1:1 solution of concentrated sulphuric acid and ethanol, followed by heating in a glass-fronted oven at 100 degrees for a few minutes. Under these conditions, different steroids give different characteristic colours which in almost all cases change with time.

#### d) High performance liquid chromatography (HPLC)

A Waters 244 Liquid Chromatograph equipped with a R401 differential refractometer and a Schoeffel model 450 variable wavelength detector was used for the HPLC

separations. The steroids were separated on either a Whatman Partisil 10/25 Column or on a Waters  $\mu$ Bondapak C18 (30cm) column. For identification of the products of the incubation, the methylene chloride extract was refiltered through a plug of cotton wool and then injected. Cholesterol and phenanthrene together with the three steroid substrates were used as standards. The peaks were identified by measuring their retention times relative to that of the solvent peak (K) and comparing them to those of the standards.

#### e) Protein Determinations

All protein determinations were carried out according to the method of Lowry et al., (1951). Bovine serum albumin was used as the standard.

#### f) Rapid spot plate technique

Initial search for  $3\alpha$ -HSD activity was done using a modified form of the technique developed by Rimsay and Orr (1982). After column chromatography, detection of enzyme activity was achieved by incubating 100  $\mu$ L samples of the fractions with solution A containing 10mg NAD, 1mg phenazine methosulphate and 2.5mg nitro blue tetrazolium made up to 10mL of 50mM phosphate buffer, pH 7.5 and solution B consisting of 5mM of the steroid substrate in t-butanol. The t-butanol was freshly distilled by refluxing with lithium aluminium hydride and testing for peroxides with starch and potassium iodide. Preliminary experiments had shown that the t-butanol did not contain a substrate for the human liver  $3\alpha$ -HSD. Incubations were done at 37°C in the dark for 30-60 minutes.

g) Iso-electric focussing

i) Preparation of the gel solutions

LKB ampholytes pH 3.5-10 and pH 8-10.5 were used.

Stock solutions were prepared as follows:

Stock Solution

Acrylamide solution	29.1% w/v,
N,N'-methylenebisacrylamide (bis)	0.9% w/v
Ammonium persulphate (prepared fresh)	1% w/v

The acrylamide and the bis were filtered and stored in a dark bottle at 4 degrees

C. For one 0.5mm thin-layer polyacrylamide gel (%T=5, where %T is the percentage total monomer i.e grams acrylamide plus bisacrylamide/100 mL; C=3%, where C is the degree of cross-linking due to the bisacrylamide/100 mL).

The composition of the gel was as follows:

Gel Composition

Acrylamide stock solution	3.5 mL
Bis-acrylamide stock solution	3.5 mL
LKB Ampholine carrier ampholyte	1.5 mL
Distilled water	12mL

This was de-aerated under vacuum for 10 minutes. Ammonium persulphate (0.5 mL) was added to the mixture and poured, and the gel allowed to stand and set.

ii) Focussing conditions

The electrode solutions consisted of 1M phosphoric acid at the anode and 1M sodium hydroxide solution at the cathode. The electrode strips were evenly soaked with the appropriate electrode solution removing excess solution with tissue paper or filter paper. Parts of the electrode strips which protruded beyond the end of the gel were cut off with sharp scissors. The Pharmacia Flat Bed Apparatus, FBE 3000 and the Pharmacia Electrophoresis Constant Power Supply, ECPS 3000/150 were used. A small amount of kerosene was spread on the template and the polyacrylamide gel laid on the template within the outermost lines, avoiding trapping of air bubbles. The cooling plate was connected to a thermostat and a Haake K 11 flow-through cooling apparatus and the temperature was set at 10 degrees C. Dry application sample strips were applied to the surface of the gel using the template as a guide. 10 $\mu$ L samples of the dialyzed protein were applied by means of a micropipette. The pressure bar with the electrodes was applied to the electrode strips and fixed into the holes of the safety lid. Focussing was done at constant power of 25W for 60 minutes.

iii) pH Gradient measurements

After focussing, the pH gradient in the gel was measured with an Ingold-R surface electrode at 1 cm intervals. The gel was then cut into strips and stained for activity and protein.

iv) Staining

For the protein stain, the staining solution consisted of Coomassie Brilliant Blue R 250, 0.35 g dissolved in 300 mL of destaining solution with stirring. The flask was sealed with foil and then heated to 60 degrees C. The destaining solution was made up of:

Destaining Solution

Ethanol	500 mL mL L
Acetic acid	160 mL L
Distilled water to	2 L

The fixing solution consisted of:

Fixing Solution

Trichloroacetic acid	34.5 g 4 g mL
Sulphosalicylic acid	10.4 g mL
Distilled water to	300 mL

Preserving solution consisted of 30 mL glycerol diluted with destaining solution and made up to 300 mL.

For activity stain, the gels were incubated with appropriate hydroxysteroid substrate dissolved in t-butanol in 50 mL of 50mM phosphate buffer, pH 7.5 containing 2.3mM NAD at 37°C for 30 minutes and then 5 mL of 50mM phosphate buffer, pH 7.5 containing 0.5mg of phenazine methosulphate and 1.25mg of nitro blue tetrazolium (the solution A of the rapid spot plate technique, but without the added NAD). The distance moved by the enzymes as determined by movement across the gel was measured and the pI found by extrapolation on the pH gradient curve obtained. Ethanol dehydrogenase activity was checked for by staining using ethanol and pentanol as substrates.



#### **h) Polyacrylamide gel electrophoresis**

Polyacrylamide gel electrophoresis was done by a modification of the method of Maurer and Allen (1972). Protean<sup>TM</sup>II polyacrylamide gel electrophoretic apparatus was used. The gels were prepared as follows:

For the running gel:

Two parts of solution A consisting of 1N KOH (8.0mL), Glycine (19.0 g), TEMED (0.08 mL) and water made up to 100 mL.

One part of solution B consisting of acrylamide (60.0 g), bisacrylamide (1.6 g) and water to 100 mL.

One part of solution C consisting of 0.56 g ammonium persulphate in 100mL and four parts of water.

These were mixed and de-gassed and poured.

The stacking gel consisted of:

One part of solution A,

One part of water,

Four parts of sucrose (40.0 g/100 mL),

One part of a solution consisting of 20.0 g of acrylamide and 3.5 g of bisacrylamide in 100 mL of water and

One part of riboflavin (4.0 g/100 mL).

This was also de-gassed and poured and allowed to set on top of the running gel with the well comb in place.

The electrode buffer solution consisted of a 10% solution of 2,6-lutidine (2,6-dimethylpyridine), 38.2 mL, and 13.7 g glycine in 1000 mL water, pH 8.3. 50 $\mu$ L samples of the various ammonium sulphate precipitates of both horse and human liver cytosol in sucrose were run. The gels were run at 150V for 24 hours and then stained for 3 $\alpha$ -HSD activity and for protein.

#### i) Starch gel electrophoresis

Starch gel (Connaught, 15% w/v) was made in 0.025M tris-Cl buffer, pH 8.6 (350 mL). The mixture was heated over a Bunsen burner with shaking until it was clear and almost boiling. This was de-gassed while still shaking (about 30 sec) under vacuum (house). This was then poured into a mould (18 x 18 cm). After cooling for about 2 minutes, a glass plate was placed on top of the gel taking care that no air bubbles were trapped. This was held in place till the gel set. A transverse strip, 1 cm wide was removed from the middle of the gel and then 10  $\mu$ L samples adsorbed on filter paper were inserted in the gel. The two halves were then brought together. The running buffer was 0.3M tris-Cl buffer, pH 8.6. Good electrode contact was made by using filter paper on the gel and then covered with cheese cloth dipping into the electrode solution. The gel was run for 15 hours at 100V constant voltage, covered with Saran Wrap<sup>®</sup> and heat dissipated by a fan. After electrophoresis, the gel was sliced laterally along its smallest (5 mm) dimension into two equal halves with a wire gel slicer and then each layer stained separately for activity and protein. Alcohol dehydrogenase activity was also stained for using ethanol and occasionally pentanol as substrates.

## CHAPTER IV

### EXPERIMENTAL RESULTS

#### a) Synthesis of Steroid Substrates (Fig 6)

The synthetic steps are outlined in Fig 6.

##### i) 17 $\alpha$ -Methyl-DHT

17 $\alpha$ -Methyltestosterone (3g, 9.93mmols) was dissolved in tetrahydrofuran. The pale straw-coloured solution was added by pipette to a blue solution of lithium in ammonia (liquid, approximately 90mL, distilled in, not poured from an inverted cylinder). The lithium and steroid solution were added alternately in portions to judge the progress of the reaction by the colour. Approximately 1.5 lithium atoms per steroid molecule were necessary to give a permanent blue colour. The reaction was fast (< 1 min., perhaps limited by the steroid solubility in the ammonia / tetrahydrofuran).

When the blue colour appeared to be much slower to disappear (dependent on the ketonization of the enolate anion or perhaps due to loss of lithium by the production of  $\text{LiNH}_2$ ), the solution was left stirring for a further 5 min. and then acetone added to the solution to remove the remaining lithium metal, followed by ether. The ammonia liquid was evaporated off in about 10 min. in a warm water bath until the solution became warm (b.p. of ether rather than of ammonia). The steroid was extracted with ether/water and the ether washed about seven times when no ammonia smell remained. The ether solution was filtered to remove approximately 5 mg of a white precipitate and evaporated to about 50 mL when it crystallized as prisms. TLC of the ether extract showed it to contain approximately 90% of one compound, later shown to be 17 $\alpha$ -

methyl-5 $\alpha$ -DHT. On attempted recrystallization, the product did not dissolve even with 200 mL ether, but dissolved upon addition of methanol and crystallized nicely upon evaporation. The melting point was 189-191°C. Results from TLC showed the product to consist of the following: 17 $\alpha$ -methyl-DHT 90%, allylic 3 $\alpha$ -alcohol 3%, allylic 3 $\beta$ -alcohol 1%, 17 $\alpha$ -methyltestosterone 6%.

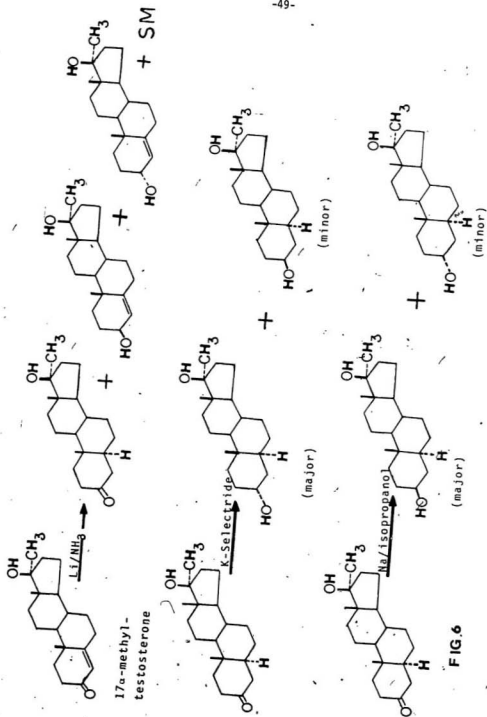
Repeated crystallization of the product from methylene chloride and isopropanol gave 17 $\alpha$ -methyl-DHT as essentially a single spot on TLC (solvent system: ether/toluene 2:1) with  $R_f$  = 0.46. HPLC retention time 10.3 min; relative to cholesterol(6.4 min): 1.61. The melting point was determined to be 187-190°C. The reported value is 189-192°C.

ii) 17 $\alpha$ -methyl-5 $\alpha$ -androstane-3 $\beta$ ,17 $\beta$ -diol

The 3 $\beta$ -alcohol was prepared from 17 $\alpha$ -methyl-5 $\alpha$ -DHT by reduction with sodium in isopropanol. 17 $\alpha$ -Methyl-5 $\alpha$ -DHT (approximately 100 mg) was dissolved in 50 mL of isopropanol and then sodium was added; it dissolved slowly. The mixture was heated to reflux and then left overnight at room temperature. The steroids were extracted with ether/water/tartaric acid mixture in a separatory funnel. The ether layer was passed through a plug of cotton wool and warmed gently on a water bath. On cooling, the solution yielded crystals which were later recrystallized from methylene chloride/isopropanol. Results from TLC showed the formation of the desired product as a more polar steroid with  $R_f$  = 0.26. HPLC retention time 14.0 min; relative to cholesterol (6.4 min): 2.19. M.p. 205-207°C; the reported value from the literature is 208-209°C.

FIG 6. Synthesis of Steroid Substrates.

(For product ratios, see section a) above.).



iii) 17 $\alpha$ -methyl-5 $\alpha$ -androsterane-3 $\alpha$ ,17 $\beta$ -diol

For the synthesis of the 3 $\alpha$ -alcohol, the method of Gondos and Orr (1982) was used. 17 $\alpha$ -Methyl-5 $\alpha$ -DHT (100 mg) in tetrahydrofuran (approximately 25 mL) and 10 mL of K-selectride (1M in tetrahydrofuran) were stirred at room temperature overnight. The mixture was extracted with ethyl acetate and washed with water. The ethyl acetate layer was rotary evaporated and then recrystallized from methylene chloride/isopropanol. There was approximately 70% conversion to the 3 $\alpha$ -alcohol, 10% 3 $\beta$ -alcohol and 20% starting material, as judged by TLC. Repeated recrystallization from methylene chloride/isopropanol gave the required 3 $\alpha$ ,17 $\beta$  diol as a pure product, the melting point of which was determined to be 179-183 C. The reported value from the literature is 182-185 C. The 3 $\alpha$ -alcohol (Rf 0.38) was less polar than the 3 $\beta$ -alcohol (Rf 0.25), and more polar than the 3-ketone (17 $\alpha$ -methyl-5 $\alpha$ -DHT, Rf 0.47) as shown by TLC. HPLC retention time 12.14 min; relative to cholesterol (6.4 min): 1.90. The order of polarity on HPLC corresponds to that for TLC. The above three products were identical in TLC and HPLC characteristics with authentic samples later purchased from Steraloids Inc.

b) Purification of the Human 3 $\alpha$ -Hydroxysteroid Dehydrogenase

The preparation of the human liver hydroxysteroid dehydrogenase was performed using 200-300 gram-samples of human liver obtained immediately after autopsy with the approval of the Human Investigations Committee. When not used immediately, the livers were stored at -70 C in a plastic bag.

Typically, if frozen, the livers were thawed in a 50mM Tris-Cl buffer containing 1mM NAD, pH 8.5 at 4 C. The thawed liver was ground in a meat grinder and homogenized in a Sorvall-Omni Mix. Further buffer was added to give a ratio of 1 gram liver/1 mL of buffer.

i) Centrifugation

The homogenate was centrifuged at  $10,000 \times g$  for 30 minutes at  $4^{\circ}\text{C}$  (Beckman J-21B centrifuge) and filtered through cheese cloth to remove the cell debris and the fatty upper layer. The resulting supernatant was centrifuged at  $100,000 \times g$  for 1 hour (Beckman L2-65 Ultra centrifuge), giving a microsomal pellet and a clear supernatant fraction. The supernatant was then passed through cheese cloth. A typical preparation using 270 g of liver gave 192 mL of cytosol. Part of the supernatant (7 mL), referred to as the cytosol, was kept for assays and incubations. The remainder of the cytosol was used for the next part of the purification.

ii) Ammonium Sulphate Precipitation

Preliminary ammonium sulphate precipitation was carried out to determine which fraction had most of the  $3\alpha$ -HSD activity. Ammonium sulphate precipitated fractions of 0-20%, 20-40%, 40-60%, 60-80% and 80-100% of saturation ( $100\% = 4.1 \text{ M}$ ) were produced using solid ammonium sulphate. The ammonium sulphate crystals were added to the cytosol slowly while it was stirred at  $4^{\circ}\text{C}$ ; the mixture was re-adjusted to pH 8.5 if necessary and left to stand for about 15 min. The precipitate was collected after a  $10,000 \times g$  spin for 30 minutes. This preliminary test showed most of the  $3\alpha$ -HSD activity to be in the 40-60% ammonium sulphate precipitated fraction (Fig 7).

For larger scale enzyme isolation, an example is as follows: 44.95 grams of ammonium sulphate were dissolved in 185 mL of the cytosol and centrifuged at  $10,000 \times g$  for 30 minutes. The precipitate (0-40% fraction) was discarded. To the supernatant (150 mL), 19.8 grams ammonium sulphate crystals were added and stirred gently till they dissolved. The suspension was allowed to stand for 15 min. and spun at  $10,000 \times g$  for 30 minutes to obtain the 40-60% fraction. This precipitate was then homogenized with a small amount of 50mM Tris-Cl buffer containing 1mM of NAD,



FIG 7 3 $\alpha$ - and 3 $\beta$ -Hydroxysteroid Dehydrogenase Specific Activity  
in Ammonium Sulphate Precipitated Cytosolic  
Fractions of one Human Liver.

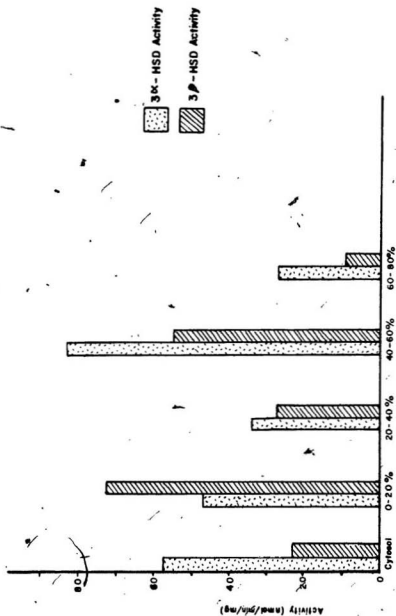


Fig. 7. 3α and 3β HSD Activity Human Liver Cytosol: Ammonium Sulphate ppid fractions

pH 8.5 in an Potter-Elvehjem homogenizer and the solution dialyzed in two 9-litre batches of 10mM Tris-Cl buffer, pH 8.0 using Spectrapor tubing with a MW cut-off of 6000-8000. The tubing had been prepared by heating in water containing a trace of disodium EDTA and sodium bicarbonate. The dialyzed solution (128 mL) was used for the next stage of the purification.

iii) DEAE-Sephacel Chromatography

A column (35 x 2.5 cm) was packed at room temperature with pre-swollen DEAE-Sephacel and then equilibrated at 4 C with 10mM Tris-Cl buffer, pH 8.0; the buffer being pumped on to the column. The dialyzed 40-60% fraction was then layered on to the column and 15 minute fractions of approximately 11 mL were collected. Preliminary experiments had indicated that the 3 $\alpha$ -HSD activity was not significantly retarded by the column and so no salt gradient was used. The bulk of the inactive protein remained on the column. Fractions 30-46 contained activity as judged by the spot plate technique described later. Using the specially prepared substrates, 3 $\alpha$ - and 3 $\beta$ -HSD as well as ethanol dehydrogenase activity assays were performed.

iv) CM-Cellulose Chromatography

The active fractions were pooled and concentrated/dialyzed using the Multiple  $\alpha$ -Micro-Pro-DiCon, Model MPDC-315 (Pierce Chemical Company, Rockford, Ill.). The concentrated fractions were then applied to a CM-cellulose column equilibrated with 10mM Tris-Cl buffer, pH 8.0. The active fractions were eluted with a linear gradient of 10-250mM Tris-Cl buffer. Approximately 10 mL fractions were collected each 15 minutes. Enzyme activity was tested for by the spot plate technique and by spectrophotometric assays.

v) Affinity chromatography

An attempt was made to purify the  $3\alpha$ -HSD by an affinity column chromatography utilizing a blue dye, MX3G, covalently-bound to Sepharose 4B-200 (prepared by Gordon Murphy in our laboratory). It was not a success, as the spot plate test showed the  $3\alpha$ -HSD activity to be spread widely throughout the fractions, however a considerable amount of protein was separated from the  $3\alpha$ -HSD activity. The elution was done as follows: after the sample was loaded on to the column, enzyme was eluted with 10mM Tris buffer, pH 8.5 and then with the same buffer containing 1mM NAD. The rest of the protein was eluted with 1M NaCl in the same buffer.

c) Solubilization of Placental and Kidney Microsomes

A solubilized microsomal fraction of each tissue (200-300g) was prepared essentially as described by Wiebe and Lärner (1977). Tris-Cl buffer, 10 mM, pH 8.6, containing DTT and EDTA (each 1mM) was prepared. NaCl was added to a portion of the buffer to give a 4M solution which was adjusted with HCl (1M) to pH 8.6. To 50 mL of the microsomal fraction suspended in the buffer, 50 mL of the NaCl-buffer was added slowly with stirring to bring the concentration to 2M NaCl. The solution was incubated on ice for 2 hours and then kept at -20 C overnight. The preparation was then thawed at 4°C and centrifuged at 145,000 x g (40K, 50.2Ti rotor). The supernatant containing solubilized enzymes was then dialyzed in two successive 5-litre batches of 10mM Tris-Cl buffer containing DTT and EDTA (0.25mM each). Enzyme assays as well as incubations using  $3\alpha$ - and  $3\beta$ -alcohols were done to check for activity. Incubations using the 3-ketone were also done which demonstrated the reversibility of the reactions.

The  $3\alpha$ -HSD activity in the human placenta was, however very unstable as it deteriorated very fast at 4°C in the absence of cofactor. The presence of added

cofactor stabilized the enzyme for up to about eight days. Due to the relatively unstable nature of the placental  $3\alpha$ -HSD, further characterization of the enzyme was not pursued.

**d)  $3\alpha$ - and  $3\beta$ -HSD Activity in other Tissues**

The cytosol obtained from human kidney had approximately a 4:3 ratio of  $3\alpha$ - to  $3\beta$ -HSD activity. The solubilized enzymes from the microsomes however had about 90%  $3\beta$ -HSD activity to 10%  $3\alpha$ -HSD activity. As with the placental cytosol studies, these results were obtained from kinetic assays of enzyme activity and were supported by characterization of the incubation products. The cytosolic enzymes from human kidney were much more stable than those of the placenta, but relative scarcity of kidney tissue did not permit further purification and characterization. Similar studies done with horse liver revealed the presence of a  $3\alpha$ -HSD activity in both the cytosol and microsomal fractions. In Table 1, the assays were not performed in duplicate unless an error was suspected.

Table 1

Enzyme Activity in Various Tissue Cytosols and Microsomes

Tissue	Fraction	3 $\alpha$ -HSD Activity	3 $\beta$ -HSD Activity
		(nmol/min. mg protein)	(nmol/min. mg protein)
Liver	Cytosol	218	291
Kidney	Cytosol	82	59
	microsomes	17	118
Placenta	Cytosol	28	42
	microsomes		328
Horse Liver	Cytosol	78	102
	Microsomes		144

e) Preliminary Investigation of the Human Liver 3 $\alpha$ -HSD

Many investigators have used ME or DTT in buffers as a means of preserving unstable enzymes. In this study, it was discovered that addition of ME to the cytosol caused appreciable reduction of NAD to NADH (Table 2).

Table 2

Observed NADH Generation in the Presence of Various Substances

Substance	Volume Used	Conc.	Activity (nmol/min.)
Sodium pyrophosphate	10 $\mu$ L	0.5 M	0
t-Butanol	10 $\mu$ L		0
Mercaptoethanol	10 $\mu$ L	0.3 M	89
NAD	100 $\mu$ L	2.3 mM	0

Glutathione (reduced)	10 $\mu$ L	1.0 mM	0
Low M.W substances (from cytosol)	40 $\mu$ L		13

#### f) Substrate Specificity of the Human Liver 3 $\alpha$ -HSD

The activity of the 3 $\alpha$ -HSD in the human liver was assessed using various steroids as substrates. The steroids were dissolved in t-butanol and 10 $\mu$ L samples of the 5 mM solutions were used in the assay (see results, later).

#### g) Effect of 4-Methyl Pyrazole on Human Liver 3 $\alpha$ -HSD

The enzyme sample was a pool of active fractions from a CM-cellulose column with protein concentration of 1.4 mg/mL. One cuvette was assayed as described in Methods. To a second cuvette, 4-methyl pyrazole was added immediately before initiating the assay by adding the steroid. In the absence of the inhibitor, the 3 $\alpha$ -HSD activity was 0.253 nmol/min. $\mu$ L while the ethanol activity was 2,436 nmol/min. $\mu$ L. With 10 $\mu$ L of the free base of the liquid 4-methyl pyrazole (0.12 mmol) in 3.0 mL of assay medium, all the ethanol dehydrogenase activity was abolished while 0.248 nmol/min. $\mu$ L of 3 $\alpha$ -HSD activity remained, representing 0% and 98% of the control, respectively.

#### h) Separation of Low Molecular Weight Substances from the Human Liver Cytosol

During the enzyme assays of early preparations of the human liver 3 $\alpha$ -HSD, it became apparent that in the unfractionated cytosol, there was an appreciable conversion of NAD to NADH evidenced by a slow increase in absorbance at 340nm in the absence of added substrate. This increase in absorbance did not occur when there was

no added NAD. It therefore appeared that there was an endogenous substrate which was slowly converting NAD to NADH. In order to examine further this hypothesis, the "enzyme" (macromolecule) and "endogenous substrate" fractions were separated.

A 10.0 mL syringe (1.5 × 8.5 cm) was packed with approximately 6.5cm of Sephadex G25 (preswollen, fractionation range 1,000-5,000 M.W) and equilibrated with a 50 mM Tris-Cl buffer, pH 8.5. A 2.0 mL sample of the red cytosol was loaded on to the column and fractions of 16 drops (approximately 0.8 mL) were collected. Blood-containing fractions (3-9) were used for the assays since these contained the bulk of the protein. The following colourless fractions (10-15), presumably containing the low molecular weight substances, were then tested for the presence of substrates responsible for the drift of the blanks in the enzyme assays. The macromolecule fractions (3-9) did not cause any increased absorbance at 340 nm when added to the assay buffer containing NAD. There was however an appreciable increase in absorbance when any of fractions 10-15 was thereafter added to the enzyme/NAD/buffer combination. The small-molecule fraction of cytosol contains one or more substrates for the cytosol NAD-dependent dehydrogenases.

#### **i) Demonstration of the Presence of a Mercaptoethanol**

##### **Dehydrogenase in the Cytosolic Fraction of Human Liver**

During the enzyme preparation, a number of reagents had been added. It was considered that these were potential substrates, or might contain substrates as impurities. These included NAD<sup>+</sup>, glutathione, t-butanol, sodium pyrophosphate, and mercaptoethanol. These were added sequentially to the reaction medium in the cuvette and their effects on the absorbance at 340 nm observed. Mercaptoethanol was included since, before this study was done, it had been included in the buffer, as it had frequently been used in previously published enzyme isolations.



Using a 50 mM sodium pyrophosphate buffer (2.9 mL), NAD, and 50 $\mu$ L of the cytosol macromolecular fraction, each of the above substances was investigated. It was observed that in the presence of the low molecular weight substances obtained from fractionation from the cytosol, there was appreciable conversion of NAD to NADH (increased 340nm absorbance). There was however a much greater increase in absorbance when mercaptoethanol was added as a substrate suggesting perhaps the presence of an enzyme capable of utilizing mercaptoethanol as a substrate. Whether the thiol or hydroxyl group of mercaptoethanol was being oxidized was not determined. No increase in absorbance was observed when glutathione, t-butanol or sodium pyrophosphate was tested for the presence of substrate. These observations led to the elimination of mercaptoethanol from the purification technique.

#### j) Stability Studies, Cofactor Requirement and pH Optima

The stability of the 3 $\alpha$ -HSD in the cytosolic fraction of the human liver was assessed in the presence and in the absence of cofactors at 4°C. Total 3 $\alpha$ -HSD activity was measured daily using 17 $\alpha$ -methyl-5 $\alpha$ -androstane-3 $\alpha$ ,17 $\beta$ -diol as substrate. Generally, higher activities were observed with NAD than with NADP and enzyme activities decreased faster in the fractions that did not contain added cofactor, suggesting that the presence of cofactors is beneficial in preserving enzyme activity. At 4°C, even with added NADH, enzyme activity was less than 50% of the initial activity after two weeks. The preparations which did not have cofactors present had corresponding lower activities (Fig 33). The ethanol dehydrogenase activity in the preparations were still high and could still be detected after four weeks.

In order to determine the pH optima for the human liver 3 $\alpha$ -HSD, the cytosol in various buffers at pH 4, 6, 7, 8, 9 and 10 was assayed for enzyme activity (Table 5). Using 17 $\alpha$ -methyl-5 $\alpha$ -androstane-3 $\alpha$ ,17 $\beta$ -diol as substrate, the preparation in buffer at

pH 9.0 had the highest  $3\alpha$ -HSD activity. Reduction of the corresponding 3-ketone was maximal at pH 7.0.

Fig 8 shows the formation of the 3-ketone, together with a larger peak of unaltered  $17\alpha$ -methyl- $5\alpha$ -androstane- $3\alpha,17\beta$ -diol. The identity of the incubation product,  $17\alpha$ -methyl- $5\alpha$ -DHT was confirmed by reduction of the ketone by sodium borohydride to the  $3\beta$ -hydroxy compound, clearly seen even in the continued presence of  $17\alpha$ -methyl- $5\alpha$ -androstane- $3\alpha,17\beta$ -diol (Fig 9). The identity of the HPLC peaks was assessed by measuring the retention times (and calculating the  $K's$ ) and comparing them to those of the authentic steroids and cholesterol, which is present in the human liver cytosol (Fig 10). A similar NADH incubation with the  $17\alpha$ -methyl DHT as substrate at pH 7.0 gave both the  $3\alpha$ - and the  $3\beta$ -hydroxysteroids (Fig 11).

#### k) Partial Purification of the Human Liver $3\alpha$ -HSD

An early indication of the charge characteristics of the  $3\alpha$ -HSD was obtained from the elution profile of a preliminary DEAE-Sephacel column. This was confirmed in the later more detailed DEAE-Sephacel column (Fig 15). Since the enzyme activity was observed in the early fractions without any salt gradient, it became clear that the enzyme did not bind to the positively charged DEAE and that the enzyme was itself positively charged at pH 8.0. No additional  $3\alpha$ -HSD activity was detected after elution of the column with a salt gradient.

A larger scale purification procedure is outlined in Fig 13. The rapid spot-plate technique was very useful in locating the fractions with the  $3\alpha$ -HSD activity, immediately after the DEAE separation. As is shown in Fig 14, the active fractions with the  $3\alpha$ -hydroxysteroid <sup>was</sup> used for enzyme assay on the Gilford spectrophotometer. The first column chromatographic step, on DEAE-Sephacel, was followed by multiple enzyme assays using in addition to the  $3\alpha$ -hydroxysteroid, ethanol and  $5\alpha$ -androstane- $17\alpha$ -

methyl-3 $\beta$ ,17 $\beta$ -diol. The results are shown in Fig 15. Partial separation of the 3 $\alpha$ - from 3 $\beta$ -HSD activity had been effected. The active fractions from the DEAE column (pooled) were further separated on a CM-cellulose column using a Tris-Cl gradient (Fig 16). The rapid spot-plate assay on these column fractions show the presence of at least three different forms of the 3 $\alpha$ -HSD activity, peaking in the regions of fraction 44, 50, and 56 (Fig 17).

The enzyme binds tightly to the CM-cellulose column and is only eluted when the Tris buffer gradient reaches about 80mM. Activity-staining for 3 $\alpha$ -HSD on polyacrylamide gels shows bands coincident with the ethanol activity (see later). All fractions which had 3 $\alpha$ -HSD activity (spot plate test) also had strong ethanol dehydrogenase activity. A later CM column (ME absent) followed by Gilford assays for 3 $\alpha$ -HSD activity, showed the presence of at least four forms (Fig 18). Here again, fractions which had predominant 3 $\alpha$ -HSD activity also had strong ethanol dehydrogenase activity. The partial purification of the cytosolic 3 $\alpha$ -HSD from a separate liver different from that of Fig 15 is summarized in Table 3.

Inhibition of the ethanol dehydrogenase activity by 4-methyl pyrazole was essentially complete, however there was no significant effect on the 3 $\alpha$ -HSD activity. After CM-cellulose chromatography, the ratio of 3 $\alpha$ -HSD activity to ethanol activity was 1 : 10. With 4-methyl pyrazole (10 $\mu$ L, 0.12 mmols) in the assay medium, all the ethanol activity (243.6 nmol/min) was abolished. The initial 3 $\alpha$ -HSD activity of 25.3 nmol/min only dropped to 24.8 nmol/min.

#### 1) Characterization of the Human Liver 3 $\alpha$ -HSD

The relative rates of transformation of several steroid 3 $\alpha$ -alcohols are shown in Table 4. They include steroid anaesthetics (Fig 19) and metabolites of naturally-occurring pregnanes (Fig 20). The cytosolic 3 $\alpha$ -HSD (pool of fractions 43-46 of the

CM-cellulose column) more rapidly oxidized the  $5\alpha$ -steroid substrate than the  $5\beta$ -substrate in the two pairs in which comparison can be made. The steroid anaesthetic Alfathesin® (Glaxo), is a 3 : 1 mixture of alfaxalone and alfadolone acetate (Fig 19). Both compounds are  $3\alpha$ -hydroxy- $5\alpha$ -pregnane derivatives; they are included in the studies which show that both are efficiently transformed in the direction of steroid oxidation.

Among the steroids tested,  $5\alpha$ -pregnan- $3\alpha$ -ol-20-one is the next best substrate to  $17\alpha$ -methyl- $5\alpha$ -androstan- $3\alpha,17\beta$ -diol for the human liver  $3\alpha$ -HSD activity (Table 4) under the assay conditions and might reflect the importance of the  $3\alpha$ -HSD in the metabolism of progesterone (Fig 20). Qualitative cofactor requirement studies by incubation and enzyme assays using NAD and NADP suggested NAD as the preferred cofactor. Most of the studies done on rat and mouse liver  $3\alpha$ -HSD report NADP as the preferred cofactor. The pI of the  $3\alpha$ -HSD activity is 8.6-9.0, the same region as ethanol dehydrogenase activity (Fig 22 and 23). Though the  $3\alpha$ -HSD activity was near the very end of the gel, the results obtained were reproducible.

In isoelectric focussing experiments on various fractions of the human liver cytosol, using ampholytes with pH ranges of 3-10.5 (Fig 24) and 8-10.5 (Fig 25), single bands stained for  $3\alpha$ -HSD activity. The protein stain however showed the presence of at least 8 protein bands (Fig 26). It is interesting to note that the band that stained for  $3\alpha$ -HSD activity was the most basic.

The optimum pH recorded during enzyme assays was 9.0 for the oxidation reaction and 7.0 for the reduction reaction (Table 5). The enzyme itself was stable at pH 6.0 and above with the optimum at pH 9.0. Even though the pH optima recorded for the reduction reaction was 7.0, this could not be ascribed to the  $3\alpha$ -HSD activity alone since the reduction of the carbonyl group was due to both  $3\alpha$ - and  $3\beta$ -HSD activities. Polyacrylamide gel electrophoresis showed that the active fractions from the

CM-cellulose column appear as a single band when stained for activity. It is possible that the different enzyme forms differ in stability or that all but one were lost from the gel. The corresponding protein stain showed about six bands. On prolonged starch gel electrophoresis, single activity bands were obtained. It was also evident that the  $3\alpha$ -HSD from *Pseudomonas testosteroni* was an entirely different enzyme. This is because the bacterial enzyme migrated in the direction opposite to that of the human liver cytosolic enzyme (Fig 27) and appeared to be positively charged at pH 8.3. The starch gel also showed that the protein bands that stained for ethanol dehydrogenase activity included that of the  $3\alpha$ -HSD (Fig 28), although the bands are somewhat diffuse. Figures 29, 30 and 31 also show the activity staining of various fractions of the human liver cytosol towards ethanol,  $5\alpha$ -androstane- $17\alpha$ -methyl- $3\alpha,17\beta$ -diol and  $5\alpha$ -androstane- $17\alpha$ -methyl- $3\beta,17\beta$ -diol after starch gel electrophoresis. From the staining results, it appears that the ethanol, and  $3\beta$ - and  $3\alpha$ -HSD activities are coincident.

The results from the polyacrylamide gel electrophoresis of different ammonium sulphate preparations from horse and human liver suggested that the ethanol dehydrogenase activity in the two tissues were different. While the 40-60% fraction of the human liver cytosol has two bands, the corresponding fraction in the horse liver cytosol has four or more (Fig 32). The  $3\alpha$ -HSD activity stain in these two tissues could not be obtained for comparison, as the enzyme became inactive before this study was carried out. Stability studies of the human liver cytosolic  $3\alpha$ -HSD activity and ethanol dehydrogenase activity were done with and without added cofactors. It was shown that at  $4^{\circ}\text{C}$  both enzyme activities are stabilized to some extent (Fig 33).

Enzyme deterioration was faster in both cases without added cofactor. The  $3\alpha$ -HSD activity however was less stable than the corresponding ethanol dehydrogenase activity. While the specific activity of the ethanol dehydrogenase remained at about 80% of the initial activity in the presence of added cofactors after a month, the  $3\alpha$ -

HSD activity dropped to less than 50% of the initial activity.

Table 3. Purification of the Human Liver Cytosolic 3 $\alpha$ -HSD  
Activity (same batch as described in Fig 13 Procedure).

STEP	TOTAL VOLUME (mL)	TOTAL PROTEIN (mg)	TOTAL ENZYME ACTIVITY (U)	SPECIFIC ACTIVITY (U/mg)	ENZYME RECOVERY (%)	PURIFICATION (fold)
1. CYTOSOL	300	13680	1505	0.11	100	1
2. AMMON. SULPH. FRACT. (40-60)	75	5540	869	0.16	57.7	1.43
3. DEAE-Sephacel COLUMN	210	231.8	751	3.24	49.9	29.45
4. CM-cellulose COLUMN						
Fract. 4-7	1.5	122.7	58.0	0.47	3.9	4.3
Fract. 23-33	1.5	76.5	37.0	0.48	2.5	4.4
Fract. 34-41	1.5	83.7	85.6	1.02	5.7	9.3
Fract. 42-46	1.0	44.1	78.5	1.78	5.2	16.2



Table 4. Substrate Specificity Studies of the  
Human Liver Cytosolic 3 $\alpha$ -HSD Activity.

The compounds in series are:

17 $\alpha$ -methyl-5 $\alpha$ -androstane-3 $\alpha$ ,17 $\beta$ -diol

5 $\beta$ -androstan-3 $\alpha$ -ol-17-one

3 $\alpha$ -hydroxy-5 $\alpha$ -pregnane-11,20-dione (alfaxalone)

5 $\beta$ -pregnan-3 $\alpha$ -ol-20-one

3 $\alpha$ ,21-dihydroxy-5 $\alpha$ -pregnane-11,20-dione-21-acetate (alfadolone acetate)

5 $\alpha$ -androstan-3 $\alpha$ -ol-17-one

5 $\alpha$ -pregnan-3 $\alpha$ -ol-20-one

17 $\alpha$ -methyl-5 $\alpha$ -androstane-3 $\beta$ ,17 $\beta$ -diol.

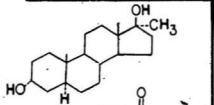
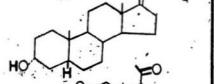
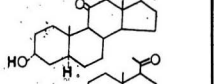
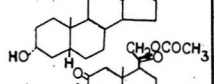
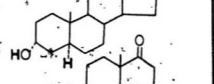
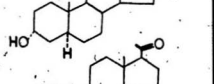
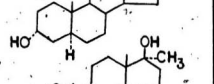
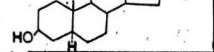
SUBSTRATE	SPECIFIC ACTIVITY (nmols/mg/min.)	Relative Spec. activity
	0.6	1
	0.08	0.13
	0.02	0.03
	0.14	0.23
	0.19	0.32
	0.20	0.34
	0.49	0.82
	0.17	0.28

FIG 8. HPLC Analysis of Products of Incubation of

17 $\alpha$ -methyl-5 $\alpha$ -androstane-3 $\alpha$ ,17 $\beta$ -diol

with Human Liver Cytosol.

(Whatman Partisil 10/25 Column; Isopropanol-Hexane (5:95)

Eluent 1.0 mL/min.).

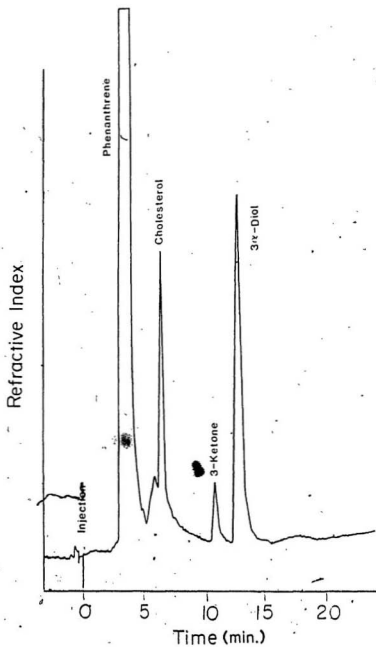


FIG 9. HPLC Analysis of Borohydride Reduction of Incubation

Products of 17 $\alpha$ -methyl-5 $\alpha$ -androstane-3 $\alpha$ ,17 $\beta$ -diol  
with Human Liver Cytosol.

(Whatman Partisil 10/25 Column; Isopropanol-Hexane (5:95)

• Eluent 1.0 mL/min.).

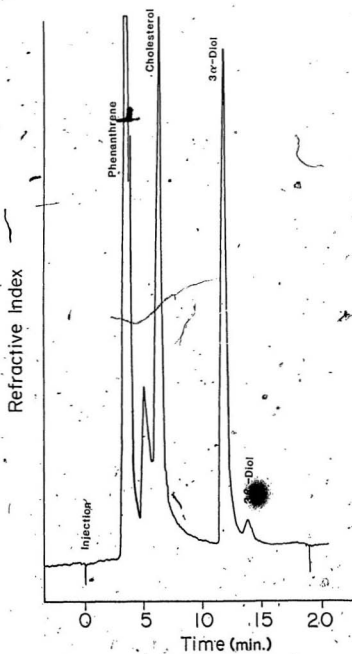


FIG 10. HPLC Analysis of Steroid Substrate Standards.

(Whatman Partisil 10/25 Column; Isopropanol-Hexane (5:95)  
Eluent 1.0 mL/min.).

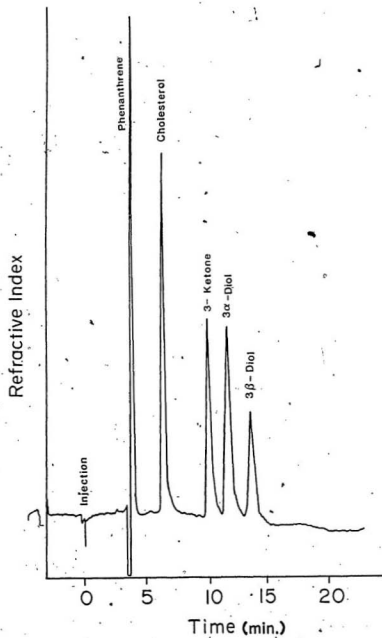




FIG 11. HPLC Analysis of Products of Incubation of

17 $\beta$ -hydroxy-17 $\alpha$ -methyl-5 $\alpha$ -androstan-3-one,

with Human Liver Cytosol.

(Whatman Partisil 10/25 Column; Isopropanol-Hexane (5:95)

Eluent 1.0 mL/min.).

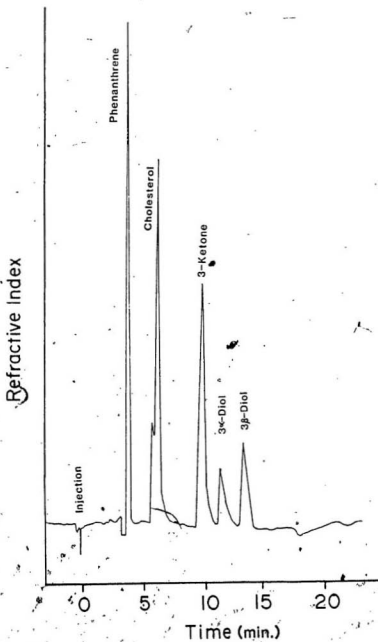


FIG 12. Protein Elution Profile from DEAE-Sephacel Column.

Sample was from dialyzed 40-60% ammonium sulphate pptd.

Human Liver Cytosol.

Buffer, 10 mM Tris-Cl, pH 8.0; Gradient, Tris-Cl 10-250 mM,

pH 8.0 started after Fraction 70. Bulk of eluted protein not shown.

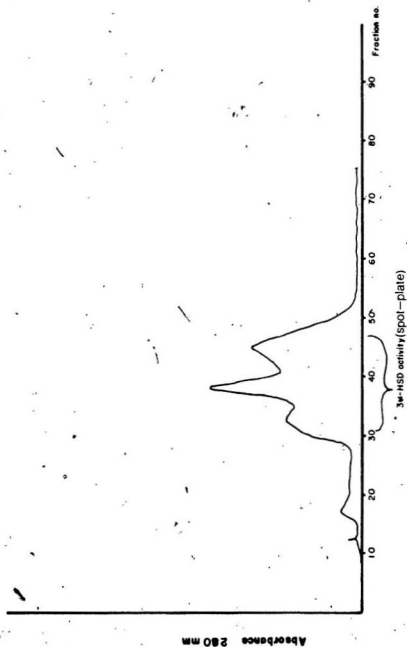


Fig. 12 Protein Elution Profile from DEAE-Sephacel Column

FIG 13. Enzyme Purification Procedure

# PURIFICATION PROCEDURE

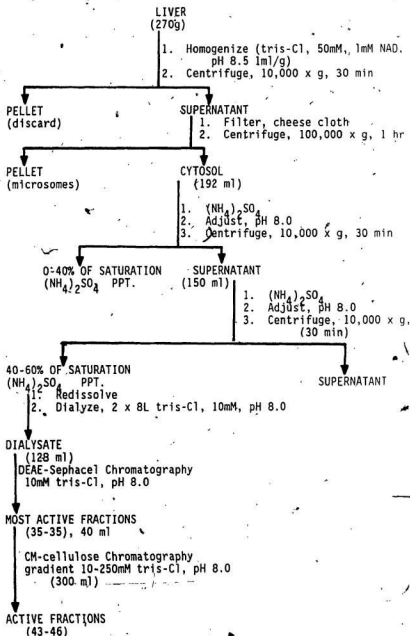


FIG 14 Rapid Spot-Plate Test (see Methods) for  $3\alpha$ -HSD Activity.

Fractions from DEAE-Sephacel Column.

(The main activity is in fractions 31-44 (C 7 to D 8)).

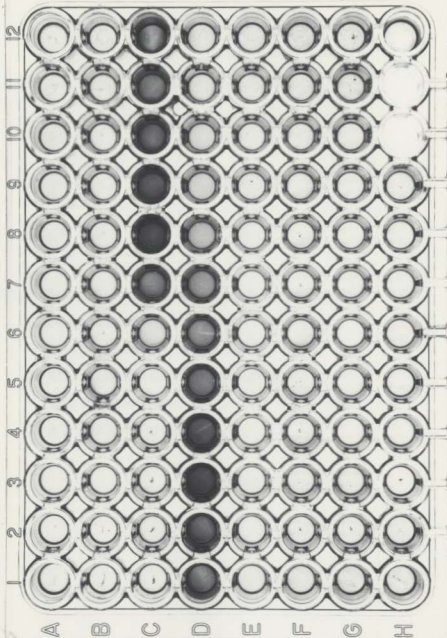
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FIG 15 Profiles of Protein Concentration and Enzyme Activity of  
DEAE-Sephacel Column.

- ( X X X ) Protein concentration (Lowry)
- ( O O O ) Ethanol Dehydrogenase Activity
- ( ● ● ● ) 3 $\alpha$ -HSD Activity
- ( ▲ ▲ ▲ ) 3 $\beta$ -HSD Activity

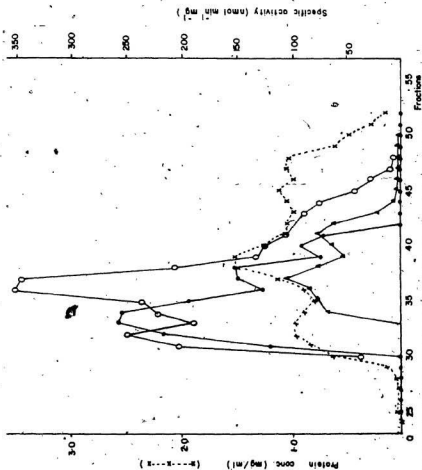


Fig.15. Protein-Activity Profile of Fractions from DEAE-Sephacal Column

**FIG 16 Protein and Dehydrogenase Activity**

**Protein Elution Profile from CM-cellulose Column.**

**The Sample loaded was Fractions 31-44 pooled from DEAE-Sephacel Column (FIG 14).**

**(Buffer, 1 mM tris-Cl, pH 8.0; Gradient: 10-250 mM Tris-Cl).**

**The region of 3 $\alpha$ -HSD activity was detected only by the spot plate test.**

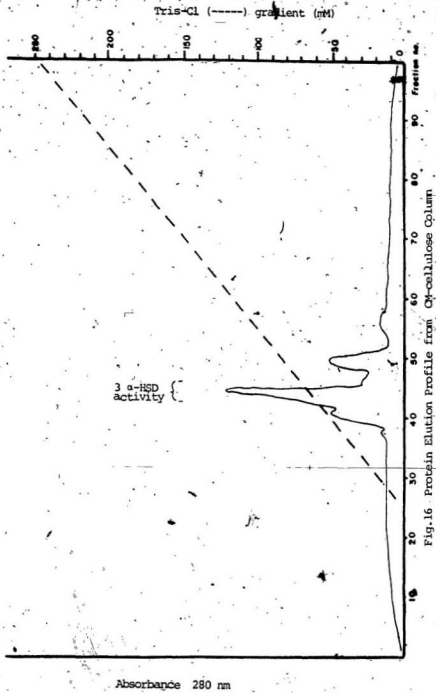


Fig.16 Protein Elution Profile from Cx-cellulose Column

FIG. 17 Rapid Spot-Plate Test for  $3\alpha$ -HSD Activity.  
Fractions from CM-cellulose Column (Fig 16).  
(Main Activities are in Fractions D8-E8).

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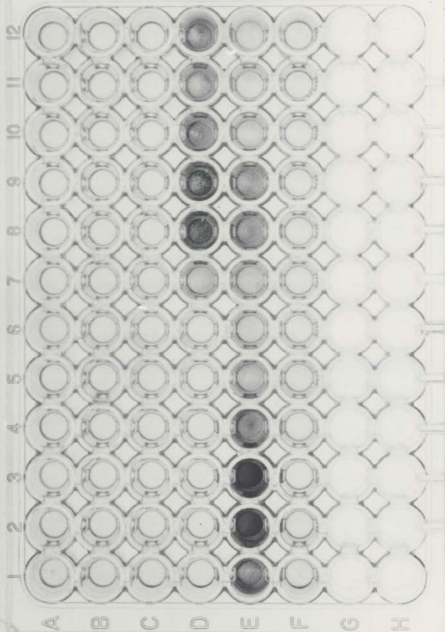


FIG 18 Protein and Dehydrogenase Activity

Profile from one CM-Cellulose Column Elution (Fig 16)

( ● ● ● ) Protein

( ○ ○ ○ ) 3 $\alpha$ -HSD Activity

The sample loaded was pooled from a previous run on a DEAE-Sephacel Column.

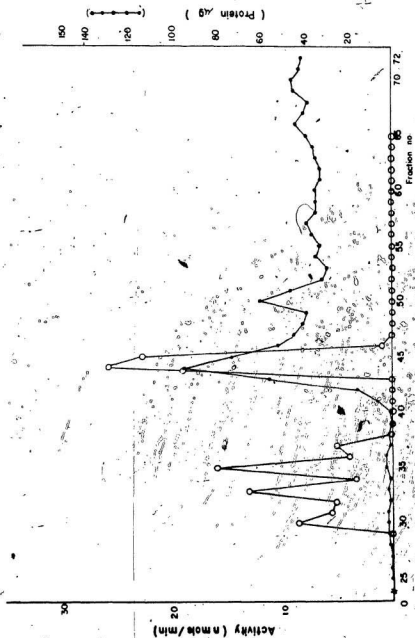


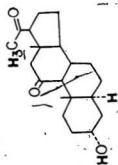
Fig. 48. Protein - 3K-HSD Activity from CM-cellulose Column



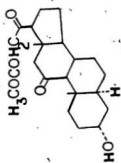
FIG 19. Structures of Alfadolone Acetate and Alfaxalone

(Alfathesin is a 1:3 mixture of alfadolone acetate and alfaxalone).

These two steroids were efficiently transformed by the Human Liver  $3\alpha$ -HSD.



Alfaxalone



Alfadolone-Acetate

FIG 19

FIG 20 A Pathway of Catabolism of Progesterone to a major excretory product, Pregnanediol.

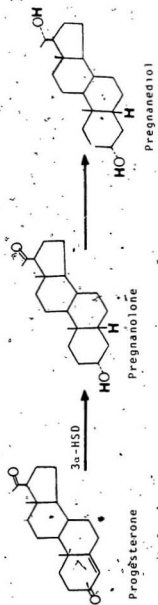


FIG 20

FIG 21 pH Gradient Measurement and Focussing Conditions.  
(Iso-electric Focussing, LKB Ampholytes)

## Experimental result form

pH range 3-10.5  
 Anode buffer solution 1M NaOH  
 Cathode buffer solution 1M  $H_3PO_4$

Sample No.	Sample description	Conc. (mg/ml)	Volume (ml)	Py. clear
1				
2				
3				
4			10	X
5				
6				
7			5	X
8				
9				
10				
11			10	X
12				
13				
14				
15				
16				
17				
18				
19				
20				
21				
22				
23				
24				

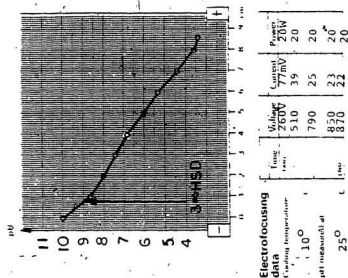


FIG 21

FIG. 22 Ethanol Dehydrogenase Activity Stain.

(Iso-electric Focussing, LKB Ampholytes, pH 3-10.5)  
pH 3-10.5)

Track 1 CM-cellulose Active Fractions (pool) F 43-46

Track 2 Ammon. sulph. ppt (40-60%)

Track 3 DEAE-Sephacel Active Fractions (pool) F 31-44

(For Visualization, see section under Activity Staining  
in text).





FIG 23 Composite Diagram Illustrating the Pattern of Human Liver Ethanol Dehydrogenase Detected by High Voltage Starch Gel Electrophoresis (A) and Isoelectric Focussing (B) summarized from many runs. (Harada et al., 1978).

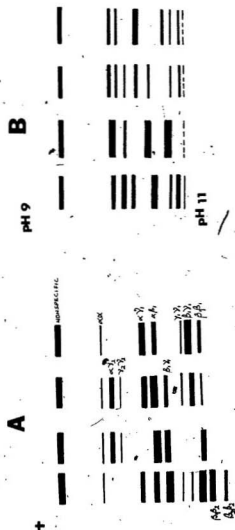


FIG 23 Scheme of the human liver alcohol dehydrogenase electrophoretic patterns.

FIG 24 3 $\alpha$ -HSD, Activity Stain: Isoelectric

Focussing (LKB Ampholyte pH 3-10.5)

Track 1 CM-cellulose Active Fractions (pool) F 43-46

Track 2 Ammonium sulphate fraction (40-60%)

Track 3 DEAE-Sephacel Active Fractions (pool) F 31-44

(For Visualization, see section under Activity Staining  
in text.).



FIG 25 3 $\alpha$ -HSD Activity Stain: Isoelectric  
Focussing (LKB Ampholyte pH 8-10.5)

(40-60% and 60-80% refer to Ammonium sulphate  
fractions precipitated from the cytosol)

(For Visualization, see section under Activity Staining in  
text.)

ISO-ELECTRIC FOCUSING(IEF)

LKB AMPHOLYTES pH 8-10.5

Activity Stain 3- $\alpha$  HSD

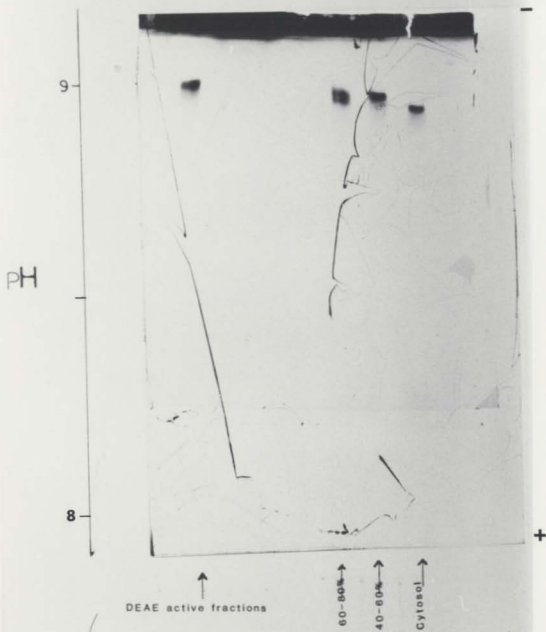


FIG 26 Protein Stain: Iso-electric

Focussing (LKB Ampholyte pH 3-10.5)

Track 1 & 2 CM-cellulose column, active fractions pool

Track 3 & 4 ammonium sulphate cytosol ppt. (40-60%)

(For Visualization, see section under Protein Stain in text)

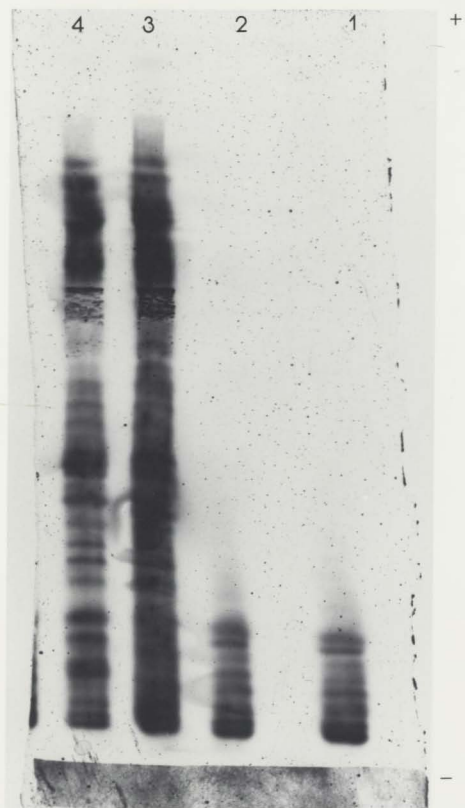




Table 5. Optimum pH for the Assay of Human Liver 3 $\alpha$ -HSD.

pH	3 $\alpha$ -HSD ACTIVITY Alcohol oxidation with NAD	TOTAL 3 $\alpha$ -HSD ACTIVITY Carbonyl group reduction with NADH
4	22.9 nmols/min.	31.3 nmols/min.
6	36.3 " "	60.1 " "
7	42.4 " "	68.7 " "
8	69.3 " "	54.3 " "
9	87.4 " "	36.7 " "
10	64.3 " "	21.1 " "

FIG 27 3 $\alpha$ -HSD Activity Stain: Starch Gel Electrophoresis

(15% Starch Gel, Electrophoresis was performed at 10V  
for 15 h. at 4 C with Tris-HCl Buffer, 0.03 M, pH 8.6,  
Well Buffer, Tris-HCl, 0.3 M, pH 8.6)

0-20%

20-40% refer to ammonium sulphate precipitates

40-60% of the cytosol.

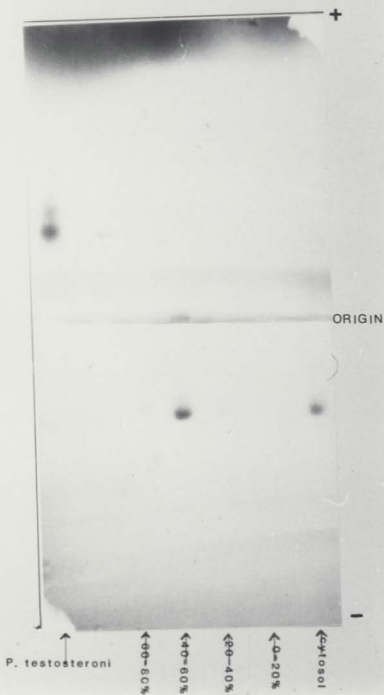
60-80%

3 $\alpha$ -HSD from *P. testosterone* was added for comparison.

(For Visualization, see section under Activity Staining  
in text.)

STARCH GEL ELECTROPHORESIS

ACTIVITY STAIN 3-ALPHA HSD



**FIG 28 Ethanol Dehydrogenase Activity Stain: Starch Gel  
Electrophoresis.**

(15% Starch Gel, Electrophoresis was performed at 100V  
for 15 h. at 4 C with Tris-Cl Buffer, 0.03 M, pH 8.6,  
Well Buffer, Tris-Cl, 0.3 M, pH 8.6).

The numbers refer to ammonium sulphate precipitates of  
the cytosol.

(For Visualization, see section under Activity Staining  
in text.)



FIG 29  $3\beta$ -HSD Activity Stain after Starch Gel Electrophoresis.

- Track 1 Active fraction from CM-cellulose column. (F 43-46 pool)
- Track 2 Cytosol
- Track 3 Active fraction from DEAE column. (Fraction 31-44 pool)
- Track 4 40-60% ammonium sulphate fraction
- Track 5 60-80% ammonium sulphate fraction

(For Visualization, see section under Activity Staining in text.)

**FIG 29**





FIG 30 3 $\alpha$ -HSD Activity Stain after Starch Gel Electrophoresis.

(For legend of tracks and Visualization procedures, refer to Fig 29).

FIG 30

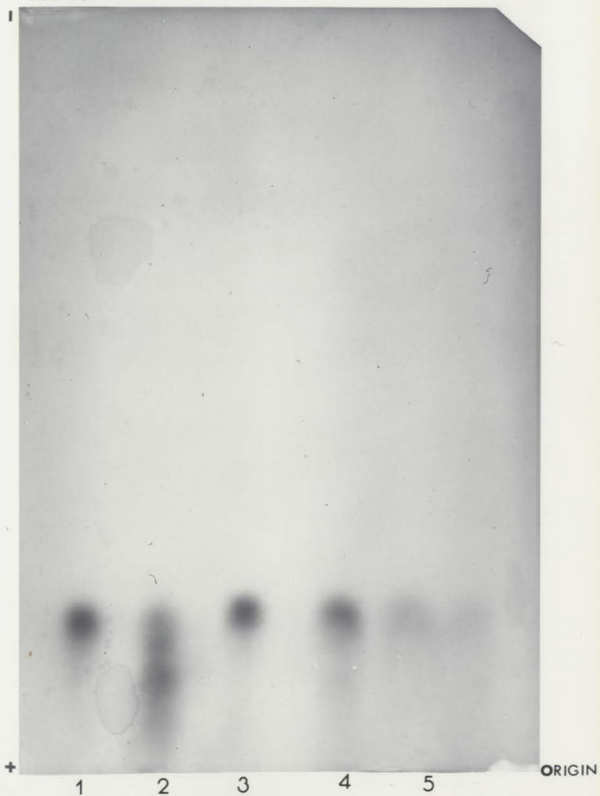


FIG 31 Ethanol Dehydrogenase Activity Stain, Starch Gel

Electrophoresis.

(15% Starch Gel, Electrophoresis was performed at 100V  
for 15 h. at 4 C with Tris-HCl Buffer, 0.03 M, pH 8.6,

Well Buffer, Tris-HCl, 0.3 M, pH 8.6)

Track 1 20-40% Ammonium sulphate fraction

Track 2 40-60% Ammonium sulphate fraction

Track 3 40-60% Ammonium sulphate fraction

Track 4 DEAE column fractions (3 $\alpha$ -HSD activity pool; F 31-44)

Track 5 CM-cellulose fractions (3 $\alpha$ -HSD activity pool; F 43-46)

Track 6 Cytosol

Track 7 DEAE column fractions (3 $\alpha$ -HSD activity pool; F 31-44)

(For Visualization procedures, see section under Activity Staining  
in text.)

**FIG 31**

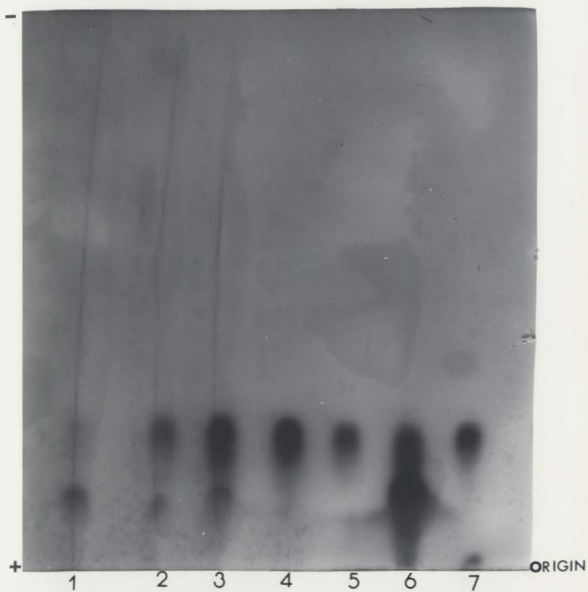


FIG 32 -Polyacrylamide Gel Electrophoresis of Fractions from  
Human and Horse Liver Cytosol (Ammonium Sulphate pptd)  
Ethanol Dehydrogenase Activity Stain.

(Electrophoresis was performed at 150V for 24 h. at 4 C  
with Glycine, 0.18 M and Luitidine, 0.54 M (10% solution),  
pH 8.3)

The numbers refer to the ammonium sulphate precipitates of  
the cytosol.

(For Staining procedures, see section under Activity staining  
in text.)

FIG 32

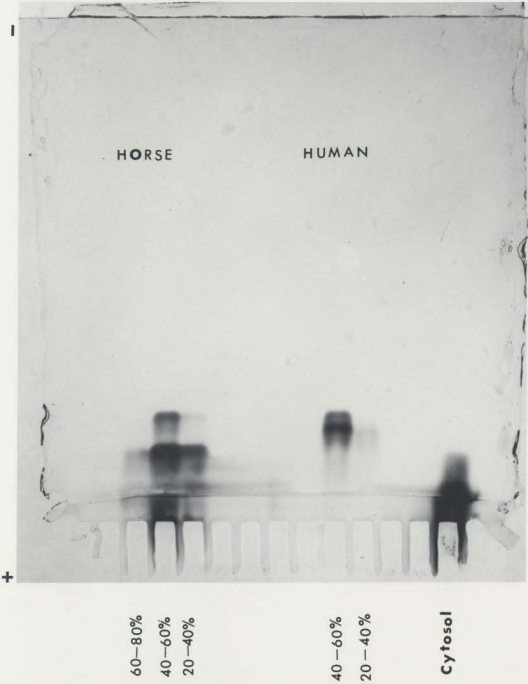


FIG 33 Stability Studies of Human Liver Cytosolic 3 $\alpha$ -HSD and Ethanol Dehydrogenase Activities.

- ( ● ● ● ) 3 $\alpha$ -HSD (with NAD)
- ( ○ ○ ○ ) 3 $\alpha$ -HSD (without NAD)
- ( ▼ ▼ ▼ ) Ethanol DH (with NAD)
- ( ▽ ▽ ▽ ) Ethanol DH (without NAD)

(3 $\alpha$ -HSD was assayed with 5 $\alpha$ -Androstan-17 $\alpha$ -methyl-3 $\alpha$ , 17 $\beta$ -diol, 5mM as substrate; Ethanol Dehydrogenase Activity was assayed with 2 M Ethanol.

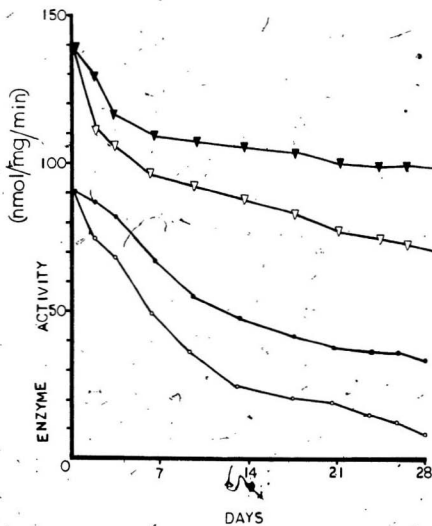


FIG 33



## CHAPTER V

### DISCUSSION

#### a) Steroid Synthesis

The synthesis of the steroid substrates with a  $17\alpha$ -methyl group was carried out as these substrates allowed the study of enzymic oxidation/reduction reactions uniquely at C-3 of the steroid molecule. The sodium/liquid ammonia reduction of the  $17\alpha$ -methyltestosterone gave about 90% yield of  $17\alpha$ -methyl- $5\alpha$ -DHT, the remaining 10% being allylic alcohols and starting material. Reduction of the saturated 3-ketone,  $17\alpha$ -methyl- $5\alpha$ -DHT, to the required axial  $3\alpha$ -alcohol was effected by K-Selectride (potassium tri(R,S-s-butyl)borohydride), and to the equatorial  $3\beta$ -alcohol by sodium/isopropanol. Both methods gave good yields. All three steroids were purified by repeated recrystallization from methylene chloride/isopropanol and their identity established by comparison of their TLC, HPLC and melting point data with authentic reference samples.

#### b) $3\alpha$ - and $3\beta$ -HSD Activity in the Human Placenta

##### i) Cytosol

Experiments on the human placenta indicated that there is indeed a cytosolic  $3\alpha$ -HSD present. This result was obtained from incubations of the initial cytosol fraction and of various ammonium sulphate precipitates with the steroid substrates. The incubations indicated that, in the presence of added NAD,  $17\alpha$ -methyl- $5\alpha$ -androstane- $3\alpha,17\beta$ -diol was converted to the corresponding 3-ketone. Both the  $3\alpha$ - and the  $3\beta$ -alcohols were produced from the 3-ketone in incubations in the presence of NADH.

They were identified by comparison with authentic samples on TLC and also by further characterization on HPLC. The crude cytosol when incubated with the 3-ketone in the presence of NADH gave a 3:2 ratio of  $3\beta$  :  $3\alpha$ -alcohol. However, in the 40-60% ammonium sulphate precipitate the ratio of  $3\alpha$  :  $3\beta$ -activity was approximately 3:1. This observation is consistent with the results obtained in the preliminary experiment done to find the ammonium sulphate precipitate with the highest  $3\alpha$ -HSD activity. Conversely,  $3\beta$ -HSD activity was mostly found in the 0-40% fractions. The incubations also suggested that NAD may be the preferred cofactor over NADP. Various investigators had suggested NADP as the preferred cofactor for the  $3\alpha$ -HSD isolated from rat and mouse liver cytosol.

#### ii) Microsomes

The method described by Wiebe and Larner (1977) was very effective in solubilizing the enzymes from the microsomes. In these two organs (placenta and kidney),  $3\beta$ -HSD activity predominate. This would seem to suggest that the  $3\beta$ -HSD is a microsomal enzyme in these tissues and that the  $3\alpha$ -HSD activity detected may have come from possible contamination from the cytosol during separation. In the placenta, the  $3\alpha$ -HSD activity is very unstable. The enzymes in both tissues, however could be stabilized by addition of NAD or NADH. It was not surprising to find that a larger proportion of  $3\alpha$ -HSD activity was found in the kidney cytosol as the kidney is a point where the steroids could be metabolized finally before they are excreted in the urine.

c) Partial Purification of the Human Liver 3 $\alpha$ -HSD

The ME-dehydrogenase did utilize NADP but more slowly than NAD. It is therefore probable that there exists in human liver cytosol a ME-dehydrogenase. Since the product of this reaction was not examined, it is not known if the hydroxyl or thiol group was being oxidized, nor indeed if the ME was instead acting as the ultimate reducing agent in a redox chain. From the practical viewpoint of this study of 3-HSD activities, however, it was clear that the presence of ME reduced the accuracy of the 3-HSD assays by increasing the background activity. It was unfortunate that this source of the high background was not discovered until relatively late in the study.

Low molecular weight substances were found in human liver cytosol by Sephadex G25 gel filtration (which separates low molecular weight substances from protein) that could act as substrates for the soluble enzymes in the preparation in the presence of NAD(P). Both 3 $\alpha$ - and 3 $\beta$ -HSD activity was detected in all the ammonium sulphate precipitates of human liver cytosol. The 3 $\alpha$ -HSD activity is, however, concentrated in the 40-60% precipitate. Even though the activities measured show significant amounts of 3 $\alpha$ -HSD activity in the other ammonium sulphate precipitates, later analysis by gel electrophoresis, and subsequent activity-staining indicated that most of the activity was localized in the 40-60% ammonium sulphate precipitate. This fraction also contained the bulk of the protein (about 50%). Even though there appears to be appreciable 3 $\beta$ -HSD activity, it was convenient to use this fraction for the next stage of the purification.

This preliminary information was very important in the subsequent purification steps to isolate the 3 $\alpha$ -HSD activity from the human liver cytosol. The working buffers were prepared without added ME or DTT. No EDTA was added; it is well known that the human liver ethanol dehydrogenase contains zinc (Bosron et al., 1979; Lange and Vallee, 1976; Pares and Vallee, 1981) and one or more of the human liver

3 $\alpha$ -HSD may be ethanol dehydrogenases and may contain zinc removable by EDTA.

Using the prepared substrates, the presence of 3 $\alpha$ - and 3 $\beta$ -HSD activities was unequivocally established. Production of 17 $\alpha$ -methyl DHT by incubation of 17 $\alpha$ -methyl-5 $\alpha$ -androstane-3 $\alpha$ ,17 $\beta$ -diol at 37°C, pH 9.0 with NAD was indicative of the presence of a 3 $\alpha$ -HSD. Since the enzyme activity was observed in the early fractions without any salt gradient, it became clear that the enzyme did not bind to the positively charged DEAE and that the enzyme was itself positively charged at pH 8.0. These properties place the human liver 3 $\alpha$ -HSD in a category corresponding to the human liver ethanol dehydrogenase class I enzymes or a class II enzyme (a small amount of class II enzymes elutes with the bulk of the positively charged proteins). The rapid spot-plate technique was very useful in locating the fractions with the 3 $\alpha$ -HSD activity immediately after the DEAE separation. This technique is very useful where very labile enzymes are involved. Apart from detecting the enzyme activity very early in the chromatography, enzyme assays and kinetic studies could be performed while the column is still being run.

The 3 $\alpha$ -HSD activity binds tightly to CM-cellulose column. Since none of the isozymes of the ethanol dehydrogenase class II enzymes binds tightly to a CM column, it is likely that the 3 $\alpha$ -HSD activity is due to one or more enzymes similar or identical to ADH class I isozymes. The reason for the reference to human liver alcohol dehydrogenase enzymes is that 3 $\alpha$ -HSD activity-staining on polyacrylamide gels shows bands coincident with the ethanol activity. All fractions which had 3 $\alpha$ -HSD activity (spot plate test) also had strong ethanol dehydrogenase activity. A later CM column (ME absent) followed by Gilford assays for 3 $\alpha$ -HSD activity, showed the presence of at least four forms. Here again, fractions which had predominant 3 $\alpha$ -HSD activity also had strong ethanol dehydrogenase activity. Inhibition of the ethanol dehydrogenase activity by 4-methyl pyrazole was essentially complete under conditions in

which there was no significant effect on the  $3\alpha$ -HSD activity.

#### d) Characterization of the Human Liver $3\alpha$ -HSD

The substrate specificity studies (Table 4) showed that a partially purified cytosolic  $3\alpha$ -HSD pool more rapidly oxidized the  $5\alpha$ -steroid substrate than the  $5\beta$ -steroid substrate in the two pairs, one of androstanes, the other of pregnanes in which comparison can be made. The steroid anaesthetic Alfathesin® (Glaxo), a mixture of  $3\alpha$ -hydroxypregnanes were also efficiently transformed.

The pI of the human liver cytosolic  $3\alpha$ -HSD is about 8.6-9.0. It therefore appears that the human liver cytosolic  $3\alpha$ -HSD is at least close to, and probably coincident with the  $\beta 1\beta 1$  isozyme of human liver alcohol dehydrogenase (class I). The considerable genetic polymorphism of human liver alcohol dehydrogenases, make the characterization only tentative. Isoelectric focussing experiments on various fractions of the human liver cytosol using ampholytes with pH ranges of 3-10.5 and 8-10.5 showed single bands staining for  $3\alpha$ -HSD activity. On the other hand, the protein stain however showed the presence of at least 8 protein bands indicating the presence of more than one isozyme with the  $3\alpha$ -HSD activity being the most basic.

For the oxidation reaction, a pH optimum of 9.0 was recorded whilst 7.0 was recorded for the reduction. Enzyme stability was observed at pH 6.0-9.0 with the optimum at 9.0. Though the pH optima recorded for the reduction reaction was 7.0, this could not be ascribed to the  $3\alpha$ -HSD activity alone since the reduction of the carbonyl group was due to both  $3\alpha$ - and  $3\beta$ -HSD activities. Lang et al., (1986), had reported a pH optimum of 10.0 for the microsomal  $3\alpha$ -HSD. A pH optimum for the cytosolic  $3\alpha$ -HSD was however not reported.

With the polyacrylamide gel electrophoresis, the active fractions from the CM-cellulose column appear as a single band when stained for activity. It is possible that

the different enzyme forms differ in stability or that all but one were lost from the gel. The corresponding protein stain showed about six bands. Single activity bands were stained for activity after prolonged starch gel electrophoresis. Also evident was the fact that the 3 $\alpha$ -HSD from *Pseudomonas testosteronei* was an entirely different enzyme. This bacterial enzyme migrated in the direction opposite to that of the human liver cytosolic enzyme and appeared to be positively charged at pI 8.3.

The results from the polyacrylamide gel electrophoresis of different ammonium sulphate preparations from horse and human liver suggested that the ethanol dehydrogenase activity in the two tissues were different. While the 40-60% fraction of the human liver cytosol has two bands, the corresponding fraction in the horse liver cytosol has four or more. The 3 $\alpha$ -HSD activity stain in these two tissues could not be obtained for comparison, as the enzyme became inactive before this study was carried out. Stability studies of the human liver cytosolic 3 $\alpha$ -HSD activity and ethanol dehydrogenase activity were done with and without added cofactors. It was shown that at 4°C both enzyme activities are stabilized to some extent by the presence of NAD.

Enzyme deterioration was faster in both cases without added cofactor. The 3 $\alpha$ -HSD activity however was less stable than the corresponding ethanol dehydrogenase activity. While the specific activity of the ethanol dehydrogenase remained at about 80% of the initial activity in the presence of added cofactors after a month, the 3 $\alpha$ -HSD activity dropped to less than 50% of the initial activity. This suggests that either the two activities reside on different proteins or that there is a mixture of unresolved isozymes.

### Concluding Remarks

$3\alpha$ -HSD plays an important part in the synthesis of bile acids, and in the degradation of testosterone, progesterone, cortisol and other steroids in man. The lack of extensive study on this enzyme in the human, unlike the  $3\beta$ -HSD, prompted this study. Surprisingly, the only characterization of the human liver cytosolic  $3\alpha$ -HSD as a protein (Lang et al., 1986) refers to activity in the crude cytosol. This M.Sc thesis therefore appears to be the first systematic attempt to isolate and characterize this important human cytosolic enzyme activity by following the  $3\alpha$ -HSD activity through a sequence of purification steps. Lang et al., 1986 reports cofactor requirement of this enzyme to be NADP with the microsomal enzyme utilizing NAD. Although it was not studied in detail, NAD was found to be the more efficient cofactor in this work.

The enzyme appears to possess ethanol dehydrogenase activity which is strongly inhibited by 4-methyl pyrazole. The fact that the steroid activity persists after such inhibition may suggest that there are different catalytic sites for steroids and ethanol or more likely may merely reflect differences in binding characteristics of the two types of substrates. The enzyme appears to coincide with the  $\beta 1\beta 1$  isozyme of the human alcohol dehydrogenase on starch and polyacrylamide gel electrophoresis. Since there are different polymorphic forms of the human liver alcohol dehydrogenases this assignment is tentative pending further study. The presence of at least four separate enzyme activities after CM-cellulose chromatography which also stain for ethanol activity gives support for the above assignment of the major one as  $\beta 1\beta 1$ , of human liver alcohol dehydrogenase activity.

The pI determined is closer to the pI of the class I isozymes of human liver alcohol dehydrogenase (pI 7.7- 8.6) than to class II or class III.

The presence of a  $3\alpha$ -HSD in the human placental cytosol has been demonstrated. The unstable nature of this enzyme may make characterization difficult. Metabolism of

3-ketosteroids to the 3-hydroxysteroid is definitely catalyzed by two enzyme activities. The presence of  $3\alpha$ -HSD activity in the human kidney and in horse liver cytosol has also been demonstrated. It is interesting to note that the  $3\alpha$ -HSD activity is twice that of the  $3\beta$ -HSD in the cytosol of the human kidney, but predominantly  $3\beta$ -HSD in the microsomal fraction.

More complete characterization of the human liver  $3\alpha$ -HSD activities is necessary before we can assess the range of substrates for each form. The relation between this rather unknown enzyme group and the widely known class I isozymes of the human liver alcohol dehydrogenases is also worthy of further study.



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