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THE SOLUBLE 17β-HYDROXYSTEROID DEHYDROGENASE OF FEMALE RABBIT LIVER: CHARACTERIZATION AND AGE DEPENDENT CHANGES OF THE MULTIPLE FORMS OF THE ENZYME

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DEDICATION

This thesis is dedicated to Nadia, Ramez and George for their love, patience and understanding were the most importance in the completion of this research work.

SUMMARY

Multiple forms of 17β-hydroxysteroid dehydrogenase (17β-HSD) have been identified in the cytosol of female rabbit liver. Three of these enzyme forms (I, II and III) have been purified from liver cytosol of the 8-week-old rabbit by a procedure involving affinity chromatography and chromatofocusing. These enzyme forms appear to be charge isomers since they differ in their isoelectric points, have similar molecular weights and have no subunit structure. All of the purified forms catalyze the oxidation of 17β-hydroxysteroids in the presence of $NADP^+$ but not NAD^+. Activity toward C_{19} steroids is higher than that toward C_{18} steroids. These enzyme forms also exhibit 3α-hydroxysteroid dehydrogenase (3α-HSD) activity toward C_{19} steroids, activity toward 5β-androstane substrates being higher than that toward 5α-androstane substrates. Kinetic analysis with the substrates testosterone and etiocholanolone indicates a competitive inhibition pattern supporting the presence of a common active site for the two substrates.

The enzyme forms I and II have considerable structural homology as indicated by their amino acid compositions and by peptide mapping. These enzyme forms have the same amino-terminal residue and carboxyl-terminal peptide pattern. Enzyme forms I and II also have similar relative activities toward a variety of 3α- and 17β-hydroxysteroid substrates. Although enzyme form III is structurally similar to forms I and II, it differs from these enzymes at the amino-terminal of the protein. Enzyme form III also differs in its relative activity toward the 3α- and 17β-hydroxysteroid substrates.

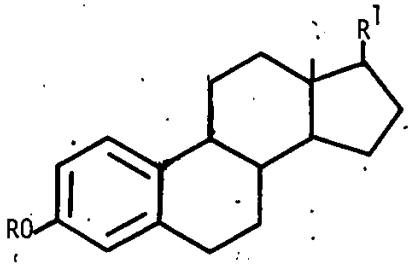
The multiple forms of the soluble 17β-HSD of female rabbit liver are age-related. The enzyme pattern obtained by chromatofocusing differs among rabbits at the ages of 28, 35, 42 and 49 days. A single major form (IIIY) of the soluble 17β-HSD is present in the liver of the 28-day-old female rabbit. This enzyme, which is more acidic than any of the enzyme forms present in the 8-week-old rabbit, has been purified and compared to the enzyme forms I, II and III. Although enzyme form IIIY has the same amino-terminal amino acid residue as enzyme form III, it is more homologous to enzyme forms I and II in terms of its peptide map and relative substrate specificity.

GLOSSARY

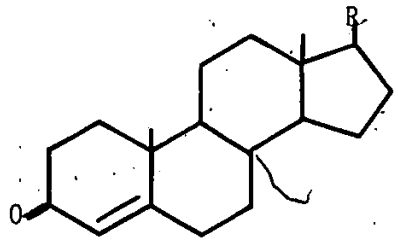
1) Trivial and systematic names of steroids:

Androstanedione	5α-Androstan-3,17-dione
Androstenedione	4-Androsten-3,17-dione
Androsterone	5α-Androstan-3α-ol-17-one
Dehydroepiandrosterone	5-Androsten-3β-ol-17-one
5α-Dihydrotestosterone	5α-Androstan-17β-ol-3-one
Epitestosterone	4-Androsten-17α-ol-3-one
17α-Estradiol	1,3,5(10)-Estratrien-3,17α-diol
17β-Estradiol	1,3,5(10)-Estratrien-3,17β-diol
17β-Estradiol 3-glucuronide	17β-Hydroxyestra-1,3,5(10)-trien-3yl-β-D-glucopyranosiduronic acid
Estriol	1,3,5(10)-Estratrien-3,16α,17β-triol
Estrone	1,3,5(10)-Estratrien-3-ol-17-one
Etiocholan-3,17-dione	5β-Androstan-3,17-dione
Etiocholanolone	5β-Androstan-3α-ol-17-one
Testosterone	4-Androsten-17β-ol-3-one

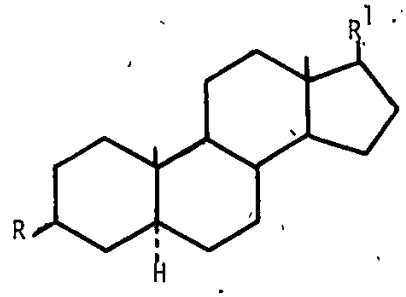
2) Structural formulae of some steroids, including those which are used as substrates:



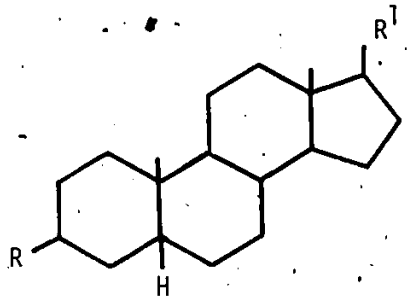
	R	R^1
17β-Estradiol	H	— OH
17α-Estradiol	H	-- OH
Estrone	H	= O



	R
Epitestosterone	---OH
Testosterone	—OH
Androstenedione	=O



	R	R^1
Androsterone	---OH	=O
Androstanedione	=O	=O
5α-Dihydrotestosterone	=O	--OH



	R	R^1
Etiocholanolone	---OH	=O
Etiocholan-3,17-dione	=O	=O

3) Abbreviations and symbols:

Bis	N,N^1-methylenebisacrylamide
Ci	curie (2.22×10^{12} d.p.m.)
DnP-F	1-fluoro-2,4-dinitrobenzene
d.p.m.	disintegrations per minute
DTT	dithiothreitol
g	acceleration due to gravity
HSD	hydroxysteroid dehydrogenase
Ki	inhibitor constant
μ	micro (10^{-6})
m	milli (10^{-3})
mA	milliampere
M	molar
mol	mole
MW	molecular weight
n	nano (10^{-9})
NAD, NADH	nicotinamide-adenine dinucleotide (oxidized and reduced forms)
NADP, NADPH	nicotinamide-adenine dinucleotide phosphate (oxidized and reduced forms)

NBT	nitroblue tetrazolium salt
nm	nanometer or millimicron
p	pico (10^{-12})
pI	isoelectric point
PMS	phenazine methosulfate
PPO	2,5-diphenyloxazole
SDS	sodium dodecyl sulfate
TEMED	N,N,N^1,N^1-tetramethylenediamine
t.l.c.	thin-layer chromatography
Tris	2-amino-2-hydroxymethylpropane-1,3-diol
UV	ultraviolet
V	voltage
Vmax	maximum velocity
v/v	by volume
w/v	weight in volume

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CHAPTER I

INTRODUCTION

A) General Introduction

In mammalian tissues steroid hormones are secreted by endocrine glands and travel in the blood stream to their target organs. These hormones are involved in a variety of biological actions in their target organs, such as sex differentiation, the maintenance of pregnancy and the regulation of energy utilization and electrolyte balance. Steroid hormones also play a role in controlling the growth of certain tumors of the breast and prostate gland. Although the regulation of steroid hormones is primarily at the level of their biosynthesis through the action of pituitary peptide hormones, the metabolic processes for the inactivation and removal of steroid hormones are also important in regulating the level of these hormones in the body and therefore affect hormonal action on target tissues.

The metabolic transformations of steroid hormones occur principally in the liver and kidney and include the following: 1) interconversions of specific hydroxyl and carbonyl functions on the steroid skeleton and side chain by pyridine-nucleotide-linked hydroxysteroid dehydrogenases; 2) reductions of steroid double bonds by Δ-reductases which require the participation of pyridine nucleotide; 3) hydroxylations of primary, secondary and tertiary carbon atoms in reactions required NADPH and molecular oxygen; 4) conjugation of steroids with sulfate or glucuronide residues.

The designation hydroxysteroid dehydrogenase (ketosteroid oxidoreductase) has been widely adopted for the group of enzymes which catalyze the interconversion of hydroxyl and carbonyl functions on the steroid skeleton

and side chain. The position and steric configuration of the reacting group is conveniently designated by an appropriate prefix. These reactions require pyridine nucleotides as hydrogen carrier and may be formulated according to the following equation:

$$\text{Hydroxysteroid} + \text{NADP}^+ \ (\text{NAD}^+) \rightleftharpoons \text{Ketosteroid} + \text{NADPH} \ (\text{NADH}) + \text{H}^+$$

Dehydrogenases which act on 3α-, 3β-, 6α-, 6β-, 7α-, 7β-, 11α, 11β-, 12α-, 15α-, 15β-, 16α-, 16β-, 17α-, 17β-, 20α-, 20β- and 21-hydroxysteroids have been identified.

Since chemical modification at C-17 of androgens and estrogens can affect their biological activity, the 17-hydroxysteroid dehydrogenases (17-HSD; EC 1.1.1) have a potentially important role in regulating the physiological action of estrogenic and androgenic steroids. As a consequence considerable attention has been directed to these enzymes with regard to their chemical and physical properties and their function in mammalian tissues. The 17-HSD consist of the 17α- and 17β-HSD groups based on the stereospecificity of the enzymatic reaction at C-17 of the steroid substrates. 17α-HSDs catalyze the interconversion of the 17-keto and 17α-hydroxysteroids while, 17β-HSDs catalyze the interconversion of the 17-keto and 17β-hydroxysteroids.

The rabbit is an appropriate model for studies on the structure-function relationships of the 17-HSDs since estrogen metabolism and likely androgen metabolism as well, involves in part the conversion of 17β-estradiol to the inactive 17α-epimer. Both 17α- and 17β-HSDs are present in a number of rabbit tissues. Previous studies in our laboratory have characterized the 17α-HSDs of liver and kidney. The studies described in this thesis were therefore undertaken to characterize the 17β-HSD of liver in order to

compare this activity to the 17α-HSDs and to establish the role of this enzyme in estrogen and androgen metabolism in the rabbit.

B) Literature Review of 17-Hydroxysteroid Dehydrogenases

1. Tissue distribution

The presence of 17β-HSD activity has been documented in a variety of mammalian tissues. 17β-HSD activity is present in the ovary (Renwick and Engel, 1967), testis (Inano and Tamaoki, 1974) and placenta (Langer and Engel, 1958). The enzyme activity is also presented in the hormone-responsive tissues such as the uterus (Ryan and Engel, 1953) and ventral prostate (Hussein and Kochakian, 1968). Moreover, 17β-HSD activity has been demonstrated in tissues such as liver and kidney (Aoshima and Kochakian, 1963), skin (Davis et al., 1972; Hodgins et al., 1982) and lung (Milewick et al., 1978).

Folkerd and James (1982) studied the 17β-HSD in human subcutaneous adipose tissue homogenate. They found that the reduction of estrone occurred more readily in this tissue although 17β-estradiol was also a substrate. They postulated that the adipose tissue could be an important site of peripheral 17β-estradiol production in particular in postmenopausal obese women where ovarian estradiol production is greatly diminished.

Farthing et al. (1982) have recently observed a 17β-HSD in the mucosal homogenates of rat and human small intestine which converts testosterone to the biologically inactive androstenedione. The estimated total 17β-HSD activity of the rat small intestinal mucosa was considerably greater than both testis and lung, suggesting that the small intestine might make a significant contribution to the peripheral oxidative metabolism of testosterone. This could explain the ineffectiveness of oral testosterone

replacement which had been previously attributed to hepatic metabolism of testosterone.

The pyridine nucleotide specificity of the 17β-HSDs can vary among tissues in the same species and among the same tissues in different species. 17β-HSDs of rabbit tissues are typical examples of this variation. Hasnain and Williamson (1975) examined the tissue distribution of the NAD^+- and $NADP^+$-dependent 17β-HSD activities in the rabbit and showed that 17β-enzyme activity was present in the homogenates of liver, kidney, small intestine, large intestine, spleen and uterus. Moreover, the 17β-enzyme activity of the liver and large intestine exhibited a preference for NAD^+, while $NADP^+$ was the preferred cofactor in the kidney and small intestine.

2. Subcellular distribution and pyridine nucleotide specificity

Studies on the subcellular distribution of the mammalian 17β-HSDs have shown that in general these enzymes are located either in the microsomal or the soluble fraction of the cell, indeed some tissues have 17β-HSD activity in both of these subcellular fractions (Williamson, 1979).

In the majority of tissues, NAD^+ is the preferred cofactor for the microsomal 17β-HSD activity and $NADP^+$ is the cofactor for the soluble enzyme activity. Examples of the microsomal 17β-HSD activities which prefer NAD^+ as cofactor include those of the dog prostate (Hussein and Kochakian, 1968), human endometrium (Pollow et al., 1972a,b,c), rat skin (Davis et al., 1972) human forehead skin (Hodgin et al., 1982), guinea pig liver (Aoshima and Kochakian, 1963) and human liver (Littman et al., 1971). Examples of soluble 17β-enzymes which prefer $NADP^+$ as a cofactor include those of rabbit liver (Williamson, 1979), chicken liver (Renwick and Engel, 1967),

guinea pig liver and kidney (Aoshima and Kochakian, 1963) and human main axillary skin (Hodgin et al., 1982).

This relationship between subcellular distribution and pyridine nucleotide specificity of the 17β-HSD is not strictly followed in all species and tissues. Inano and Tamoki (1974) have purified a $NADP^+$-dependent 17β-HSD from the microsomal fraction of porcine testis and an NAD^+-dependent 17β-HSD activity has been identified in the soluble fraction of the sheep ovary (Kautsky and Hagerman, 1970; Michel et al., 1975). Recently Kochakian (1982a,b) examined the 17β-HSD of the liver and kidney of the adult hamster. NAD^+ was the preferred cofactor for the soluble activity of both tissues and the liver microsomal activity while $NADP^+$ was the preferred cofactor for the kidney microsomal enzyme. 17β-HSDs that can utilize either NAD^+ or $NADP^+$ as cofactor have also been reported. The best example is the soluble 17β-estradiol dehydrogenase of human placenta. Karavolas et al. (1970) determined the kinetic constants for both pyridine nucleotides and the values for maximal velocity obtained with $NADP^+$ were 70% to 80% of those obtained with NAD^+.

3. Physical and chemical properties of 17β-hydroxysteroid dehydrogenase

The 17β-HSD that has been examined most throughly in terms of its physical properties is the soluble 17β-estradiol dehydrogenase of human placenta.

(a) Molecular weight and subunit structure

A dimeric structure for the human placental 17β-estradiol dehydrogenase has been suggested by various molecular weight determination experiments. Burns et al. (1971) showed that this enzyme sedimented as a single

band by SDS-polyacrylamide gel electrophoresis, corresponding to a molecular weight of approximately 33,500. Ultracentrifugation of the enzyme in the presence of 20% glycerol gave a molecular weight of 67,000 suggesting that in solution the enzyme exists as a dimer (Burns et al., 1971). Ultracentrifugation of the enzyme in the presence of 8 μM 17β-estradiol gave a molecular weight of 68,000 (Burns et al., 1972). Jarabak and Street (1971) obtained a subunit molecular weight of 35,000 by SDS-polyacrylamide gel electrophoresis, when the native enzyme preparation was first reacted with the cross-linking agent dimethyl-suberimide, in addition to a second protein band with a molecular weight of approximately 73,000. Jarabak and Street (1971) also obtained values in the range of 72,000 when they used different methods, including gel filtration, sedimentation coefficient in order to measure the molecular weight of the placental enzyme, 17β-estradiol dehydrogenase.

Studies have been carried out to establish whether the dimeric form of the enzyme consists of similar subunits. Burns et al. (1972) have shown that the two subunits are identical in their amino acid composition and the amino acid sequences at the amino-terminal ends for a distance of at least five residues are similar. Moreover, peptide mapping studies on the chymotryptic digests of the enzyme revealed only six different cysteinyl peptides from a total of twelve cysteine residues (Pons et al., 1977). Each subunit of the enzyme was shown to have an essential cysteine residue in its active site.

The molecular weights of a number of other 17β-HSDs have been determined. Sucrose density gradient centrifugation of the 17β-HSD of sheep ovary gave a molecular weight of 104,000 for this enzyme (Kautsky and Hagerman, 1970).

There was no evidence of monomer-polymer interconversion, although such an equilibrium has been found with the human placental 17β-estradiol dehydrogenase. Two values for the molecular weight of the 17β-HSD from human red blood blood cells have been reported. Jacobsohn and Hochberg (1968) obtained the value of 75,500 estimated by gel filtration and employing the same technique, Mulder et al. (1972) obtained a value of 64,000. Pollow et al. (1976a) determined the molecular weight of the 17β-HSD of human secretory endometrium to be 50,000 to 54,000 by gel filtration.

A number of 17β-HSDs have molecular weights similar to that of the subunit of the human placental 17β-estradiol dehydrogenase. The molecular weight of the microsomal $NADP^+$-17β-HSD of porcine testis was estimated as 35,500 from gel filtration data (Inano and Tamaoki, 1974). This value is coincident with that for 17β-HSD of rabbit liver cytosol (Thaler-Dao et al., 1972) and guinea pig kidney (Liu and Kochakian, 1972a). None of these enzymes appear to have subunit structures. Renwick et al. (1981) purified two forms of 17β-HSD of chicken liver cytosol. The molecular weights of the two forms were 43,000 and 97,000 as determined by SDS-polyacrylamide gel electrophoresis.

(b) pH optimum

The pH optima of various 17β-HSDs show striking similarities. For example the 17β-HSD of rabbit liver (Thaler-Dao et al., 1972) and chicken liver (Renwick and Engel, 1967) exhibit broad pH optima ranging from 9.0 to 9.5. The guinea pig kidney 17β-HSDs have pH optima from 9.4 to 10.2 (Liu and Kochakian, 1972a) and similarly the 17β-HSD from human erythrocytes is optimally active within a pH range of 8.5 to 10.0 (Mulder et al., 1972). Human placental 17β-estradiol dehydrogenase also exhibits a pH optimum at

pH 10.0 (Langer and Engel, 1958). An exception to this alkaline pH optimum is the microsomal 17β-HSD of porcine testis which has a pH optimum between 6.5 and 7.5 (Inano and Tamaoki, 1974).

Often, the pH optimum of the 17β-HSDs differs for the oxidations and reductions. Jutting et al. (1976), in studies on the 17β-HSD of rabbit uterus, observed that the pH optimum for the enzymatic oxidation of 17β-estradiol to estrone was pH 9.5 and for reduction of estrone to 17β-estradiol was 6.4. In another study, Breuer and Dahm (1964) showed that the oxidation of testosterone to androstenedione by a partially purified soluble 17β-HSD of rat kidney was maximum at pH 8.7, whereas the reverse reaction was maximum at pH 6.6

(c) Substrate specificity

The substrate specificity of the 17β-HSDs has been studied in a variety of mammalian species and tissues. These studies have revealed that there is a broad variation in specificity depending on the species and/or tissues that are the sources of the enzyme. Therefore this review will not attempt to discuss the specificity of all 17β-HSDs but will describe examples of enzymes that exhibit high specificity and others that are less specific. The possible physiological significance of this substrate specificity will also be discussed.

The soluble human placental 17β-estradiol dehydrogenase has been examined most extensively in terms of substrate specificity. Langer et al. (1959) showed that the enzyme had an absolute stereochemical specificity for the 17β-hydroxyl group and that it did not react with the following substituents on steroid molecules: 3α-hydroxy, 3β-hydroxy, 3-keto, 11α-hydroxy, 11β-hydroxy, 16α-hydroxy, 16β-hydroxy and 21-hydroxy. They noted also that an unsubstituted phenolic hydroxyl group at C-3 was not essential.

Although some nonbenzenoid steroids were acted upon slowly, an aromatic A or B ring or both appeared to be characteristic of highly reactive substrates. The most reactive substrate was 17β-estradiol and the relative rate of testosterone oxidation was less than 1% that of 17β-estradiol. Because of this high activity toward 17β-estradiol, this enzyme was called 17β-estradiol dehydrogenase or EDH.

Engel and Groman (1974) showed that 16α-hydroxyestrone was readily reduced to estriol in the presence of NADH or NADPH, while estriol was not a substrate for the 17β-estradiol dehydrogenase. They concluded that one of the physiological roles of placental 17β-estradiol dehydrogenase was to carry out the final step in estriol synthesis. The inability of estriol to be oxidized by the enzyme could be due to the formation of a nonreactive complex, probably a consequence of the steric effect exerted by the oxygen function at C-16 (Blomquist et al., 1981). A similar irreversible reaction catalyzed by 17β-estradiol dehydrogenase is its interaction with 18-hydroxyestrone. The placental enzyme is unable to catalyze the oxidation of 18-hydroxy-17β-estradiol whereas 18-hydroxyestrone is readily reduced (Findlay and Breuer, 1974).

The ovarian 17β-HSD has been studied from sheep (Kautsky and Hagerman, 1970; Michel et al., 1975), human (Pittaway et al., 1977), rabbit (Davenport and Mallette, 1966; Rodway and Rahman, 1978) and in immature mouse (Lerner et al., 1972). In all of these species, the enzyme activity is relatively specific for estrogens and minimal activity is detected with androstenedione, dehydroepiandrosterone, testosterone and androstenediol as substrates (Pittaway et al., 1977). The 17β-HSD of the myometrium from rabbit uterus (Jutting et al., 1976) and human endometrial microsomes (Ryan

and Engel, 1953; Tseng et al., 1981) oxidize 17β-estradiol at a rate much higher than that observed with testosterone as substrate.

Leinonen (1982) studied 17β-estradiol oxidation in the human testis in order to characterize a potentially important physiological regulatory step in the action of estradiol on testicular steroidogenesis. Administration of 17β-estradiol is known to inhibit testicular androgen production (Kalla et al., 1980). 17β-Estradiol was shown to be metabolized to estrone, a metabolite with lower estrogenic activity, in a reaction requiring NAD^+. This reaction was not inhibited by testosterone to any great extent. Leinonen (1982) suggests that in the human testis the oxidation of 17β-estradiol to the weaker estrogen prevents the deleterious effect of this hormone on androgen biosynthesis. On the other hand, 17β-oxidation of testosterone by testicular 17β-HSD required a different coenzyme ($NADP^+$), had a different pH optimum and was inhibited by 17β-estradiol and testosterone. This data suggests that in the human testis different enzymes catalyze the oxidation of 17β-estradiol and testosterone (Leinonen, 1982).

In the rat testis, the presence of two distinct 17β-HSDs have been described, one located in the interstitial tissue and the other in the seminiferous tubules (Murono and Payne, 1976). These two enzymes catalyze the reduction of androstenedione to testosterone, have different pH optima, Km values and respond differently to testosterone and its 5α-metabolites. The interstitial tissue enzyme is inhibited by testosterone and its 5α-reduced metabolites and 17β-estradiol. On the other hand, testosterone and 5α-androstan-3α,17β-diol activate the 17β-HSD activity in seminiferous tubules and 5α-dihydrotestosterone in high concentrations inhibits this

enzyme activity. The different effects of testosterone and its 5α-metabolites on the 17β-HSD activities of the interstitial and seminiferous tubules led Murono and Payne (1976) to postulate that the testosterone biosynthesis in the rat testis might be regulated locally in the two testicular compartments without any direct involvement of the pituitary gonadotrophin hormones.

The 17β-HSDs of liver and kidney generally exhibit a broader substrate specificity than that observed with this enzyme in endocrine tissues. For example studies with human and guinea pig liver have shown that both C_{18} and C_{19} steroids are substrates for the 17β-HSD of these tissues (Joshi et al., 1963; Littman et al., 1971; Blomquist et al., 1981). Thaler-Dao et al. (1972) resolved three 17β-HSD activities from rabbit liver cytosol and partially purified two of these enzymes. One of the enzyme activities utilized NAD^+ or $NADP^+$ as cofactor and catalyzed the oxidation of both testosterone and 17β-estradiol. The other enzyme required $NADP^+$ and had higher activity toward testosterone than 17β-estradiol. This enzyme activity also possessed 3α-HSD activity and to lesser extent 3β-activity with substrates of both the 5α- and 5β-androstane series. It was suggested that a single protein was responsible for both the 17β- and 3α-HSD activities. Renwick et al. (1981) were able to purify two soluble $NADP^+$-linked 17β-HSDs from chicken liver with molecular weights of 43,000 and 97,000. The lower molecular weight form was the more active toward 17β-estradiol and C_{19} steroids were poor substrates.

The soluble 17β-HSD of guinea pig kidney is the best characterized enzyme of this tissue. Purification of the enzyme activity by Liu and Kochakian (1972a,b) indicated the presence of several soluble 17β-HSDs in

this tissue. All of these enzymes exhibited dual pyridine nucleotide specificity although higher activity was observed with $NADP^+$ than with NAD^+. With steroid substrates, highest activity was obtained with testosterone, only a trace of enzyme activity was observed with 17β-estradiol. The 5α- and 5β-reduced C_{19} steroids were also substrates.

17β-HSD activity toward both estrogens and androgens has been demonstrated in the blood of several mammalian species. Enzyme activity catalyzing the interconversion of androstenedione and testosterone in the peripheral venous blood of the human, sheep, dog and rabbit has been observed (Van der Molen and Groen, 1968). A 17β-HSD catalyzing the interconversion of 17β-estradiol and estrone has been detected in the peripheral blood of pregnant woman early in gestation (Plotti et al., 1972). The 17β-HSD activity of human erythrocytes has a broad substrate specificity and catalyzes the conversion of both androgens and estrogens with a 17β-hydroxyl group or 17-oxo group. In addition, the sulfate derivatives of estrone, 17β-estradiol and dehydroepiandrosterone are also substrates.

Milewich et al. (1982) showed that, in the human lymphocytes the major androstenedione metabolizing activity was 17β-HSD. These authors suggested that in human, lymphocytes were a potential site of peripheral metabolism of androstenedione to more potent androgens.

(d) Bifunctional enzyme activity

Studies in a number of purified 17β-HSDs have shown that these enzymes may catalyze oxidation-reduction at more than one position of steroid substrates. For example, four forms of a 17β-HSD purified from crude extracts of Pseudomonas testosteroni have been shown to possess enzymatic activity toward both 3β- and 17β-hydroxysteroid substrates (Schultz et al.,

1977a). The evidence suggested that in each of the forms, both the 3β- and 17β-activities were associated with the same protein molecule.

In an early study, Purdy and colleagues (1964) reported that the 17β-estradiol dehydrogenase and 20α-HSD activities of the human placenta were indistinguishable in their physical properties. They possessed the same degree of stability in glycerol solution, the same heat inactivation and the same degree of inhibition by p-hydroxymercuribenzoate. These authors also obtained kinetic evidence that supported the assumption that 17β-estradiol dehydrogenase and 20α-HSD represented a bifunctional enzyme activity on a single protein. This early study has been confirmed by Strickler and Tobias (1980; 1981a,b), who were able to purify an enzyme from human placental cytosol that possessed both 17β-estradiol dehydrogenase and 20α-HSD activities, with a ratio of specific activities of about 100:1 respectively. To evaluate whether catalysis of the estrogen and progestin substrates occurred at a single active site on a single protein or at two different sites, alkylation studies using 16α-bromoacetoxyprogesterone were carried out (Strickler et al., 1981). This affinity alkylating steroid bound at the enzyme active site, inactivated the enzyme in an irreversible and time dependent manner and caused a loss of both 17β- and 20α-activities. Strickler et al. (1981) showed by affinity radioalkylation studies using 16α[2'-^{3}H]bromoacetoxyprogesterone that 2 moles of steroid were bound per mole of inactivated enzyme dimer. Amino acid analysis of the acid hydrolyzate of radioalkylated enzyme showed that 16α-bromoacetoxyprogesterone dicarboxymethylated a histidyl residue at the active site. These results were identical with those reported for this enzyme when 16α[2'-^{14}C]bromoacetoxyestradiol-3-methyl ether was used as the alkylating agent (Tobias

and Strickler, 1981). These studies clearly demonstrate that the catalysis of estrogen and progestin substrates could be occurring at a single enzyme active site. Tobias et al. (1982) also demonstrated the bifunctional activity of human placental 17β-estradiol dehydrogenase using the affinity labels 17β[1-(R)-1-hydroxy-2-propynyl] androst-4-en-3-one (α-HPA) and 17β[1-oxo-2-propynyl] androst-4-en-3-one (OPA). OPA was a substrate and a very rapid irreversible affinity alkylator in the absence of NADH and caused simultaneous inactivation of the 17β- and 20α-activities in a time dependent manner which followed pseudo-first order kinetics. The enzyme was protected against OPA inactivation by the substrates, estrone, 17β-estradiol, progesterone and 20α-hydroxyprogesterone. On the other hand, incubation of α-HPA in the absence of cofactor did not result in inactivation of the enzyme. When α-HPA was enzymatically oxidized to OPA in the presence of NAD^+, identical time-dependent, irreversible inactivation of both the 17β- and 20α-activities was observed. Tobias et al. (1982) concluded that the simultaneous inactivation of both 17β-estradiol dehydrogenase and 20α-HSD activities by OPA and by enzymatic oxidation of α-HPA clearly demonstrates the bifunction activity of the single enzyme active site of the human placental 17β-estradiol dehydrogenase.

Bifunctional enzyme activity has also been reported for the 17β-HSD of human endometrium (Tseng and Gurpide, 1979) and rabbit ovary (Rodway and Rahman, 1978) and liver (Thaler-Dao et al., 1972). Morevoer, bifunctional enzyme activity is not restricted to 17β-HSD. 3β- and 20β-HSD activities have been observed in Streptomyces hydrogenans. Different studies have shown that both activities are due to one enzyme (Edwards and Orr, 1978; Sweet and Samant, 1980, 1981; Sweet et al., 1980).

(e) Heterogeneity of mammalian 17β-hydroxysteroid dehydrogenases

The purification of 17β-HSDs from a variety of tissues and species has revealed the heterogeneity of this enzyme. The first indication of the isozymic nature of a 17β-HSD of mammalian origin was demonstrated by polyacrylamide disc gel electrophoresis of the cytosol of guinea pig kidney by Liu and Kochakian (1972a,b). When gels were stained for 17β-HSD activity, three strong and two weak bands which possessed both NAD^+-$NADP^+$-linked 17β-HSD activities were observed. Electrophoresis at a series of gel concentrations indicated that the enzyme forms were charge isomers rather than size isomers. Shen and Kochakian (1978) have isolated some of the multiple forms of the guinea pig kidney 17β-HSD by an extensive combination of anion and cation exchange chromatography. The amino acid composition, Km values and coenzyme and substrate specificities of all but one of the enzyme forms were very similar.

One major and six minor 17β-hydroxy C_{19}-steroid dehydrogenases have been purified from male adult guinea pig liver by a combination of gel filtration and ion exchange chromatography (Kobayashi and Kochakian, 1978). The amino acid compositions, Km values and substrate specificities indicated two separate groups of enzymes. The first group was specific for 5β-androstanes series and could utilize either NAD^+ or $NADP^+$ as cofactor. The second group was specific for 5α-androstanes and $NADP^+$.

Isoelectric focusing of the soluble 17β-HSD from normal human endometrium and endometrium carcinoma indicated a heterogeneity of the enzyme (Pollow et al., 1976a,b). The enzyme from normal human endometrium yielded three enzymatically active bands with either 17β-estradiol or testosterone as substrate. In contrast, the activity patterns from endometrial carcinoma

consisted of 2, 4 or 7 bands of enzyme activity, depending on the substrate (17β-estradiol or testosterone) and the cofactor (NAD^+ or $NADP^+$). This finding indicated that in normal and neoplastic endometrium, the 17β-HSD existed in different molecular forms.

Inano et al. (1980) separated two forms of the porcine testicular 17β-HSD by isoelectric focusing. This heterogeneity did not appear to be due to protein methylation or the presence of carbohydrate on the protein. The two enzyme molecules were very similar in their amino acid composition. The two enzymes were also immunologically similar. More recently, Inano et al. (1981) separated the porcine testicular 17β-HSD into at least three distinct bands of enzyme activity, by polyacrylamide gel electrophoresis. The heterogeneity was mainly due to difference in net charge of the enzyme forms and not a difference in the molecular size.

Engel and Groman (1974) subjected the human placental 17β-estradiol dehydrogenase, a dimeric enzyme, to isoelectric focusing in 20% glycerol on polyacrylamide gels, between pH 4.0 and 6.0. They obtained a pattern of three prominent and two faint bands upon staining for protein or enzyme activity. Isoelectric focusing carried out in the presence of 8 M urea revealed the presence of three protein bands. Engel and Groman (1974) concluded that there were three different monomers of the enzyme present in unequal amounts that could interact to form six dimers in the native enzyme. Heterogeneity of the 17β-HSD has also been observed in the rabbit and chicken liver (Thaler-Dao et al., 1972; Renwick et al., 1981).

(f) Mechanism of reaction

The kinetic parameters for the interaction of steroid substrates and pyridine nucleotides with a number of 17β-HSDs have been used to establish

the molecular mechanism of the enzyme reaction. Warren and Crist (1967) carried out a kinetic analysis of the site specificity of purified human placental estradiol 17β-dehydrogenase in terms of cofactor (NAD^+ and $NADP^+$) and steroid substrates (17β-estradiol and estradiol 3-sulfate). This analysis indicated that both 17β-estradiol and estradiol 3-sulfate bound at a single active site. NAD^+ and $NADP^+$ had an active site in common and a second NAD^+ binding site of lower binding affinity was also present. Warren and Crist (1967) postulated an ordered mechanism in which either steroid or cofactor bound first to the enzyme. The steroid and cofactor formed a ternary complex with the enzyme before the catalytic step occurred (bi bi mechanism).

Betz (1971) investigated the reaction mechanism of human placental estradiol 17β-dehydrogenase by measuring the rate of isotopic exchange between substrate-product pairs while varying the concentrations of unlabeled reactants (Betz, 1971). He found that either the steroid or nucleotide formed a binary complex with the enzyme. This data was consistent with a random addition of substrates to the enzyme in contrast to an ordered mechanism in which the binding of one substrate was prerequisite for the binding of the second. In contrast to the initial velocity studies of Warren and Crist (1967) no abortive ternary complex of enzyme and two oxidized or two reduced substrates were noted. The results of Betz (1971) are supported by an affinity labelling study by Pons et al. (1973) which showed that $NADP^+$ exerted a protective effect against inactivation of enzyme by the oxidized steroid inhibitor iodoacetoxyestrone and NADPH prevented the inactivation by the reduced inhibitor iodoacetoxyestradiol. Apparently, neither the ternary complexes iodoacetoxyestrone-enzyme-$NADP^+$ nor iodoacetoxyestradiol-enzyme-NADPH were formed.

Michel et al. (1975) carried out a kinetic study of the reaction catalyzed by the 17β-HSD of sheep ovary, in the presence of different concentrations of coenzyme and substrates. The data obtained suggested a compulsory order mechanism with the binding of NAD^+ as the first substrate. This mechanism was supported by the observation that the binding of the coenzyme enhanced the affinity of the ovarian enzyme for the steroid substrates by 2- to 30-fold. This effect was not observed with the placental enzyme.

Further studies on the mechanism of the 17β-HSD reaction have involved an examination of the stereospecificity of hydride transfer from the pyridine nucleotide coenzyme to steroid substrates. Jarabak and Talalay (1960) carried out the reaction with 17β-estradiol and NAD(P^+)-nicotinamide-4-^{3}H. The placental 17β-estradiol dehydrogenase was a "β" stereospecific enzyme, meaning that the enzyme transfers hydride between the substrate and the β (Pro-S) side of the dihydropyridine ring. This was subsequently confirmed in studies by Warren et al. (1967).

Groman et al. (1975) used affinity labeling of the 17β-estradiol dehydrogenase with bromoacetoxyestrone to study the stereospecificity of hydride transfer. They observed that, treatment of the inactivated enzyme with (4S)-[4-^{2}H]NADH resulted in the formation of covalently bound [17β-^{2}H]-estradiol-17β, which was released by hydrolysis and identified by gas chromatography-mass spectrometry. When (4R)-[4-^{2}H]-NADH was used, deuterium was not transferred. This experiment also illustrated that the normal stereochemistry of hydride transfer was preserved for both partners even when the enzyme was covalently bound to ligand.

(g) Studies on the active site of the soluble human placental 17β-estradiol dehydrogenase

Affinity labeling has been used extensively to study the active site and the mechanism of action of 17β-estradiol dehydrogenase. Affinity labels are substrate analogs or competitive inhibitors which react specifically and irreversibly with amino acid residues at the active site of an enzyme. Direct evidence for binding of an affinity label at the active site of an enzyme would be obtained if, following covalent modification, the enzyme could catalyze its normal chemical reaction by using its covalently bound ligand as a substrate. Steroids are ideal carriers of reactive groups that can form covalent bonds with amino acid residues in proteins. Their rigid skeletons and the fact that reactive groups can be attached to them at known positions and with known stereochemistry permit them to be used as probes of high precision for the delineation of the geometry of binding sites on a protein molecule.

A number of modified estrogens have been synthesized and used as labels for 17β-estradiol dehydrogenase in different laboratories. These alkylating steroids have been radiolabeled either on the steroid skeleton or on the side chain. Examples of such reagents include: 3-bromoacetoxyestrone (Groman et al., 1975), 16α-bromoacetoxyestradiol 3-methyl ether (Chin and Warren, 1975), 12β-bromoacetoxy-4-estrene-3-17-dione (Warren et al., 1977), 4-bromoacetoxidoestrone 3-methyl ether (Bhatanager et al., 1978), 3-iodoacetoxyestrone and 16α- and 16β-estroneiodoacetate or bromoacetate (Pons et al., 1976). Some representative structural formula of these modified steroids are provided in Appendix II.

Groman et al. (1975) showed that incubation of 17β-estradiol dehydrogenase in the presence of excess 3-bromoacetoxyestrone at pH 6.5 resulted in rapid inactivation of the enzyme. The inactivation was accompanied by incorporation of 1 mole of 3-bromoacetoxyestrone per mole of enzyme subunit. The covalently bound estrone could still be reduced by NADPH with the same stereospecificity, both for the steroid and for the coenzyme, as noncovalently bound estrone. Groman et al. (1975) state that this property of the modified enzyme demonstrates its catalytic competence, i.e. the ability of an enzyme to catalyze its normal reaction with a covalently bound substrate.

Chin and Warren (1975) studied the inactivation of 17β-estradiol dehydrogenase by 16α-bromoacetoxyestradiol 3-methyl ether and they were able to identify a histidyl residue in the catalytic region of the active site of the enzyme. 17β-Estradiol, NADH and NADPH slowed the rate of inactivation. The protective effect of the pyridine nucleotide was interpreted as resulting from cofactor induced changes in the enzyme steroid binding site. When the enzyme was inactivated with 16α-bromoacetoxyestradiol 3-methyl ether, amino acid analysis of acid hydrolyzates revealed the presence of both 3-carboxymethyl histidine and 1,3-dicarboxymethyl histidine. Comparison of enzyme samples that had been inactivated to the extent of 28 or 51% indicated that, as inactivation proceeded, the total amount of 3-carboxymethyl histidine decreased while, 1,3-dicarboxymethyl histidine increased, suggesting that the former was converted to the latter by a second alkylation step.

It was reported earlier by Pons et al. (1973a,b) and Boussioux et al. (1973) that two cysteinyl residues and one histidyl residue were located at or near the binding site when 17β-estradiol dehydrogenase was inactivated by 3-iodoacetoxyestrone (IAE) and that the histidyl residue was not essential

for enzyme activity. Chin and Warren (1975), suggested that the histidine residue identified by Pons et al. (1973b) could be located near position 3 of the bound steroid and was a different residue from that which Chin and Warren (1975) identified near position 16 of the bound steroid.

More recently Murdock and Warren (1982) isolated a single carboxymethyl-histidine-bearing peptide from the steroid binding site of human placental 17β-estradiol dehydrogenase when the enzyme was inactivated with 3-bromo-acetoxyestrone. The alkylation of this peptide was markedly increased in the presence of cofactor and a second carboxymethyl histidine-bearing peptide was also isolated. Murdock and Warren (1982) suggested that the first peptide was actually at the catalytic site and that cofactor binding oriented the steroid or moved the histidyl residue closer to the steroid in the active site. The second peptide was either a second histidyl-bearing region that was moved closer to the reagent binding region of the steroid or it was derived from the first peptide by chemical modification, i.e. deamination, cyclization or incomplete hydrolysis during the purification process.

Murdock et al. (1982) identified two histidyl residues at the active site of human placental 17β-estradiol dehydrogenase. They conducted the affinity labeling studies at pH 6.3, where considerably less cysteinyl alkylation was noted and histidyl residues were better detected. 12β-(Bromo[2-^{14}C]acetoxy)-4-estrene-3,17-dione and 16α-(bromo 2-^{3}H acetoxy)-estratriene-3,17β-diol 3-methyl ether were used as affinity labeling steroids. Both of them were substrates and inactivated the enzyme in a time dependent, irreversible manner. When samples of the enzyme inactivated with these two affinity labeling steroids were submitted to acid hydrolysis, both yielded 1-(carboxymethyl)-histidine, (3-carboxymethyl)histidine, 1,3-bis(carboxymethyl)-

histidine, S-(carboxymethyl)cysteine, as well as small amounts of (carboxymethyl) lysine. When the two inactivated enzyme samples were combined, reduced, carboxymethylated and hydrolyzed with trypsin, three radioactive (carboxymethyl) histidyl-bearing peptide fractions containing ^{14}C and ^{3}H were isolated. One of these peptides contained only 1,3-bis(carboxymethyl)-histidine, the second peptide contained only 3-(carboxymethyl)-histidine and the third only 1-(carboxymethyl) histidine. Papain digestion of each of the three isolated peptides produced two subpeptides bearing the [^{14}C] carboxymethylated and the other bearing the [^{3}H] carboxymethylated histidyl residue. Murdock and coworkers (1982) concluded that the histidyl residue carboxymethylated at position 3 by 12β-(bromo[2-^{14}C]acetoxy)-4-estrene-3,17-dione was different from the one carboxymethylated by 16α-(bromo[2-^{3}H]acetoxy)-estradiol 3-methyl ether, and therefore might be at a different region of the active site. Furthermore, the 3-(carboxymethyl)-histidyl-bearing peptide generated by affinity labeling with 12β-(bromo[2-^{14}C]acetoxy)-4-estrene-3,17-dione had identical mobility on electrophoresis with that of the 3-(carboxymethyl) histidyl-bearing peptide generated on affinity labeling with 3-(bromo[2-^{14}C] acetoxy) estratriene-17-one. Thus it appeared that the histidyl residue alkylated by both of these steroids was located in the upper-A-B-ring region of the steroid, some distance from the site of the catalytic event. Therefore, a topographic map of the steroid-binding site was proposed in which two histidyl residues were present. One is affinity labeled by 12β(bromo [2-^{14}C]acetoxy)-4-estrene-3,17-dione, and the other by 16α-(bromo[2-^{3}H] acetoxyestratriene-3,17β-diol 3-methyl ether, and neither residue was pinpointed at the site of the catalytic event.

4. Regulation of 17β-hydroxysteroid dehydrogenase

(a) Development and sexual differentiation

The 17β-HSD activity in some tissues of mammalian species has been shown to be age-dependent. Bongiovanni and Marino (1972) examined the enzyme activity in liver tissue homogenates of rabbits at various developmental stages. While 17β-HSD activity was absent in fetal rabbit liver (30 to 33 days of gestation), it was present in the livers of young (20-day-old) and mature animals (17- to 19-months-old).

Baldi and Charreau (1972) studied the 17β-HSD activity in rat submaxillary glands in relation to the sex and age of the animal. The rate of conversion of testosterone to androstenedione was higher in the female gland than in the male. This higher activity was not observed when 17β-estradiol was the substrate. Androstenedione production from testosterone was maximal during fetal life and decreased gradually with increasing age of the animal. Baldi and Charreau (1972) suggested that the sex differences in the 17β-HSD activity were related to histological and functional differences in the tubular ducts of the submaxillary glands. These ducts were taller and more columnar in adult male rats than in females. Since these histological changes were caused by testosterone, the diminished oxidation of this androgen in the submaxillary glands of the male rat would lead to a greater effect of testosterone on the male gland.

In some species and tissues the age-dependent development of 17β-HSD is accompanied by a sex specific differentiation of this enzyme activity. Ghraf et al. (1975a,b,c) investigated the development and sexual differentiation of 17β-HSD activities in the liver, kidney and gonads of rats ranging in age from 15 to 120 days. In all organs investigated, 17β-HSD

activity was found in both the cytoplasmic and microsomal fractions. The activity was very low in the microsomal fraction of the kidney and in the cytosol of the testis. In all of the tissues studied, the enzyme activity of at least one of the subcellular fractions displayed an age-dependent development, reaching significantly higher levels in 60-day-old animals. The only exceptions noted were the cytosol of the adrenal, the microsomes of the kidney and the cytosol of female liver, in which no increases in enzyme activity were observed. 17β-HSD activities in the kidney, adrenal and gonads were significantly higher in female rats than in male rats, whereas the enzyme activity in the cytosol of male liver was higher than that observed in the cytosol of female liver.

In studies on the golden hamster, Furubayashi et al. (1982) showed that in the testis, microsomal 17β-HSD activity was found to be equally distributed between tubules and interstitial tissues in immature animals. In adult testis this activity was approximately 10 times that found in immature testis. Similar observations were made by Inano et al. (1967) and Ghraf et al. (1975c) in studies on the 17β-HSD of rat testis. The activity of 17β-HSD in the tissue increased gradually up to the 50th day after birth and then increased markedly by the 60th day. This increase of 17β-HSD activity appeared to be correlated with the growth of accessory organs during this period.

Macartney and Thomas (1969) showed an age-dependent increase in the conversion of estrone to 17β-estradiol in rabbit uterine preparations. The levels of this 17β-HSD activity in the myometrium of the immature rabbit were increased by injection of 17β-estradiol.

(b) Hormonal regulation

The effects of castration and/or hormone administration on 17β-HSD activity has been examined by a number of investigators. Kochakian et al. (1973) have reported that the activity of 17β-hydroxy-C_{19}-steroid dehydrogenase of the male guinea pig kidney cytosol was decreased after castration and restored by testosterone treatment. The difference in activity was attributed to the disappearance and appearance of an enzyme form detectable by substrate staining of polyacrylamide gel electrophoresis. However, in more recent studies, Shen and Kochakian (1978), could not confirm this gel pattern although they did observe a decrease in the 17β-HSD activity after castration. Shen and Kochakian (1978) showed that the soluble and microsomal 17β-HSD activities of female guinea pig kidney were very low for several hydroxy-C_{19}-steroids. The administration of testosterone induced a gradual increase in the 17β-hydroxy-C_{19}-steroid dehydrogenase activity of the cytosol but not the microsomes to the normal male kidney level after 50 days. In contrast, the liver did not respond to the testosterone treatment indicating that the regulation of the liver enzyme in the female of guinea pig is under a different regulatory mechanism than the kidney enzyme.

In other studies on the hormonal regulation of 17β-HSD activity, Thaler-Dao and Breuer (1974) studied the gonadal factors regulating the 17β-HSD activity in rat liver cytosol. In adult animals, the enzyme activity was about 60% higher in male than in female rat liver. The 17β-HSD activity was low in young animals of both sexes and it gradually increased between 20 to 90 days of age. The time course of the development of enzyme activity during maturation revealed a difference between male and female animals. In females, 17β-HSD activity reached a plateau of 100 days,

whereas, in males, the activity was still increasing after six months. When female animals were gonadectomized, the time course of development of enzyme activity was almost identical with that observed in male rats. Thaler-Dao and Breuer (1974) concluded that female hormone might have a repressive effect on the activity of the 17β-HSD in adult rats. Further evidence for the "repressing effect" of 17β-estradiol was provided by variations of the activity of 17β-HSD during the estrus cycle in rats where high enzyme activity was observed during metoestrus and diestrus and low enzyme activity during pro-estrus due to increase secretion of estrogen during this period (Thaler-Dao and Breuer, 1974). In contrast 17β-estradiol has been reported to have a stimulatory effect on 17β-HSD activity of rabbit uterus (Jutting et al., 1967; Macartney and Thomas, 1969).

Lax et al. (1974) and Ghraf et al. (1975b), examined the role of the gonads and the hypophysis in the regulation of the soluble 17β-HSD of rat liver. Activity was significantly higher in intact male rats than in female rats and was not decreased in the male by castration. Hypophysectomy or administration of 17β-estradiol to male rats decreased the enzyme activity to the female liver level, while gonadectomy or testosterone administration to female rats increased the soluble hepatic 17β-HSD activity to the male level. These authors termed the enzyme in rat liver cytosol as "estrogen dependent", since gonadectomy of male animals had no effect on the enzyme activity. Ghraf et al. (1975a) conducted similar studies on the soluble 17β-HSD of rat kidney and concluded that this activity was androgen dependent. In intact animals, enzyme activity was higher in females than in males. Castration of the male rat increased the activity to the female level, an effect reversible by administration of testosterone. The

administration of testosterone to either intact or ganodectomized female animals decreased the enzyme activity to the male level. In male and female rats, hypophysectomy resulted in a decrease in the 17β-HSD activity of the kidney to levels far below that observed with intact animals and no sex difference in the activity was observed.

In other studies on the hormonal regulation of 17β-HSD activity, Hansson (1982) observed that the administration of testosterone, 11-oxotestosterone, and dihydrotestosterone to juvenile rainbow trout Salmo gairdnerii increased hepatic 17β-HSD activity. Furthermore, 17β-estradiol diminished the effect of testosterone on 17β-HSD activity. In male trout, the liver microsomal 17β-HSD increased significantly during the late stages of sexual maturation. The quantitative differences in liver microsomal 17β-HSD activity between female fish and male fish may be related to different levels and/or types of steroid hormones in the plasma. The marked increase in 17β-HSD observed in liver microsomes from androgen-treated fish indicated that the testicular androgens, testosterone and 11-oxotestosterone, account for the high 17β-HSD activity in fish. Furthermore, since 17β-estradiol seemed to suppress the testosterone-induced 17β-HSD activity, Hansson (1982) concluded that the low level of this enzyme activity in female fish when compared with that in male fish may be related to high plasma levels of 17β-estradiol known to be present in the female. Hansson (1982) also noticed that the effects of hypophysectomy and testosterone together on the 17β-HSD activity were greater than that of each treatment alone, which suggests that at least some of the testosterone effects are direct and independent of the hypophysis gland. These results are in constrast with the situation in the rat where the hypophysis

has an obligatory role in the steroid hormonal control of hepatic steroid metabolism (Lax et al., 1974; Ghraf et al., 1975b).

Musto et al. (1972) observed that the 17β-HSD activity in the mouse testis was increased by treatment with prolactin. The hereditary sterile dwarf male mouse; which is deficient in prolactin while having normal levels of LH and FSH was used in this study. The dwarf male mice were treated with prolactin by implantation of a normal mouse pituitary gland under the kidney. The 17β-HSD activity in the testis homogenate increased up to 244% that of the control animal, while, subcutaneous injection of prolactin increased enzyme activity to 186% that of the control animal. These authors suggested the possible involvement of this hormone in sexual maturation.

In studies carried out by Murono and Payne (1979) hypophysectomized immature rats were treated with LH or FSH to assess the role of these hormones on the activity of testicular steroidogenic enzymes during sexual maturation. Enzyme activity was decreased after hypophysectomy. Treatment with LH increased 17β-HSD activity 1.5-fold, while treatment with FSH stimulated the enzyme activity for more than 2-fold. This result was in agreement with an earlier hypothesis by Payne et al. (1977) which suggested that the increase in 17β-HSD activity during sexual maturation may be regulated by FSH and that this might be one mechanism by which FSH increases testicular responsiveness to LH.

Kondon et al. (1980) estimated the 17β-HSD activity in ovaries of the androgen-sterilized adult rat. They observed that the enzyme activity decreased significantly 6 days following hypophysectomy and was stimulated by FSH treatment but not by LH or 17β-estradiol. These results confirmed

an earlier study by Fukuda et al. (1979) which showed that in ovary homogenates of the immature rat, the 17β-HSD was regulated by FSH but not LH.

5. Clinical applications and implications

The soluble human placental 17β-estradiol dehydrogenase has transhydrogenase activity due to the dual pyridine nucleotide specificity of the enzyme (Talalay and Williamson-Ashman, 1958). This transhydrogenase activity has been used in the routine clinical determination of estrogens either on plasmal extracts or directly on urine without extraction (Nicolas et al., 1979). The enzymatic assay makes use of the transhydrogenase function of the human placental 17β-estradiol dehydrogenase whereby transhydrogenase activity is directly related to the estrogen concentration. The specificity of this method is based on the selectivity of the human placental 17β-estradiol dehydrogenase which is highly specific for C_{18} steroids. Moreover, the transhydrogenase activity of the enzyme has a higher specificity than the dehydrogenase activity since estradiol 3-sulfate or 3-glucuronide are not substrates for the transhydrogenase. This method measures the total of estradiol plus estrone in biological fluids. Estradiol alone can be determined by reacting the estrone contained in the sample with hydrazine, to form the hydrazone which is not a substrate for the transhydrogenase reaction. The sensitivity of the method was reported to be near 10 picogram in the plasma.

The activity of the 17β-HSD in the serum of pregnant women has been examined for possible diagnostic value in disturbed pregnancies since it originates almost exclusively from the placenta and the foetus (Lubbert, 1977). The enzyme activity is first observed in the 7th and 8th week of gestation and generally increases with the progression of gestation. The

activity of the 17β-estradiol dehydrogenase is highest at the expulsion of the placenta and then rapidly decreases. In normal twin births, enzyme activity is considerably higher than in single births. In acute damage of the placenta, the serum enzyme activity increases while in chronic damage the activity decreases (Lubbert, 1977).

Studies on normal endometrium at different phases of the menstrual cycle have shown that progesterone and synthetic progestins reduce the level of 17β-estradiol receptors in the tissue and increase the activity of 17β-estradiol dehydrogenase (Gurpide et al., 1977). The 17β-HSD activity is low in the proliferative endometrium and it is higher in the secretory endometrium. This result could be anticipated since progesterone appears to be necessary for the induction of this enzyme activity. Therefore, an increase in 17β-HSD would lower the 17β-estradiol concentration in the tissue. This could be one mechanism by which progestins might act as antiestrogens.

17β-HSD has been detected in some human breast cancer cells (MacIndoe and Woods, 1981). Characterization of the enzyme indicated that the intracellular location, temperature and pH optima, cofactor requirements and apparent substrate affinities were generally similar to those described for several other mammalian systems. The presence of 17β-HSDs, aromatase, 5α-reductase and 3α-HSD activities in estrogen-dependent human breast cancer cells suggest the possibility that hormonal regulation of these cells might be mediated in part by intracellular estrogen and/or androgen metabolism.

A congenital deficiency of testicular 17β-HSD has been shown to contribute to male pseudohermaphroditism (Saez et al., 1971, 1972; Pittaway et al., 1976; Viridis et al., 1978). In general, male pseudohermaphroditism (MPH) is due to abnormal testosterone synthesis, abnormal

testosterone metabolism or defective androgen receptors in the target organs. Some common features of (MPH) patients include ambiguity of internal or external genitalia and incomplete masculinization. Because of the marked ambiguity of the external genitalia at birth, these patients are frequently raised as females. The incomplete masculinization may be accompanied by gynecomastia (breast development) at puberty (Saez et al., 1971, 1972; Akesoda et al., 1977). Akesoda et al. (1977) and Pittaway et al. (1976) studied the 17β-HSD deficiency in MPH and found that there was a marked elevation of plasma androstenedione and low plasma testosterone. Furthermore, incubation of testis slices with androstenedione or testosterone demonstrated the enzymatic defect.

CHAPTER II

MATERIALS AND GENERAL METHODS

A) Materials

1. Isotopically labeled compounds

[1,2-^{3}H (N)] Androstenedione (SA, 41.0 Ci/mmol), [1,2-^{3}H (N)] androsterone (SA, 40.8 Ci/mmol), [1,2-^{3}H (N)] dehydroepiandrosterone (SA, 40.2 Ci/mmol), [1,2-^{3}H (N)] dihydrotestosterone (SA, 50.5 Ci/mmol), [1,2-^{3}H (N)] epitestosterone (SA, 50.0 Ci/mmol), [1,2-^{14}C] ethanolamine hydrochloride (SA, 6.3 mCi/mM), [1,2-^{3}H (N)] etiocholanolone (SA, 45.0 Ci/mmol), 17β-[6,7-^{3}H (N)] estradiol (SA, 40.0 Ci/mmol), [6,7-^{3}H (N)] estrone (SA, 44.0 Ci/mmol), and [7-^{3}H (N)] testosterone (SA, 25.0 Ci/mmol) were purchased from New England Nuclear, Lachine, Quebec. [1-^{14}C] Acetic anhydride (SA, 115 mCi/mmol) and 1-Fluoro-2,4-dinitro[3,5-^{3}H] benzene (SA, 26.0 Ci/mmol) were purchased from Amersham Corporation, Oakville, Ontario.

2. Unlabeled steroids

Androstenedione, epitestosterone, 17α-estradiol, 17β-estradiol, estrone, testosterone, 5α-dihydrotestosterone, androstanedione, etiocholan-3,17-dione were obtained from Sigma Chemical Co., St. Louis, Missouri.

Androsterone, and 5α-androstan-3β,17β-diol were purchased from Research Plus Steroids Lab. Inc., Denville, New Jersey.

3. Coenzymes

NAD^{+}, (Grade III from yeast) and $NADP^{+}$ (mono sodium salt) were purchased from Sigma Chemical Co., St. Louis, Missouri.

4. Chromatographic and electrophoretic chemicals

Sephadex G-75 superfine grade, Polybuffer exchanger PBE 94 and Polybuffer 74 for chromatofocusing were obtained from Pharmacia Fine Chemical, Montreal, Quebec. Matrex Gel Red A was purchased from Amicon Co., Lexington, Massachusetts and Ampholyte pH range 5-7 from LKB, Sweden (distributed by Fisher Scientific Co. Ltd., Ottawa,Ontario).

Acrylamide, bisacrylamide and TEMED were from Bio-Rad Laboratories, Richmond, California. Ammonium persulfate was obtained from Fisher Scientific Co. Ltd., Ottawa, Ontario.

Silica gel (Kieselgel N) was from Macjerey Nagel & Co., Germany.

5. Protein standards, enzymes and related compounds

Ribonuclease, chymotrypsinogen A, aldolase and ovalbumin (molecular weight calibration kit) were from Pharmacia, Montreal, Quebec. Bovine serum albumin, coomassie brilliant blue R-250, NBT and PMS were from Sigma Chemical Co., St. Louis, Missouri. Ketodase (β-glucuronidase) was from General Diagnostic, Warner-Lambert Co., Morris-Plains, New Jersey. Papain was from Worthington Biochemical Corporation, Freehold, New Jersey. Dithiothreitol was obtained from Chemalog Chemical Dynamics Corporation, South Plainfield, New Jersey. SDS (sequanal grade) was from Pierce Chemical Co., Rockford, Illinois. Urea (ultra pure) was from Schwarz Mann, Orangberg, New York. ε-DNP-L-Lysine HCl was from United States Biochemical Corporation, Cleveland, Ohio.

6. Scintillation fluor and fluids

PPO was purchased from Kent Laboratories Ltd., Vancouver, British Columbia. Aquasol-2 was from New England Nuclear, Lachine, Quebec.

7. Buffer salts and solvents

All salts and solvents were of high purity preparations or of reagent grade. They were purchased either from Fisher Scientific Co., Ottawa, Ontario or from Canadian Lab. Supplies, Ottawa, Ontario.

B) General Methods

1. Preparation of radioactive substrates

Radioactive steroids obtained from commercial sources were monitored for purity by t.l.c. (silica gel N) in benzene - acetone (4:1, v/v). 17β-[6,7-^{3}H] Estradiol 3-glucuronide (E_2βGA) was prepared by incubation of 17β-[6,7-^{3}H] estradiol (specific activity 40 to 55 Ci/mmol) with rabbit liver microsomes in the presence of UDP-glucuronic acid under the conditions described by Collins et al. (1968, 1970). The reaction mixture was extracted first with benzene or ethyl acetate to remove the free steroids and then with ethyl acetate after the aqueous layer had been adjusted to pH 2.0, with 1N HCl, to extract 17β-[6,7-^{3}H] estradiol 3-glucuronide. Final purification of the substrate was achieved by t.l.c. (silica gel N) in benzene-ethanol-methylethyl ketone-water (3:3:3:1, by volume).

For enzyme assays, the stock solutions of the radioactive steroids were diluted with radioinert steroid in methanol to give a final specific activity of 4.5 μCi/μmol. The final steroid concentration was 0.1 μmol/ml. These stock solutions were stored in the freezer, but warmed to room temperature before use.

2. Assay for dehydrogenase activity

For assays with hydroxysteroid substrates, the incubation mixtures contained the following: steroid substrate, 3 nmol in 30 μl of methanol;

$NADP^+$, 0.5 µmol in 2 ml of 0.1 M glycine-NaOH buffer, pH 9.5 and enzyme fraction, 1-100 µl. The total volume was made up to 3 ml by the addition of 0.15 M KCl. Samples were incubated at 37°C for 30 minutes. The amount of enzyme added to each incubation mixture was adjusted so that the extent of substrate oxidation was less than 40%. Control incubations in which the enzyme was omitted were also carried out.

For assays with ketosteroid substrates, the conditions and the concentrations were the same as above except that the cofactor used was NADPH and 0.1 M Tris-maleate buffer, pH 7.6, was used in place of glycine buffer.

Incubations were terminated by extraction of the steroid substrate and product from the incubation medium with 5 ml of ethyl acetate. The ethyl acetate layer was removed and evaporated to dryness under a stream of nitrogen. In incubations with 17β-[6,7-^{3}H] estradiol 3-glucuronide, the steroids were extracted with ethyl acetate after adjusting the incubation mixtures to pH 2.0 with 1 N HCl. The ethyl acetate layer was removed and evaporated to dryness under a stream of nitrogen. The steroid conjugates were then hydrolyzed by incubation with Ketodase (1000 units), in 3 ml of 0.1 M sodium acetate buffer, pH 5.0, overnight in an incubation chamber maintained at 37°C. The free steroids were then extracted with 5 ml of benzene and the benzene layer evaporated under a stream of nitrogen. The recovery of incubated radioactivity after extraction or extraction and hydrolysis was greater than 80%.

Appropriate radioenert steroid standards (10 µg) were added to each ethyl acetate or benzene residue in a small volume of methanol and the mixture was then separated by t.l.c. (silica gel N) in the solvent system benzene-acetone (4:1, v/v). The t.l.c. was carried out on 20 x 10 cm

plates coated with 0.25 mm of silica gel N. The plates were activated in an oven at 110° for 3 hours before use. After development, the plates were allowed to air dry. The steroids were then detected by spraying the plates with a solution of 20% sulfuric acid in ethanol followed by heating the plates for 5-10 minutes at 110°C. The spots were then scraped off the thin layer plate into scintillation vials and 0.4 ml methanol was added to each vial. Ten ml of scintillation fluid toluene containing 40 mg PPO (2,5-diphenyl oxazol) was added to each vial and the radioactivity was measured in a liquid scintillation counter (Searle Mark III).

3. Expression of enzyme activity and protein determination

The percent conversion of substrate (A) to product (B) was calculated by the formula $\frac{dpm\ (B)}{dpm\ (A) + dpm\ (B)} \times 100$. Enzyme activity was expressed as units/ml or units/mg protein. One unit of activity is defined as the amount of enzyme catalyzing the oxidation or reduction of 1 μmole of steroid substrate per minute under the specific conditions of the assay.

Protein concentration was determined by the coomassie blue method (Bradford, 1976).

4. Crude enzyme preparation

All procedures for the purification of the 17β-HSD were carried out at 0-4°C. A female New Zealand White rabbit was sacrificed by cervical dislocation. The liver was removed and immersed in an ice cold solution of 0.15 M KCl. The gallbladder was removed, the liver was minced with scissors and a 20% (w/v) homogenate was prepared in 10 mM Tris-HCl buffer, pH 8.0, containing 0.15 M KCl, 0.5 mM dithiothreitol (DTT), using a Sorval Omni-Mixer for one minute at maximum speed. The homogenate was centrifuged at 10,000 x g for 30 minutes in a Sorval RC2-B centrifuge and the supernatant obtained was centrifuged at 105,000 x g for 90 minutes in a

Beckman ultracentrifuge. The 105,000 x g supernatant (designated as cytosol in this thesis) was concentrated by ultrafiltration in an Amicon Diaflo apparatus equipped with a YM-10 membrane. The concentrated sample was left at 4°C overnight to remove any dissolved nitrogen. Any insoluble material was removed from the fraction by centrifugation (105,000 x g) prior to gel filtration on Sephadex G-75.

5. Sephadex G-75 (superfine) gel filtration

The concentrated enzyme fraction obtained after ultrafiltration was applied to a 5 x 95 cm column of Sephadex G-75 (superfine), that had been previously equilibrated with 10 mM Tris-HCl, pH 8.0, 0.5 mM DTT. The column was eluted with the same buffer at a flow rate of 20 ml/hour. Ten ml fractions were collected and assayed for 17β-HSD activity. Protein was monitored at 280 nm. Fractions having 17β-HSD activity were pooled and concentrated by ultrafiltration. Insoluble material was removed by centrifugation (10,000 x g).

6. Affinity chromatography

An affinity column (1.5 x 40 cm) of agarose immobilized Procion Red HE3B was equilibrated by washing with ten bed volumes of 10 mM Tris-HCl-0.5 mM DTT, pH 8.0. The enzyme fraction was applied to the affinity column at a very slow rate of 6-8 ml/hour. Subsequent elution was carried out at a flow rate of 15 ml/hour.

The column was eluted first with 150 ml of 10 mM Tris-HCl-0.5 mM DTT, pH 8.0, containing 1 mM EDTA and 5 mM $MgCl_2$; followed by a 600 ml linear gradient of NaCl from 0-1 M in 10 mM Tris-HCl-0.5 mM DTT, pH 8.0, containing 1 mM EDTA and 5 mM $MgCl_2$ and then by a 200 ml linear gradient of NaCl from

1-2 M in 10 mM Tris-HCl-0.5 mM DTT, pH 8.0, containing 5 mM $MgCl_2$. Finally the column was eluted with 400 ml of 2.5 M NaCl in 10 mM Tris-HCl-0.5 mM DTT, pH 8.0, containing 10 mM $MgCl_2$ to elute 17β-HSD activity. Fractions (3.6 ml) were collected and assayed for 17α- and 17β-HSD activities. The fractions having 17β-HSD activity (2.5 M NaCl) were pooled, glycerol was added to give a final concentration 20% (v/v) and the fraction was concentrated by ultrafiltration. The concentrated sample was dialyzed against two changes of 4 liters of 10 mM Tris-HCl-0.5 mM DTT, pH 8.0, containing 20% glycerol and then centrifuged (10,000 x g) to remove insoluble material.

7. Chromatofocusing chromatography

The fraction containing 17β-HSD activity was applied to a column (1 x 40 cm) packed with Polybuffer Exchanger PBE 94 that had been previously equilibrated with 10 mM imidazole-HCl buffer, pH 6.15, containing 0.5 mM DTT. The column was then eluted with a solution of Polybuffer 74 prepared in the following manner: 50 ml of the Polybuffer 74 was first adjusted to pH 5.15 with 1 N HCl and then diluted to 400 ml with deareated distilled water. DTT was added to give a final concentration of 0.5 mM. Elution of the column with this Polybuffer 74 solution was carried out at a flow rate of 15 ml/hour, 1.5 ml fractions were collected and assayed for 17β-HSD activity. To monitor the pH gradient developed during elution of the column, appropriate samples of the eluant were placed in an ice bath and the pH was measured within two hours of collection using a pH meter (Fisher Accumet model 520) fitted with a combination microelectrode (Brinkman Instruments). Fractions comprising each peak of enzyme activity were pooled and concentrated by ultrafiltration. The concentrated peaks were each again subjected to

chromatofocusing under the same conditions described above except the column size was 1 x 10 cm, and the eluting volume of Polybuffer solution was 100 ml. Fractions comprising each peak of enzyme activity were pooled and concentrated by ultrafiltration.

To remove Polybuffer from each pooled fraction, solid ammonium sulfate was added to each to give 100% saturation. The sample was kept at 4°C for 2 hours. The precipitate was collected by centrifugation, washed twice with a saturated ammonium sulfate solution and then dialyzed against distilled water to remove any remaining ammonium sulfate.

This procedure to remove Polybuffer was only necessary for samples required for amino acid analysis, peptide mapping, carboxyl-terminal and amino-terminal identification.

8. Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was carried out as described by Davis (1964). The acrylamide monomer concentration was 10.5%. The gels (0.6 x 9 cm) were pre-run for 45 minutes at a current of 3 mA/tube. The buffer used for electrophoresis was 0.02 M Tris, 0.2 M glycine pH 8.3. Bromophenol blue (0.01%) was used as the tracking dye. With all protein samples except the 105,000 x g supernatant fraction, glycerol was added (20% final concentration, v/v) to increase the sample viscosity. Electrophoresis was carried out at 4°C at an initial current of 2.5 mA/tube until the tracking dye had travelled to within 0.5 cm of the gel bottom.

9. Protein staining

The gels were first fixed in 12.5% trichloroacetic acid for 30 minutes in a water bath at 65°C and then stained with 0.2% aqueous Coomassie brilliant blue R-250 in 45% ethanol-10% acetic acid for 30 minutes at 65°C.

Destaining was carried out first in 25% ethanol-10% acetic at 65°C for 30 minutes and then at room temperature overnight. Gels were stored in 10% acetic acid.

10. Isoelectric focusing polyacrylamide gel electrophoresis

Isoelectric focusing in polyacrylamide gel was carried out according to Hayes and Wellner (1969) with some modifications. Polyacrylamide gels (0.6 x 9 cm) were prepared from a solution containing the following: acrylamide (8%, w/v), N, N-methylene bisacrylamide (0.24%, w/v), ampholytes LKB 40% w/v, pH 5-7 (5%, v/v), ammonium persulfate (0.024%, w/v) and N,N,N',N'-tetramethylethylenediamine (0.24%, v/v). The gels were placed in a gel electrophoresis chamber, the upper compartment (cathode) of the electrophoretic apparatus contained 0.02 M sodium hydroxide while the lower compartment (anode) contained 0.01 M phosphoric acid. Prior to application of the protein sample, the gels were pre-run under constant voltage for 15 minutes at 200 V followed by 30 minutes at 300 V and finally for another 30 minutes at 400 V at 4°C. This was carried out in order to remove the excess of ammonium persulfate. Following the pre-run, the sodium hydroxide solution was removed from the top compartment and the surface of the gel was rinsed with distilled water. Protein sample, containing 20% glycerol (at final concentration) was applied to the top of the gels and overlayed with sodium hydroxide. Viscous samples such as the 105,000 x g supernatant fraction were applied without addition of glycerol. Isoelectric focusing was carried out under constant voltage at 400 V for 30 minutes followed by 3-4 hours at 500 V. The pH gradient was determined by slicing the gel in one cm sections, placing each section in one ml of distilled water at 4°C and measuring the pH of the solution.

11. 17β-Hydroxysteroid dehydrogenase activity in isoelectric focusing polyacrylamide gels

Protein fractions subjected to isoelectric focusing electrophoresis in polyacrylamide gels were stained for 17β-HSD activity by the method of Lau et al. (1982a). In this procedure, gels were first equilibrated in 0.1 M glycine-NaOH buffer, pH 9.5, for approximately 30 minutes at 22°C (Gabriel, 1971). Each gel was then placed in 15 ml of staining solution containing the following components: 5 µmol $NADP^+$, 0.27 µmol phenanzine methosulfate (PMS), 5 µmol nitroblue tetrazolium (NBT) and 925 µmol glycine-NaOH buffer, pH 9.5. To this mixture, 6 µmol of steroid substrate dissolved in 0.8 ml methanol was added. For control gels, the steroid substrate was omitted. The staining reagent also contained 2 mM sodium cyanide to inhibit cyanide sensitive superoxide oxygen dismutase (SOD). The above staining mixture was prepared under subdued light at room temperature. The gels were incubated in the staining mixture in the dark at 37°C. Enzyme activity was detected by the formation of purplish-blue diformazan band(s). Gels were incubated until a band of suitable intensity was obtained. The gels were then removed and washed throughly with distilled water. The gels were scanned with a Gilford 240 spectrophotometer equipped with a linear transport attachment at a wavelength 540 nm and a slit width 0.25 mm. The scanning speed was 0.5 cm/minute and the recording chart speed was 0.5 inch/minute. Gels were stored in 7.5% acetic acid in the dark.

12. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis

Acrylamide gels (10%) were prepared as described by Weber et al. (1972) in 0.6 x 8 cm internal diameter glass columns. For molecular weight determination, the following protein standards were used: ribonuclease (MW, 13,000),

chymotrypsinogen A (MW 25,700), aldolase (MW 40,000), ovalbumin (MW 45,000) and bovine serum albumin (MS 68,000). These protein standards as well as the protein samples (approximately 100 µg) were mixed with 1 ml of 0.01 M sodium phosphate, pH 7.0, containing 1% SDS and 1 mM DTT and heated in a boiling water bath for 3 minutes. Aliquots of the treated protein samples (2-10 µg protein) were mixed with a small volume of glycerol to increase the viscosity of the sample and applied to the gel with 3 µl of tracking dye (0.1% bromophenol blue in 0.01 M phosphate buffer). Electrophoresis was carried out at room temperature at an initial current of 4 mA/tube for about 30 minutes and then at 8 mA/tube until the dye front had reached the bottom of the gel. After electrophoresis the length of each gel and the distance to the centre of the dye band on each gel were measured. Staining was carried out in a solution containing 0.25% Coomassie brilliant blue R-250-45% methanol-9% acetic acid for two hours at room temperature. The gels were destained in a solution of 25% methanol 7.5% acetic acid by the diffusion method at room temperature. After destaining the length from the top of the gel to the stained protein bands were measured and the mobility of each band calculated accordingly by using the equation:

$$\text{Mobility} = \frac{\text{Distance migrated by protein}}{\text{Distance migrated by tracking dye}} \times \frac{\text{Length of gel before staining}}{\text{Length of gel after staining}}$$

Gels were stored in 7.5% acetic acid.

13. Amino acid composition

Protein samples (approximately 100 µg) were dialyzed against distilled water and lyophilized. Protein samples were subjected to performic acid oxidation by the method of Hirs (1956), in order to oxidize cysteine and methionine residues in the protein samples to the more stable derivatives

cysteic acid and methionine sulfone respectively. The oxidized protein samples were lyophilized and solubilized in 2 ml of 6 N HCl containing 1 mM β-mercaptoethanol and 1 mM phenol in a heavy wall pyrex tube. The samples were subjected to hydrolysis in vacuo, at 105-110°C for 16 hours. The hydrolysate was lyophilized. The amino acid analysis was carried out using a Durrum D-500 single column multisample amino acid analyzer equipped with an automatic integrator. Amino acid analysis was carried out by Dr. M. Yaguchi of the National Research Council, Ottawa.

14. Amino sugar analysis

Amino sugar analysis of the 17β-HSD was carried out using the enzyme fraction obtained from the affinity chromatography column (1 mg) as well as each individual form of the purified enzyme (0.4 mg). The enzymes were dialyzed against distilled water and then lyophilized. Hydrolysis was carried out under the same conditions previously described except 4 N HCl was used and the sample was digested for 5 hours at 105-110°C. Amino sugar analysis was carried out by Dr. M. Yaguchi of the National Research Council, Ottawa.

15. Peptide mapping

In order to obtain peptide maps of the small quantities of 17β-HSD multiple forms, the proteins were first acetylated with [1-^{14}C] acetic anhydride and the peptides generated by proteolytic digestion and separated by electrophoresis were detected by autoradiography.

a) Labeling procedure and enzyme digestion

Protein acetylation was carried out by the method described by Kaplan et al. (1971). Each lyophilized protein sample (approximately 400 µg) was

first dissolved in 1 ml of 8 M urea previously acidified to destroy any cyanate ions formed. The mixture was transferred to a reaction vessel and sodium borate was added as a buffer. The pH was adjusted to 9.0 with 5 N KOH. Acetonitrile (200 µl) containing 0.2 mCi of [1-^{14}C] acetic anhydride (specific activity 115 mCi/mmol) was added to the reaction mixture in two equal portions at 10-minute intervals. The reaction mixture was stirred throughout the procedure and a pH of 9.0 was maintained using a pH-stat with 5 N KOH as titrant. Unlabeled acetic anhydride (20 µl) was then added to the reaction mixture in four equal portions at 10-minute intervals. After acetylation, each sample was acidified with concentrated HCl.

Acetylation of bovine serum albumin was carried out as described above by using unlabeled acetic anhydride. One mg of the unlabeled acetylated bovine serum albumin was added to each [^{14}C] acetylated sample in order to limit procedural losses during subsequent steps. The acetylated sample was placed in a pre-soaked dialysis tubing and dialyzed against several changes of distilled water until no radioactivity was detected in the dialyzate. The sample was lyophilized and then dissolved in 1 ml of 0.5% ammonium bicarbonate. An aliquot of each sample was then digested for 6 hours at 37°C with papain in the presence of β-mercaptoethanol (50 µl). The amount of papain used for digestion was equivalent to 10% of the total protein content of the sample. The digestion was terminated by freezing and the frozen sample was lyophilized. Ammonium bicarbonate was removed from the lyophilized sample by adding 2 ml of distilled water followed by lyophilization. This last step was performed twice.

b) High-voltage electrophoresis

The proteolytic digest of each acetylated sample was dissolved in a small volume of pH 6.5 buffer (acetic acid-pyridine-water, 0.3:10:90, by volume) and spotted as an approximately 3 cm band, 20 cm from the anode on a sheet of Whatman 3 mm chromatography paper. A drop of a mixture of the dye markers xylene cyanol FF and ε-Dnp-lysine-HCl was also spotted along the origin 1 cm from the edge of the paper chromatography. The fluorescent markers dansyl arginine and dansyl sulfonic acid were also applied to the sheet along the entire length of the origin. The sheet was then buffered and the first dimension electrophoresis was carried out in the pH 6.5 buffer at 3000 V for 45 minutes. Peptides were detected by autoradiography.

Electrophoresis of each protein sample in the second dimension was carried out by cutting out the strip of radioactive peptides obtained from the first dimension electrophoresis and stitching this strip to a sheet of Whatman 1 mm paper (45 x 56 cm), 15 cm from the anode end. The dye markers were applied as described before and electrophoresis was carried out at pH 2.1 (acetic acid-formic acid-water, 4:1:45, by volume) at 3000 V until the ε-Dnp-lysine-HCl marker had migrated 15 cm from the origin.

The band of peptides designated as neutral after the second dimensional electrophoresis was cut and stitched onto a sheet of Whatman 1 mm paper 20 cm from the anode end and electrophoresis was carried out at pH 3.5 (water-acetic acid-pyridine, 19:1:0.1, by volume). For better resolution of the neutral peptides, a longer run (approximately 90 minutes) at 3000 V was necessary.

c) Autoradiography

After electrophoresis, the chromatogram was air dried. The midpoints of the dye markers plus other identification marks was made with radioactive ink and the electrophoretogram placed under an x-ray film between folders. This was stored in the dark under even pressure overnight or for a few days. After development, the radioactive markers were used to align the film with the origin electrophoretogram.

16. Amino-terminal identification

The identification of the free amino-terminal amino acid for each of the multiple forms of the 17β-HSD was carried out according to the method of Kaplan et al. (manuscript in preparation). Figure 1 shows the outline of the procedure used in amino-terminal identification.

a) Preparation of [^{3}H] Dnp-protein

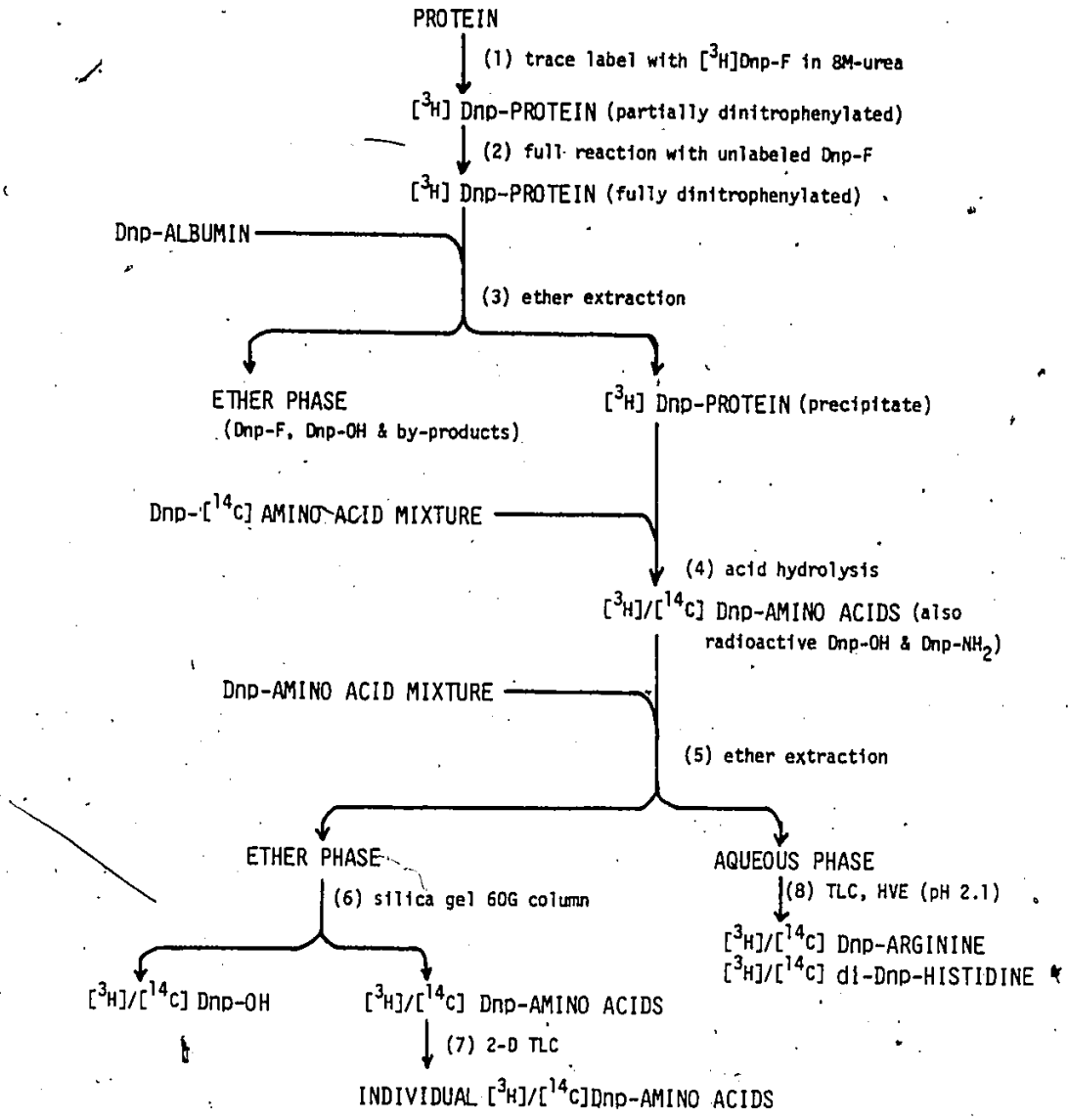
A protein sample (150-250 µg) was dialyzed against distilled water and lyophilized in a tube with screw-cap top. A 8 M urea (250 µl, pH 2.0) was added to the protein sample. After 10 minutes, sufficient solid sodium bicarbonate was added to a final concentration of 5%.

Acetonitrile (50 µl) containing 50 µCi of [^{3}H] Dnp-F (specific activity 26 Ci/mmol) was added to the sample with vigorous stirring. A clean glass bead (0.3 mm in diameter) was added and the reaction mixture was placed on a wrist-action shaker for 16 hours at 22°C in the dark. The sample was then fully reacted with unlabeled reagent by adding 25 µl of a solution of 50% Dnp-F in acetonitrile and reacting for a further 16 hours under the same conditions as described above.

FIGURE I

General Outline of the Procedure Used in Identification of the Amino-Terminus of the Multiple Forms of Soluble 17β-Hydroxysteroid Dehydrogenase of Female Rabbit Liver

In step 1, undiluted [^{3}H] Dnp-F is added in acetonitrile to the protein in 8 M urea. The partially dinitrophenylated protein is reacted with excess unlabeled Dnp-F (step 2). Dnp-albumin in 8 M urea is added as carrier and precipitates along with the labeled protein on ether extraction (step 3). A mixture of Dnp-[^{14}C] amino acids containing an equal number of disintegrations per minutes (dpm) for each amino acid is added to the protein prior to acid hydrolysis (step 4). In step 5, ether extraction separates the labeled Dnp-amino acid into ether soluble and water fractions. The ether soluble fraction is adsorbed to a silica gel 60G column and eluted with basic chloroform to remove the Dnp-OH (step 6). The labeled Dnp-amino acids from the ether phase are separated by two-dimentional thin layer chromatography (2-D TLC, step 7) and those from the aqueous phase by a combination of TLC and high voltage electrophoresis (HVE, step 8).


PROTEIN
(1) trace label with [3H]Dnp-F in 8M-urea
[3H] Dnp-PROTEIN (partially dinitrophenylated)
(2) full reaction with unlabeled Dnp-F
[3H] Dnp-PROTEIN (fully dinitrophenylated)
Dnp-ALBUMIN
(3) ether extraction
ETHER PHASE
(Dnp-F, Dnp-OH & by-products)
[3H] Dnp-PROTEIN (precipitate)
Dnp-[14C] AMINO ACID MIXTURE
(4) acid hydrolysis
[3H]/[14C] Dnp-AMINO ACIDS (also radioactive Dnp-OH & Dnp-NH2)
Dnp-AMINO ACID MIXTURE
(5) ether extraction
ETHER PHASE
AQUEOUS PHASE
(6) silica gel 60G column
(8) TLC, HVE (pH 2.1)
[3H]/[14C] Dnp-ARGININE
[3H]/[14C] di-Dnp-HISTIDINE
[3H]/[14C] Dnp-OH
[3H]/[14C] Dnp-AMINO ACIDS
(7) 2-D TLC
INDIVIDUAL [3H]/[14C]Dnp-AMINO ACIDS

b) Precipitation of [^{3}H] Dnp-protein

Dnp-albumin (2 mg) in 8 M urea (250 µl) was added to the fully reacted Dnp-protein and the final volume was adjusted to 2 ml with distilled water. The solution was ether extracted (3 x 5 ml) to remove Dnp and other ether soluble by-products. The precipitated Dnp-protein was separated by centrifugation and washed with distilled water (3 x 1 ml) to remove any residual urea. Finally the sample was washed twice with acetone and dried under a stream of nitrogen.

c) Preparation of Dnp-[^{14}C] amino acid standards

A standard [^{14}C] amino acid mixture containing aspartic acid, serine, glutamic acid, proline, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, histidine, lysine and arginine all with a specific activity of 10 mCi/mmol and methionine (specific activity 13.5 mCi/mmol) was prepared so that the individual amino acids had the identical number of counts per minute (cpm). This mixture was reacted with an excess of Dnp-F. For example, 20 µl of the [^{14}C] amino acid standard (2000 cpm) was made up to 100 µl with 5% sodium bicarbonate. Unlabeled 50% Dnp-F in acetonitrile solution (5 µl) was added and reacted as previously described.

d) Isolation of [^{3}H/^{14}C] labeled amino-termini

The Dnp-[^{14}C] amino acid standard mixture (2000 cpm of each amino acid) was added to the dried [^{3}H] Dnp-protein sample. The sample was hydrolyzed in 6 N HCl, in vacuo, at 110°C for 16 hours. To the hydrolyzate, a solution (10 µl) containing 1 µg of each Dnp-amino acid (unlabeled) was added. The hydrolyzate was transferred to a tube with a screw-cap top and extracted with ether (3 x 5 ml). The ether phase contained most of the amino acid derivatives as well as free Dnp-OH released during the acid hydrolysis while the

aqueous phase contained Dnp-arginine, ε-Dnp lysine, O-Dnp-tyrosine, imidazol-Dnp-histidine and, usually, di-Dnp-histidine.

The ether soluble Dnp-amino acids were separated from the excess Dnp-OH and Dnp-NH_2 by the method of Steven (1962). A silica gel G type I (60-200 mesh) column (0.5 x 8 cm) was equilibrated with basic chloroform (chloroform saturated with 0.067 M sodiumdibasic phosphate). The dried ether soluble Dnp-extract was dissolved in 100 µl of basic chloroform solution (if the sample was not completely soluble, 50 µl of 95% ethanol was added). The artifacts and the by-products were eluted by basic chloroform. The Dnp-amino acid derivatives were then eluted with 3 ml of 80% acetone, 0.2% acetic acid. The eluate was dried under a stream of nitrogen. To remove traces of sodiumdibasic phosphate salt, the dried sample was solubilized in 0.5 ml of 6 N HCl, ether extracted (3 x 2 ml) and dried under a stream of nitrogen.

Dnp-amino acid derivatives were solubilized in 50 µl of 95% ethanol and separated by descending two dimensional chromatography on silica gel plates (Eastman 20 x 20 cm) by the method of Kaplan et al. (1978). Chromatography in the first dimension was carried out using the upper phase of a solvent system containing toluene-pyridine-2-chloroethanol-0.8 M ammonia, (100:30:60:60, by volume). The solvent system benzene-pyridine-acetic acid, (80:20:2, by volume) was used for the second dimension. The chromatogram was subsequently air dried and Dnp-amino acids spots were visualized under U.V. light. Each Dnp-amino acid was separated from the silica gel by, first scraping the area of the plate containing Dnp-amino acid and drawing up the material under vacuum into a pasteur pipette plugged loosely with glass wool. Dnp-amino acid was eluted off the pasteur pipette with 3 ml of 80% acetone containing 0.2% acetic acid into scintillation vials.

The eluate was evaporated and the residue was counted in Aquasol-2 with an LKB Rackbeta scintillation counter, set up for double label counting of ^{3}H and ^{14}C.

The aqueous phase of the acid hydrolysis containing Dnp-arginine and mono-side chain Dnp-derivatives of histidine, lysine and tyrosine was lyophilized and then chromatographed on silica gel 60G thin layer plates (0.5 mm thickness), in a solvent system n-propanol-ammonia, (70:30 by volume). Dnp-arginine and di-Dnp-histidine were spotted as standards. The thin layer plate was developed as previously described using the Gelman descending chromatographic chamber. Dnp-arginine and di-Dnp-histidine bands were eluted off the silica gel as described above and dried down.

The procedure described above did not resolve Dnp-arginine and ε-Dnp-lysine. The following steps were carried out to separate these two amino acid derivatives. The Dnp-arginine and ε-Dnp-lysine was further reacted with unlabeled Dnp-F as previously described and the resulting di-Dnp-lysine was extracted with ether (3 x 5 ml). The aqueous phase from this ether extraction, as well as the di-Dnp-histidine samples were dried down and desalted using a Porapak Q column (Neiderwieser, 1970) and purified by electrophoresis. Prior to this electrophoresis, a small amount of standard Dnp-arginine or di-Dnp-histidine was added to the appropriate sample in order to facilitate the detection on the electrophoretogram. Each sample was subjected to high voltage electrophoresis on Whatman 3 mm paper chromatography at pH 2.1 (acetic acid-formic acid-water, 4:1:45, by volume), 1500 V for 3 hours on a Phetograph flat-plate apparatus.

The zones containing Dnp-arginine and di-Dnp-histidine were each eluted separately, with 10 mM ammonia and assayed for radioactivity as previously described.

17. Comparison of carboxyl-terminal peptides of the purified 17β-Hydroxy-steroid dehydrogenase multiple forms

The carboxyl-terminal peptides of the purified multiple forms of 17β-HSD were compared using a modification of the method described by Duggleby and Kaplan (1975).

Each protein sample (400 µg) was dissolved in 2 ml of acidified 8 M urea. Potassium chloride (100 µg) was added and the sample was stirred for 30 minutes. The pH of the sample was adjusted to pH 4.75 using 1 N KOH and 200 µl of ethanol containing 50 µCi of [1,2-^{14}C]-ethanolamine hydrochloride (specific activity 6.3 mCi/mM) was added. The coupling agent, 5 mg of 1-ethyl-3- (3-dimethyl-aminopropyl)-carbodiimide (Pierce Chemical Company, Rockford, Illinois, USA) was added and the reaction mixture was stirred for 30 minutes at room temperature. This step was repeated twice. Unlabeled ethanolamine (5 µl) and 10 mg of carbodiimide were added and the reaction mixture was stirred for 30 minutes. Finally, a last addition of carbodiimide (10 mg) was made and the reaction continued for 30 minutes. The pH for the reaction mixture was maintained at 4.75 throughout the entire procedure by using a pH stat with 1 N KOH as titrant.

Albumin (2.5 mg) was added to the reaction mixture - to precipitate the radioactive protein - and each protein sample was placed in pre-soaked dialysis tubing and dialyzed against several changes of distilled water (6 litres) until no radioactivity could be detected in the dialyzate.

Papain digestion of the derivatized proteins was performed in 0.5% (w/v) ammonium bicarbonate at 37°C for 8 hours, using an enzyme to protein ratio of 1:10 by weight. Digestion was terminated by lyophilization.

The peptide mixture for each protein sample was dissolved in 100 µl of pH 4.4 buffer, (pyridine-acetic acid-water; 6:10:120, by volume) and applied as a 3-cm wide band on Whatman 3 mm chromatography paper, together with the markers taurine and glycinamide (50 nmol/cm). The peptide mixture was subjected to electrophoresis at pH 4.4 for 2 hours at 1500 V on a Pherograph flat plate apparatus. The electrophoretogram was dried and the strip, containing the radioactive peptides and the two markers was stitched onto a full sheet of Whatman 1 mm and subjected to second dimensional electrophoresis at pH 2.1 buffer (formic acid-acetic acid-water; 1:4:45, by volume) for 45 minutes at 3000 V. The electrophoretogram was autoradiographed to locate the radioactive peptide (as described in the peptide mapping) and then dipped in a solution of cadmium-ninhydrin to locate the two markers (taurine and glycinamide). The peptides falling on the line joining the position of taurine and glycinamide are the carboxyl-terminal peptides (Duggleby and Kaplan, 1975).

CHAPTER III

PURIFICATION OF THE 17β-HYDROXYSTEROID DEHYDROGENASE FROM THE LIVER CYTOSOL OF 8-WEEK-OLD FEMALE RABBITS

The cytosol prepared from female rabbit liver homogenates was assayed for both 17α- and 17β-HSD activities in the presence of $NADP^+$ as coenzyme. Table 1 shows the enzyme activities toward the estrogen substrate, 17β-estradiol and the androgens, epitestosterone and testosterone. The enzyme activity toward these substrates decreased in the order: epitestosterone > testosterone > 17β-estradiol.

1. Sephadex G-75 (superfine) gel filtration

The 17β-HSD activity eluted as a single peak on gel filtration of the concentrated cytosol fraction on a column of Sephadex G-75 superfine (Fig. 2). A 1.5 fold purification of the 17β-HSD was achieved (Table 2) and 83% of the total activity was recovered. This fraction also contained the 17α-HSD that was present in the cytosol fraction.

2. Affinity chromatography

Further purification of the 17β-HSD activity was carried out by affinity chromatography on agarose immobilized Procion Red HE3B (Amicon Corp.) which has been shown to specifically bind $NADP^+$-dependent dehydrogenases (Watson et al., 1978 ; Inano et al., 1981). Fig. 3 shows a typical elution profile for the 17α- and 17β- HSD activities obtained by this purification step. A portion of the 17α- and 17β-HSD activities was not retained on the affinity column and was eluted with the major protein peak in the washing buffer. This activity represents approximately 8% of the total 17β-HSD activity and 5% of the total 17α-HSD activity applied to the affinity chroma-

Table 1

17-Hydroxysteroid Dehydrogenase Activity of Female Rabbit Liver Cytosol

Substrate	Enzyme Activity (μunits/mg protein)	Total Units
Testosterone	560	5.30
17β-Estradiol	60	0.57
Epitestosterone	685	6.84

The cytosol of rabbit liver was assayed for 17-HSD activities as described in General Methods. For the assay with testosterone and epitestosterone the cytosol was diluted 10 fold and 3 μl was used. With the substrate 17β-estradiol 2 μl of the cytosol was assayed. The steroid concentration was 1 μM and the assay was carried out in 67 mM glycine-NaOH buffer pH 9.5.

Figure 2

Sephadex G-75 (superfine) Column Chromatography of the Soluble 17β-Hydroxysteroid Dehydrogenase of Female Rabbit Liver.

The concentrated cytosol fraction (50 ml) was applied to a column of Sephadex G-75 (superfine), 5 x 90 cm. The equilibrating and eluting buffer was 10 mM Tris-HCl-0.5 mM DTT, pH 8.0. 10 ml Fractions were collected and 2 µl aliquots were assayed for 17β-HSD activity with the substrate testosterone (•). Protein was measured by absorption at 280 nm (o)

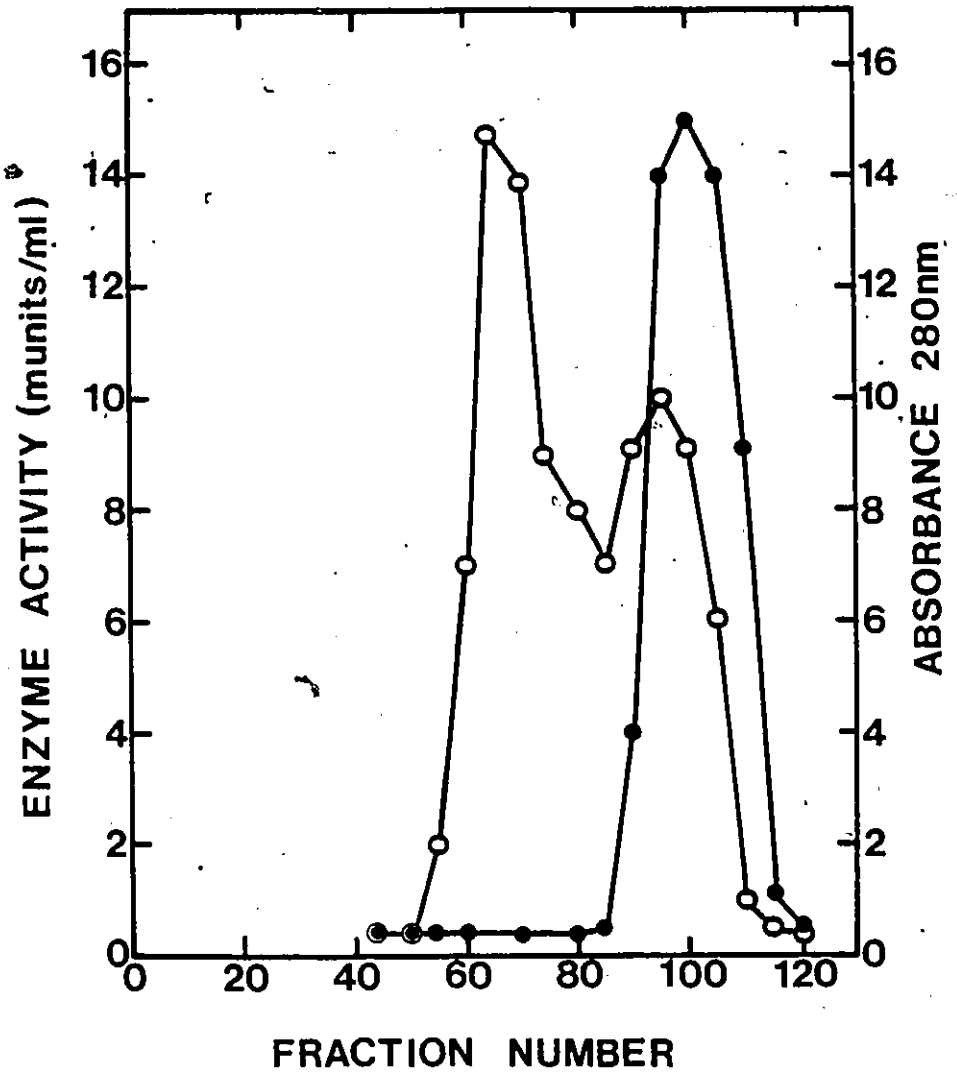
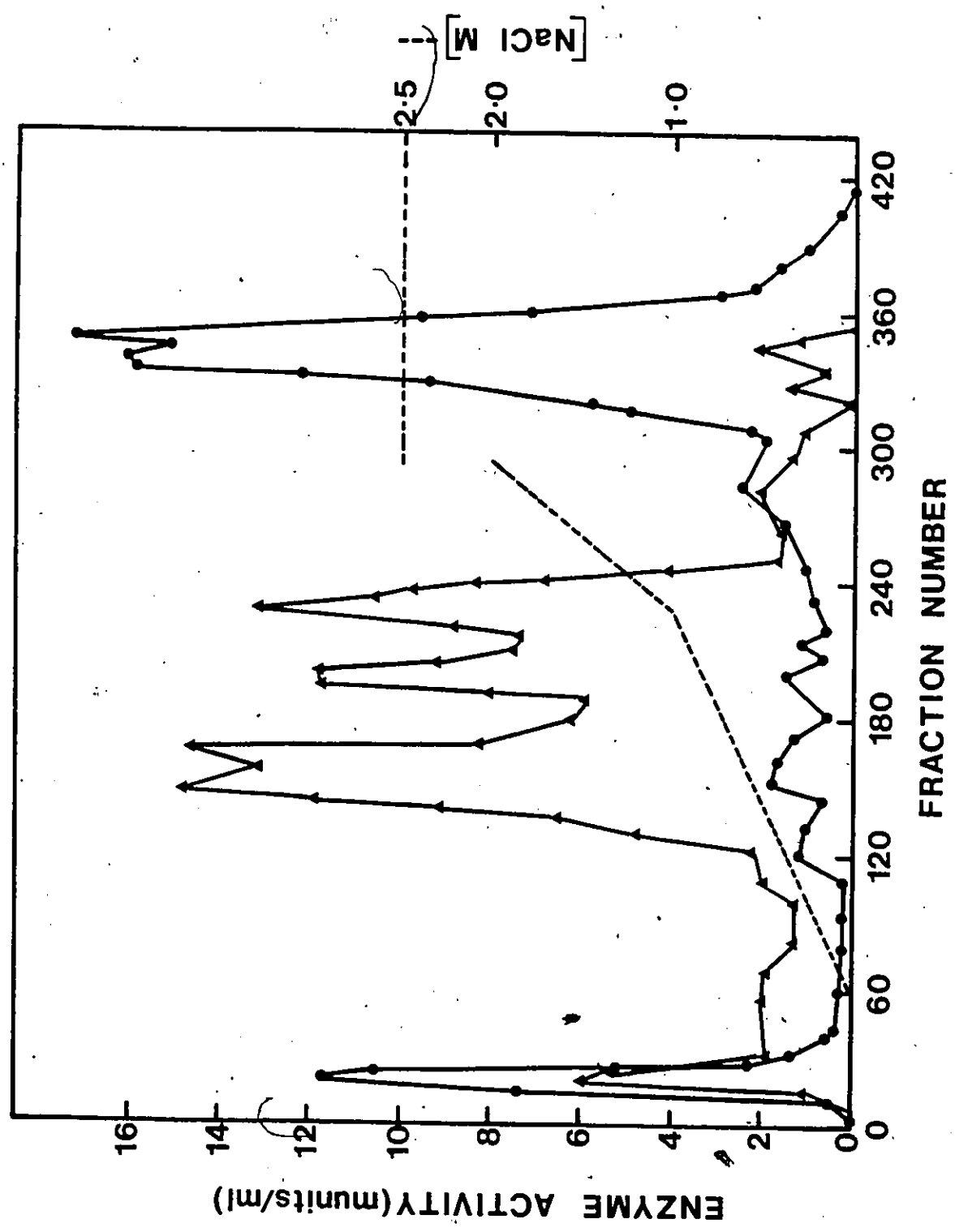

ENZYME ACTIVITY (munits/ml)
ABSORBANCE 280nm
FRACTION NUMBER
0
2
4
6
8
10
12
14
16
0
20
40
60
80
100
120

Figure 3

Affinity Chromatography of the Soluble 17β-Hydroxysteroid Dehydrogenase of Female Rabbit Liver.

The pooled eluate from the gel filtration step was concentrated to 30 ml by ultrafiltration and applied at a flow rate 6-8 ml/hour to a column of agarose immobilized Procion Red HE3B (1.5 x 40 cm) previously equilibrated with 10 mM Tris-HCl-0.5 mM DTT, pH 8.0. The column was eluted first with 10 mM Tris buffer, pH 8.0, containing 0.5 mM DTT, 1 mM EDTA and 5 mM $MgCl_2$ (150 ml), followed by a NaCl linear gradient from 0-1 M in the same buffer (600 ml) and a second NaCl linear gradient from 1-2 M in the same buffer without EDTA (200 ml). Finally the column was eluted with 10 mM Tris buffer, pH 8.0, containing 2.5 M NaCl, 0.5 mM $MgCl_2$. The flow rate was 15-20 ml/hour and 3.6 ml fractions were collected. Aliquots (2 µl) of the column fractions were assayed for 17α- (▲) and 17β-HSD activities (●) with epitestosterone and testosterone as substrates respectively.


ENZYME ACTIVITY (munits/ml)
0
2
4
6
8
10
12
14
16
0
60
120
180
240
300
360
420
FRACTION NUMBER
[NaCl M]
2·5
2·0
1·0

tography column. The majority of the 17α-HSD activity was eluted with a linear NaCl gradient from 0-1 M. Trace amounts of 17β-HSD were eluted with this gradient. A second linear NaCl gradient from 1-2 M eluted the remaining 17α-HSD enzyme activity. The 17β-HSD activity was then eluted with 2.5 M NaCl-10 mM $MgCl_2$.

Table 2 summarizes the recovery of 17α- and 17β-HSD activities during the first two purification steps. Affinity chromatography resulted in an almost complete separation of the 17α- and 17β-HSD activities, 75% of the 17α-activity applied to the column was eluted with the first salt gradient, whereas approximately 80% of the 17β-activity was eluted with 2.5 M NaCl-10 mM $MgCl_2$. Affinity chromatography resulted in a 5.4- and 95-fold increase in the specific activity of the 17α- and 17β-HSD activities respectively.

As shown in Fig. 3, a portion of the 17β-HSD activity was not retained on the affinity chromatography column and was eluted with the major protein peak in the washing buffer. The results shown in Table 3 indicate that this fraction contains the NAD^+-dependent 17β-HSD activity present in the cytosol and gel filtration fractions. NAD^+-dependent 17β-HSD activity was absent in the 2.5 M NaCl-10 mM $MgCl_2$ fraction of the affinity chromatography column which contained only $NADP^+$-dependent 17β-HSD activity.

3. Chromatofocusing chromatography

17β-HSD activity eluted from the affinity chromatography column with 2.5 M NaCl was stabilized by the addition of glycerol (20% final concentration by volume) prior to concentration by ultrafiltration and desalting. It was found that in the absence of glycerol considerable 17β-HSD activity was lost during the desalting procedure. In the presence of glycerol, a small amount of protein precipitate formed but essentially 100% of 17β-HSD activity was retained.

Table 2

Recovery of the Soluble 17-Hydroxysteroid Dehydrogenase Activities of Female Rabbit Liver During Purification

Step	Total Protein (gm)	17α-HSD Specific Activity (munits/mg protein)	17α-HSD Total Units	17β-HSD Specific Activity (munits/mg protein)	17β-HSD Total Units
Cytosol (105,000 x g)	9.98	0.68	6.84	0.56	5.30
Sephadex G-75	5.36	1.07	5.72	0.80	4.30
Affinity Chromatography					
Washing buffer	1.72	0.31	0.78	0.19	0.41
0.0 - 1.0 M NaCl	1.16	3.25	3.77	0.19	0.22
1.0 - 2.0 M NaCl	0.10	4.10	0.40	1.58	0.15
2.5 M NaCl- 10 mM $MgCl_2$	0.06	1.50	0.08	53.0	3.0

Table 3

Separation of the Soluble NAD^+- and $NADP^+$-Dependent 17β-Hydroxysteroid Dehydrogenase Activities of Female Rabbit Liver

Purification Step	Specific Activity (m units/mg protein)	
	$NADP^+$	NAD^+
Cytosol (105,000 x g supernatant)	0.56	0.23
Sephadex G-75	0.80	0.31
Affinity Chromatography		
- washing buffer	0.20	2.82
- 17β-HSD Fraction	53.0	0.0

17β-HSD activity was measured using testosterone as substrate. From the cytosol fraction, 0.3 µl and 4 µl were assayed in the presence of $NADP^+$ and NAD^+ respectively. The enzyme aliquots used from the other fractions were adjusted accordingly. The concentrations of both substrate and coenzyme were 1 µM and 167 µM respectively. The assay was carried out in 67 mM glycine-NaOH buffer pH 9.5.

Chromatofocusing chromatography of the 17β-HSD fraction gave the enzyme activity pattern shown in Fig. 4. Three enzyme activities were resolved by this procedure and were designated as enzymes I, II and III with isoelectric points of 5.60, 5.45 and 5.35 respectively. Each of the enzyme peaks was pooled as marked in Fig. 4 and each pooled fraction was concentrated by ultrafiltration and subjected to polyacrylamide disc gel electrophoresis. When the gels were stained for protein considerable cross contamination among the enzyme peaks was evident. Therefore each peak was again subjected to chromatofocusing under the same conditions and the results are shown in Fig. 5. The fractions of each peak were pooled and concentrated by ultrafiltration.

The purity of each form of the 17β-HSD was established by polyacrylamide disc gel electrophoresis (Davis, 1964) and SDS-polyacrylamide gel electrophoresis (Weber et al., 1972). The electrophoretic profiles of isolated enzymes I, II and III are presented in Fig. 6. By these two methods the enzymes were shown to be homogeneous, single bands being observed when the polyacrylamide gels were stained for protein. The molecular weights were determined using SDS-polyacrylamide gel electrophoresis by comparison of the mobility of the purified enzymes with proteins of known molecular weight. Molecular weights of 37,000, 40,000 and 39,000 were estimated for the forms, I, II and III respectively (Fig. 7).

The recovery and specific activity of the soluble 17β-HSD of female rabbit liver throughout the purification procedure is summarized in Table 4. The combined activities of the forms I, II and III was approximately 11% of the total 17β-HSD activity present in the cytosol.

Figure 4

Chromatofocusing Chromatography of the Soluble 17β-Hydroxysteroid Dehydrogenase of Female Rabbit Liver.

The desalted 17β-HSD activity fraction of the affinity chromatography in 20% glycerol was concentrated by ultrafiltration and then applied to a chromatofocusing chromatography column (1 x 40 cm) packed with PBE 94 Polybuffer Exchanger previously equilibrated with 10 mM imidazole-HCl, 0.5 mM DTT, pH 6.15. Protein was eluted with Polybuffer 74, pH 5.10 (diluted 8 times with deaerated distilled water) and 1.5 ml fractions were collected. An aliquot (2 µl) of every third fraction was assayed for 17β-HSD activity toward testosterone. The pH of every tenth tube was measured.

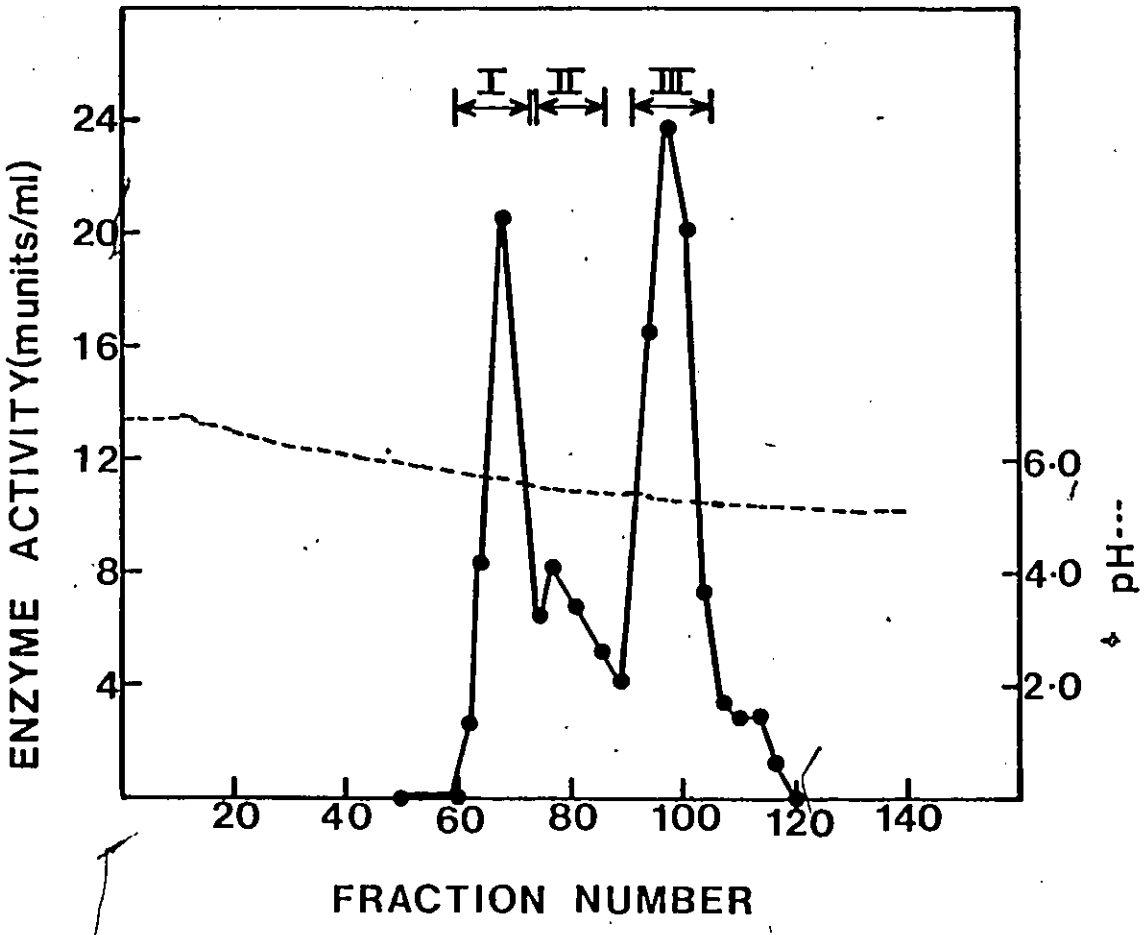

I
II
III
24
20
16
12
8
4
ENZYME ACTIVITY(munits/ml)
6·0
4·0
2·0
pH
20
40
60
80
100
120
140
FRACTION NUMBER

Figure 5

Re-Chromatofocusing of Peaks I, II and III of the 17β-Hydroxysteroid Dehydrogenase of Female Rabbit Liver Cytosol.

Each separated peak was concentrated by ultrafiltration and chromatofocused under the conditions described in Fig. 4 except that a smaller column (1 x 10 cm) was employed.

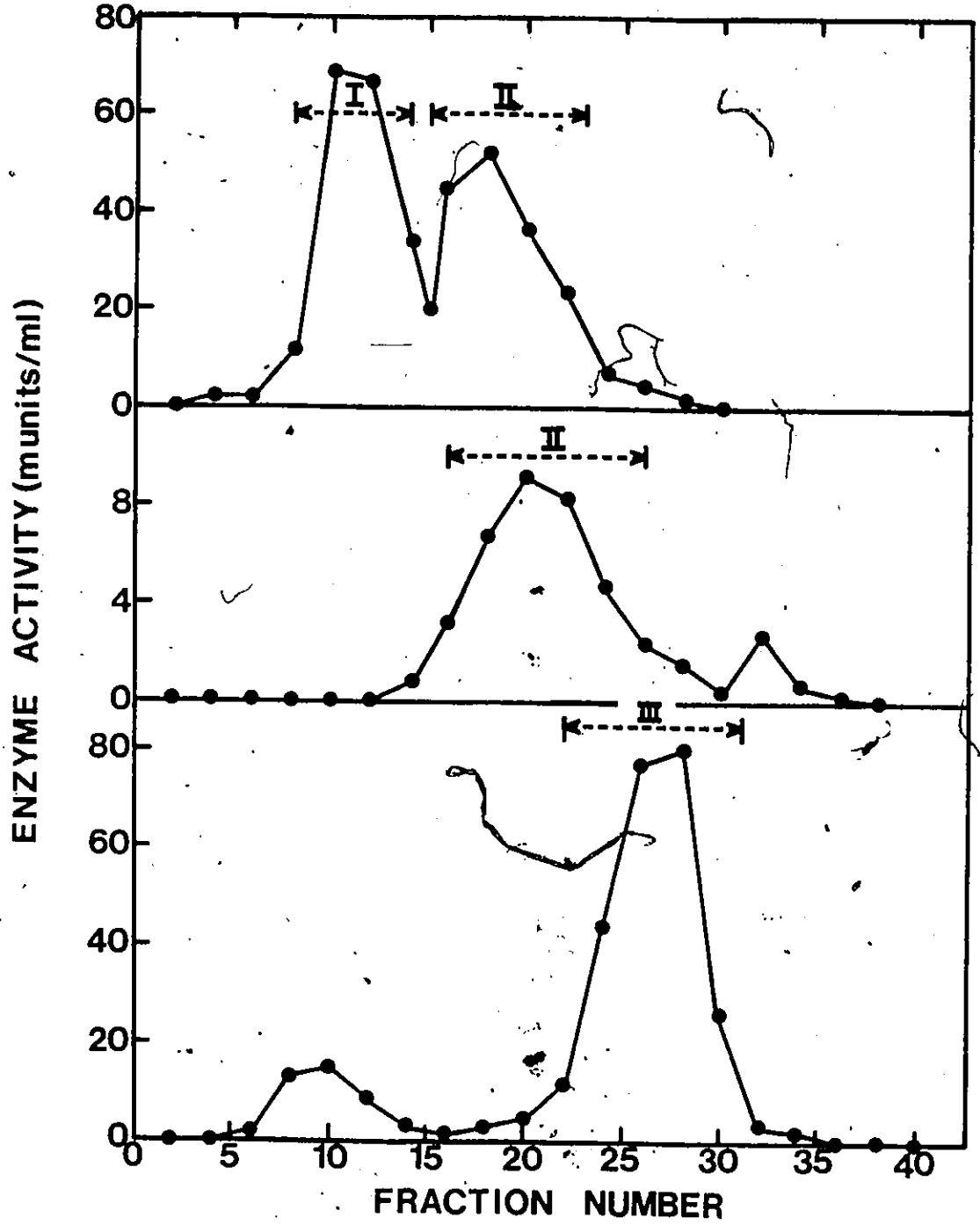

ENZYME ACTIVITY (munits/ml)
80
60
40
20
0
I
II
8
4
III
0
5
10
15
20
25
30
35
40
FRACTION NUMBER

Figure 6

Polyacrylamide Gel Electrophoresis of the Purified 17β-Hydroxysteroid Dehydrogenase.

Electrophoresis and protein staining were carried out as described in General Methods (p. 39) in 10.5% acrylamide gels. 10-20 µg of protein were applied to each gel. Gels a, b, and c represent enzymes I, II and III respectively.


-
+
a
b
c

Figure 7

SDS-Polyacrylamide Gel Electrophoresis of the Purified 17β- Hydroxysteroid Dehydrogenase.

SDS-polyacrylamide gel electrophoresis was carried out as described in General Methods (p.41). 5-10 µg protein was applied to each gel. Gels a, b and c represent enzymes I, II and III respectively.


−
+
a
b
c

Table 4

Purification of Female Rabbit Liver Soluble

17β-Hydroxysteroid Dehydrogenase

Step	Protein (mg)	Total Enzyme Activity (Units)	Specific Activity (μ units.mg/protein)	fold purification
Cytosol (105,000 x g supernatant)	9980	5.3	560	
Sephadex G-75 gel filtration	5360	4.3	800	1.40
Affinity Chromatography	57.0	3.0	53,000	95.0
Chromatofocusing Chromatography				
Enzyme I	3.1	0.30	386,000	690
Enzyme II	1.3	0.10	121,000	216
Enzyme III	6.3	0.20	333,000	595

4. Isoelectric focusing polyacrylamide gel electrophoresis of the multiple forms of 17β-hydroxysteroid dehydrogenase of female rabbit liver.

Since it is possible that the forms of the female rabbit liver 17β-HSD observed on chromatofocusing could have been formed during the purification procedure, the enzyme fraction obtained at each step of purification was monitored for the presence of multiple forms of the 17β-HSD. Fig. 8 shows densitometric scans of the polyacrylamide gels of the enzyme fractions at each step in the purification procedure stained for 17β-enzyme activity with the substrate testosterone. At least four bands of enzyme activity are present in the initial cytosol fraction (Fig. 8b). Three of these enzyme activities have very similar pI's while one form is considerably more acidic. The pI's of the 4 forms of the 17β-HSD were determined to be 6.30, 6.20, 6.05 and 5.05.

A colourless band with a pI close to that of the acidic form of the 17β-HSD was observed. This band appears as a trough in the densitometric scan (Figs 8b and d) and is likely due to a cyanide-insensitive superoxide dismutase (SOD) activity (Weisiger and Fridorish, 1973). The action of SOD activity has been shown to inhibit the reduction of nitroblue tetrazolium (NBT), thus preventing the formation of the purplish-blue diformazan and causing instead the formation of an achromatic band (Beauchamps and Fridorish, 1971).

All forms of the 17β-HSD were present after gel filtration (Fig. 8c). The acidic form was not retained on the affinity chromatography column and was present in the washing buffer (Fig. 8d). The cyanide-insensitive SOD was also present in the washing buffer and likely produced the minor peaks that appeared on either side of the through in the densitometric scan.

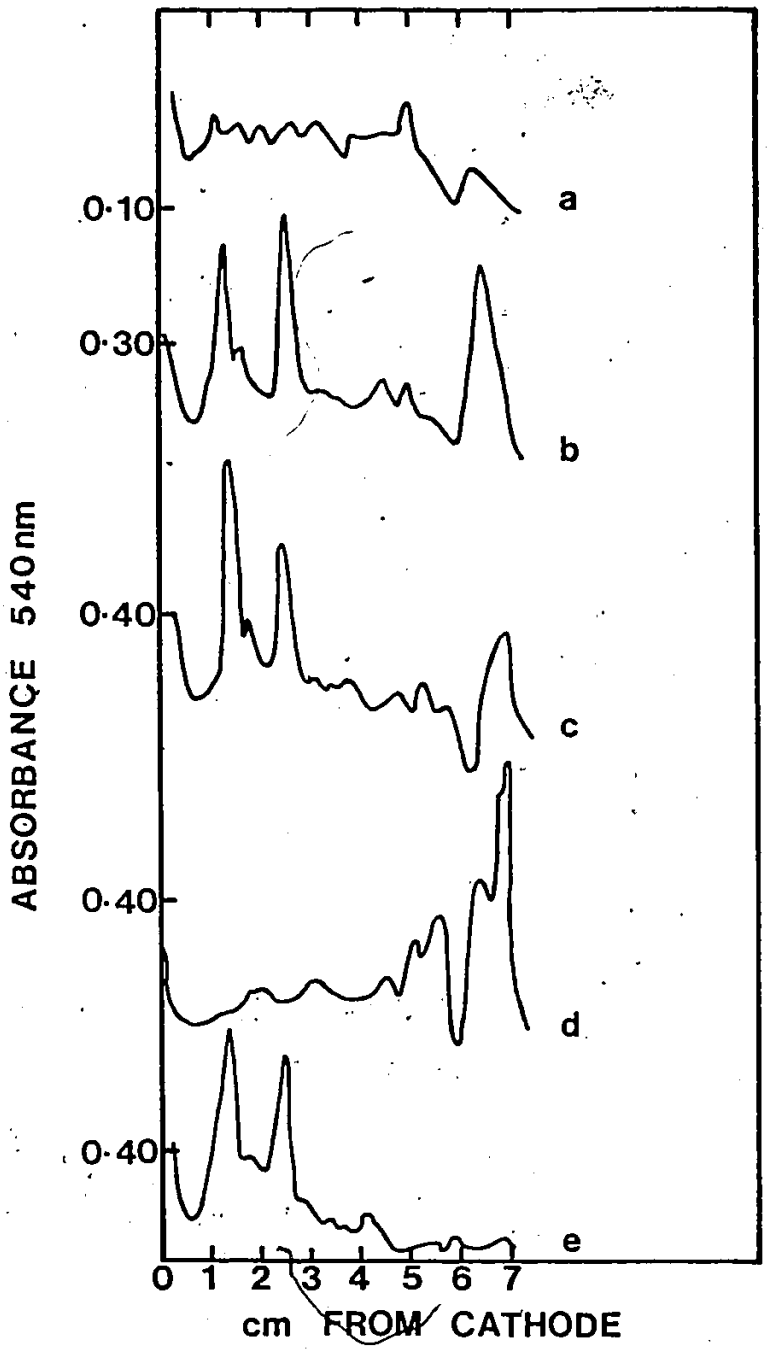
Finally, isoelectric focusing polyacrylamide gel electrophoresis of the fraction retained on the affinity chromatography column shows the three less acidic forms of the 17β-HSD (Fig. 8e).

The results in Fig. 8 show that the multiple forms of 17β-HSD present in cytosol fraction are recovered during purification and no additional or artifactural forms of the enzyme are produced by this purification procedure.

Figure 8

Isoelectric Focusing Polyacrylamide Gel Electrophoresis of the Soluble 17β-Hydroxysteroid Dehydrogenase of Female Rabbit Liver During the Steps of Purification.

Electrophoresis and substrate staining were carried out as described in General Methods (p.40). Each enzyme sample was dialyzed against 10 mM Tris-HCl-0.5 mM DTT, pH 8.0, to remove any salt and approximately 2000 μunits of enzyme activity toward testosterone was applied to each gel. Focusing was carried out initially at 400 V for 30 minutes and then at 500 V for 3.5 hours. Each gel was scanned from the top (cathode end) to the bottom (anode end). Densitometric scans are shown for the 17β-HSD activities of the cytosol (b), gel filtration fraction (c), affinity column wash fraction (d) and the affinity column 2.5 M NaCl fraction (e). Gel (a) is a control gel of the cytosol stained in the absence of the substrate testosterone.


ABSORBANCE 540nm
0·10
0·30
0·40
0·40
0·40
a
b
c
d
e
0 1 2 3 4 5 6 7
cm FROM CATHODE

DISCUSSION

The results of the present studies clearly demonstrate that the $NADP^+$-dependent soluble 17α- and 17β-HSDs of female rabbit liver are distinct enzymes. This confirms the earlier work of Hasnain and Williamson (1974) and contrasts the work of Ball and Breuer (1970). These investigators had concluded that a single enzyme was responsible for both 17α- and 17β-HSD activities in rabbit liver cytosol. Distinct 17α- and 17β-HSD activities have also been demonstrated in chicken liver (Renwick and Engel, 1967).

The specific binding properties of the affinity gel Procion Red HE3B were used in the present studies to purify the 17β-HSD from the rabbit liver cytosol. This affinity gel has a higher affinity for $NADP^+$-dependent dehydrogenases than for NAD^+-dependent dehydrogenases (Watson et al., 1978). Chromatography of the relatively crude 17-HSD on this gel both separated and partially purified the 17α- and 17β-HSD activities (Fig. 2). The high retention of 17β-HSD on the affinity column is consistent with high specificity of this activity for $NADP^+$ (Table 3).

Three forms of the 17β-HSD of female rabbit liver cytosol were resolved by chromatofocusing, a preparative protein separation method that separates the proteins according to their isoelectric points (Sluyterman, 1982). Therefore the multiple forms of 17β-HSD of the female rabbit liver cytosol are charge isomers. The molecular weights of these multiple forms are all within ± 3% of the average molecular weight of 38,700, evidence against their being size isomers. The majority of the multiple forms of guinea pig kidney (Liu and Kochakian, 1972a and b; Shen and Kochakian, 1978) and guinea pig liver (Kobayashi and Kochakian, 1978) and the multiple forms of porcine testicular 17β-HSD (Inano et al., 1981) have also been shown to differ in

the net electrical charge rather than molecular size. The molecular weights of the guinea pig and porcine testis enzymes, estimated as 31,000 to 34,000 by disc gel electrophoresis, are slightly lower than the average value of 38,700 found in this study for three forms of the 17β-HSD of female rabbit liver cytosol. The 17β-HSDs of female rabbit liver, guinea pig liver and kidney and porcine testis do not appear to have subunit structure, eliminating the possibility that the multiple forms might arise from a combination of non identical subunits. However, human placental 17β-estradiol dehydrogenase has been shown to have a dimeric subunit structure, the subunit molecular weight being approximately 34,000 (Burns et al., 1971 and 1972). Moreover, a tetrameric composition has been reported for the 3(17)β-HSD of Pseudomonas testosteroni (Schultz et al., 1977a,b). The native enzyme is believed to be formed by a combination of two subunits having similar molecular weights but different amino acid compositions. The active forms of the 3(17)β-HSD of Pseudomonas testosteroni, which exhibit both 3β- and 17β-HSD activities are the two subunits within the tetrameric structure. In contrast, the active form of the human placental 17β-estradiol dehydrogenase is the dimer (Pons et al., 1977).

The molecular heterogeneity of the soluble 17β-HSD of rabbit liver could result from multiple genes, postsynthetic modification of the protein or conformation changes (Kaplan, 1968; Epstein and Schechter, 1968). It is unlikely that the multiple forms of 17β-HSD of female rabbit liver cytosol are artifacts produced during the purification procedure. The enzyme forms resolved by chromatofocusing are also present in the initial cytosol and the same relative amounts of the different enzyme forms are obtained from different rabbit liver preparations. Heterogeneity of the 17β-HSD has been

described for this enzyme in a number of species. Thaler-Dao et al. (1972) separated the 17β-HSD of rabbit liver into several enzyme activities by DEAE-cellulose chromatography and polyacrylamide gel electrophoresis. Multiple forms of the 17β-HSD of guinea pig kidney have been observed by Liu and Kochakian (1972a and b). Moreover, Engel and Groman (1974) have reported that multiple forms of the 17β-estradiol dehydrogenase of human placenta are obtained by isoelectric focusing of the native enzyme on polyacrylamide gel electrophoresis. Multiple forms of 17β-HSD have been also observed in adult guinea pig liver (Kobayashi and Kochakian, 1978), in chicken liver (Renwick et al., 1981) and in porcine testis (Inano et al., 1980). More recently, two forms of 17β-HSD have been found in human skin (Hodgins et al., 1982) and in human testis (Leinonen, 1982).

The present studies have also shown that 17β-HSD activity in addition to the three forms separated by chromatofocusing is present in female rabbit liver cytosol. A portion of the total 17β-HSD activity present in the cytosol and after gel filtration is not retained on affinity chromatography (Fig. 3 and Table 3). This activity differs from that retained on the affinity column in that it is NAD^+-dependent (Table 3) and has a more acidic PI (Fig. 8d). Disc gel electrophoresis also indicates that there may be more than one of these acidic forms. Affinity chromatography also showed a 17α-activity associated with this acidic 17β-HSD. Whether this activity is due to distinct enzymes in this fraction must await further purification studies. Lau et al. (1982a) have recently shown that rabbit kidney also has an acidic HSD with activity toward 3α-hydroxysteroid substrates. 17β-HSD activity was also associated with this fraction.

CHAPTER IV

PROPERTIES OF THE MULTIPLE FORMS OF THE 17β-HYDROXYSTEROID DEHYDROGENASE OF FEMALE RABBIT LIVER CYTOSOL

The properties of the multiple forms of the soluble 17β-HSD of female rabbit liver were compared in order to establish whether the individual forms have a specific role in hepatic steroid metabolism in vivo. Initial studies were carried out to establish the optimum conditions for the measurement of enzyme activity.

1. Enzyme activity as a function of pH

The activities of the purified forms of the 17β-HSD of the female rabbit liver cytosol as a function of pH are shown in Figs. 9 and 10. Enzyme activities were measured in the oxidative direction using testosterone and etiocholanolone as substrates. As shown in Figs. 9 and 10 there are no significant differences in the pH profiles among the different enzyme forms. Each enzyme exhibits a relatively broad pH optimum at approximately pH 9.5 for both 3α- and 17β-HSD activities. The pH profile of enzyme form III in the reductive direction with the substrate androstenedione is shown in Fig. 11. A sharp pH optimum at 7.6 is observed in Tris-maleate buffer. With phosphate buffer the pH optimum is slightly lower, at pH 7.3 and the maximal enzyme activity with this buffer is only one-half that observed in Tris-maleate buffer.

2. Enzyme activity as a function of time

In order to establish the appropriate conditions for kinetic analysis enzyme activity was measured as a function of time. With all enzyme forms 17β-HSD activity toward the substrate testosterone, at the lowest concentra-

Figure 9

17β-Hydroxysteroid Dehydrogenase Activity as a Function of pH.

The enzyme forms I, II and III of the 17β-HSD of rabbit liver cytosol were assayed over the pH range 5.2 - 10.6. Each point shown is the average from triplicate incubations. In each incubation 0.2 - 0.4 µg of enzyme was assayed with the substrate testosterone in the following buffers; 0.1 M Tris-maleate (o), 0.01 M Tris-HCl (▲) and 0.1 M glycine-NaOH (●).

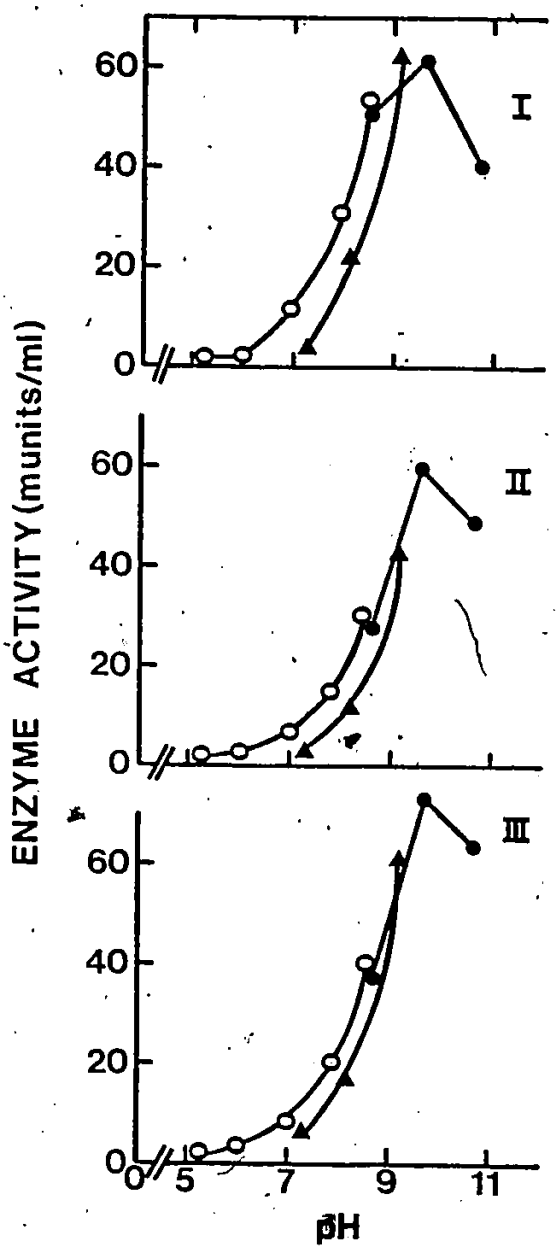

ENZYME ACTIVITY (munits/ml)
60
40
20
0
I
II
III
0
5
7
9
11
pH

Figure 10

3α-Hydroxysteroid Dehydrogenase Activity as a Function of pH

3α-HSD activity of enzyme form III toward the substrate etiocholanolone was assayed over the pH range 5.0 - 10.6 using 0.1 M Tris-maleate buffer (o) and 0.1 M glycine-NaOH buffer (•). Each incubation contained 0.8 μg of enzyme and each point shown is the average from triplicate incubations.

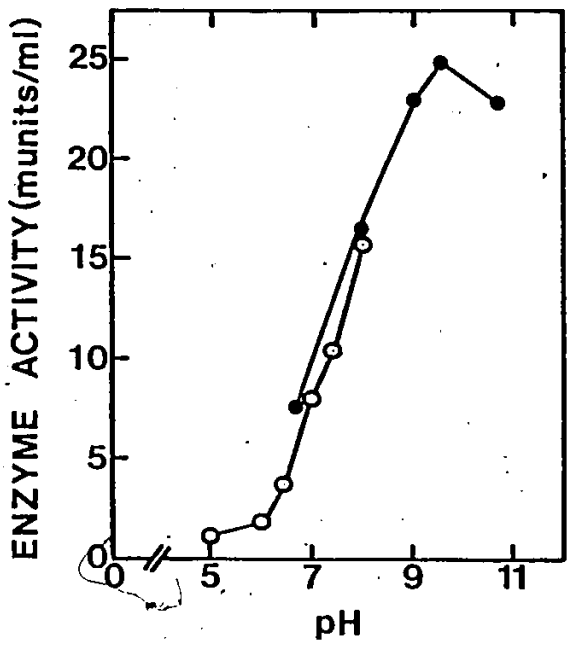
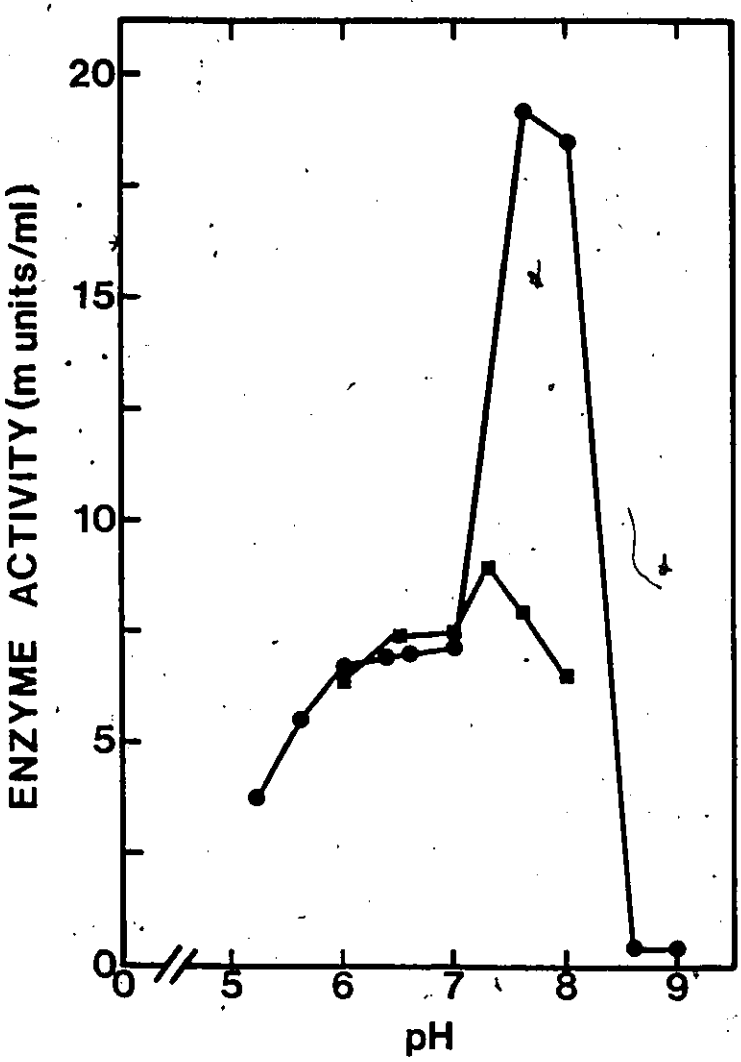

ENZYME ACTIVITY (munits/ml)
25
20
15
10
5
0
0
5
7
9
11
pH

Figure 11

The Reduction of Androstenedione as a Function of pH

17β-HSD activity in the reductive reaction with the substrate androstenedione was measured for enzyme form III over the pH range 5.2 to 9.0 with Tris-maleate (0.1 M) buffer (●) and phosphate (0.1 M) buffer (■). The assay mixture consisted of 0.5 µmol of NADPH, 2 ml of buffer at the indicated pH, 3 nmol of radioactive androstenedione and 0.8 µg of enzyme III. The volume was made up to 3.0 ml by the addition of 0.15 M KCl. The data are from triplicate incubations.


ENZYME ACTIVITY (m units/ml)
20
15
10
5
0
0
5
6
7
8
9
pH

tion of this steroid employed in the kinetic studies, is linear up to 30 minutes (Fig. 12). Similar results were obtained with the other two forms of the 17β-HSD (data not shown).

3. Enzyme activity as a function of $NADP^+$ concentration

17β-HSD activity of each of the enzyme forms was measured as a function of $NADP^+$ concentration using testosterone as the steroid substrate and the data is shown in Fig. 13. Maximum 17β-HSD activity was observed at a $NADP^+$ concentration of 100 μM with all enzyme forms. An $NADP^+$ concentration of 167 μM was routinely employed in the 17β-HSD assays.

4. Substrate specificity of the purified 17β-hydroxysteroid dehydrogenase

The activities of the purified forms of the 17β-HSD of female rabbit liver cytosol toward a number of steroid substrates were examined and the results are summarized in Table 5. With all enzyme forms the activity toward androgens is higher than toward estrogens in both the oxidative (testosterone vs. 17β-estradiol) and reductive (androstenedione vs. estrone) reactions. This is particularly evident in the oxidative reaction where activity toward testosterone is at least 10-fold higher than toward 17β-estradiol with all enzyme forms. The data in Table 5 also show that the pure forms of the 17β-HSD have 3α-HSD activity. This activity is relatively specific for androgen substrates of the 5β-androstane series, the activity toward 5β-substrate, etiocholanolone being at least 10 times higher than toward the 5α-substrate androsterone. With all enzyme forms, 3α-activity is lower than the 17β-activity. The enzyme forms also have 3β-HSD activity toward the substrate dehydroepiandrosterone. Although with enzyme I and III the 3β-activity is only 5% and 3% respectively of their 3α-activity toward etiocholanolone, with enzyme II the 3β-activity is 40% of

Figure 12

17β-Hydroxysteroid Dehydrogenase as a Function of Time

The activity of enzyme form III toward the substrate testosterone was measured as described in the General Methods (p.34) after 10, 20, 30 and 40 minutes of incubation. Each point shown is the average from triplicate incubations. Each incubation contained 0.4 μg of enzyme.

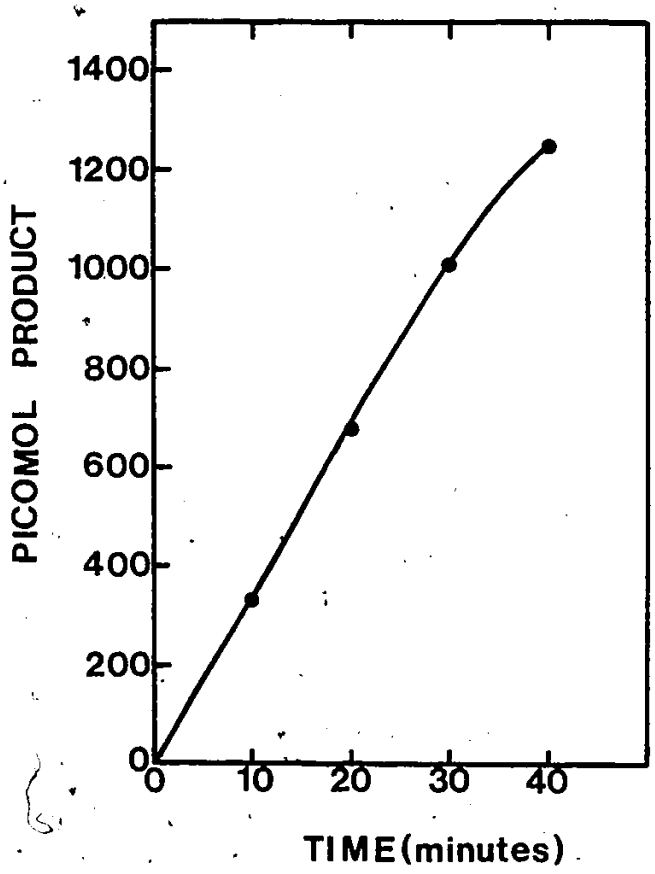

PICOMOL PRODUCT
1400
1200
1000
800
600
400
200
0
0
10
20
30
40
TIME(minutes)

Figure 13

Effect on $NADP^+$ Concentration on 17β-Hydroxysteroid Dehydrogenase Activity.

The multiple forms of 17β-HSD of rabbit liver cytosol were incubated with testosterone and increasing concentrations of $NADP^+$. Enzyme I, (●); enzyme II (▲) and enzyme III (■). Incubation conditions were as described in the General Methods (p.34).

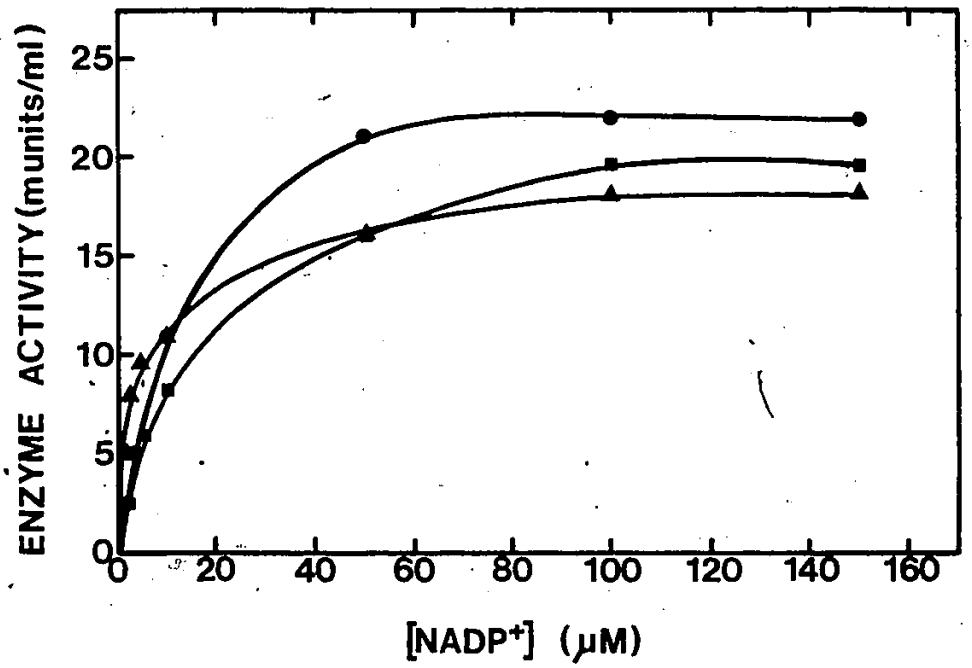

ENZYME ACTIVITY (munits/ml)
25
20
15
10
5
0
0
20
40
60
80
100
120
140
160
[NADP+] (μM)

Table 5

Substrate Specificities of the Multiple Forms of the Soluble 17β-Hydroxysteroid Dehydrogenase of Female Rabbit Liver.

Substrate	Specific Activity (munits/mg protein) Enzyme I	II	III
Testosterone	386	121	333
Androstenedione	296	100	75.7
17β-Estradiol	15.0	11.0	7.0
Estrone	50.0	18.3	35.0
Etiocholanolone	101	48.0	220
Androsterone	12.0	2.0	33.0
Dehydroepiandrosterone	5.0	19.0	6.0
Epitestosterone	9.0	0.7	1.0
17α-Estradiol	0.0	0.0	0.0

Incubations were carried out as described in the general Methods. 0.1 -0.2 μg of enzyme (final concentration 0.9 - 1.8 nM) was assayed with testosterone. The amount of enzyme used for other substrates was such that the extent of substrate conversion did not exceed 30%. The steroid concentration was 1 μM. Assays were carried out in 67 mM glycine-NaOH buffer pH 9.5 in the presence of 167 μM $NADP^+$ for the oxidation reactions and in 67 mM Tris-maleate-NaOH pH 7.6 in the presence of 167 μM NADPH for the reduction reactions

the 3α-activity. Enzyme I is the only form with significant 17α-activity, this activity is approximately 2% of the 17β-HSD activity toward testosterone.

The data in Table 5 was used to determine the relative rates of oxidation/reduction of various substrates by the purified enzymes. For each enzyme the rate of oxidation of 17β-estradiol or reduction of estrone was considered to be 1.00 and the rate of oxidation or reduction of the other substrates was calculated relative to the activity with the estrogen substrates. This gives a substrate specificity profile for each enzyme and allows a comparison of substrate specificities among the purified forms of the 17β-HSD. As shown in Table 6, no two enzyme forms exhibit the same substrate specificity profile. For example, when 17β-estradiol is modified by the attachment of glucuronic acid to the phenolic hydroxyl group the oxidation of this derivative by enzyme forms I and II is only one fifth of that observed with 17β-estradiol while enzyme form III exhibits approximately equal activity toward both substrates. Table 6 also confirms that all forms of the 17β-HSD have much higher activity toward androgen substrates than toward estrogens, in particular the activity of enzyme form III toward testosterone is approximately 50 times greater than toward 17β-estradiol. Enzyme form III also exhibits the highest relative 3α-HSD activity toward the 5β-androstane, etiocholanolone.

The oxidation and reduction at C_{17} of both the androgen and estrogen substrates are compared in Table 7. Oxidation of testosterone and 17β-estradiol is expressed relative to the reduction of androstenedione and estrone respectively. With the androgen substrates the relative rate of oxidation exceeds that of reduction with all enzyme forms. In particular with enzyme form III the rate is approximately 4 times higher in the oxidative direction. In contrast, with the estrogen substrates the rate in the reduction direction is greater with all enzyme forms, again in particular enzyme form III

Table 6

Rate of Oxidation/Reduction of Substrates as Compared to Oxidation/ Reduction of Estrogens by the Soluble Multiple Forms of Female Rabbit Liver 17β-Hydroxysteroid Dehydrogenase

Substrate	Enzyme		
	I	II	III
Oxidation			
17β-Estradiol	1.00	1.00	1.00
17β-Estradiol-3-glucuronide	0.20	0.20	1.10
Testosterone	25.7	11.0	47.6
Androsterone	0.80	0.18	4.71
Etiocholanolone	6.73	4.36	31.4
Dehydroepiandrosterone	0.33	1.73	0.85
Reduction			
Estrone	1.00	1.00	1.00
Androstenedione	5.92	5.46	2.16

Table 7

Comparison of the Oxidation and Reduction of Androgens and Estrogens at C-17 by the Multiple Forms of the Soluble 17β-Hydroxysteroid Dehydrogenase of Female Rabbit Liver

Enzyme	Substrates	
	Testosterone:Androstenedione	17β-Estradiol:Estrone
I	1.3:1	0.3:1
II	1.2:1	0.6:1
III	4.4:1	0.2:1

Data is expressed as the ratio oxidation:reduction for the substrate pairs testosterone - androstenedione and 17β-estradiol - estrone.

shows 5 times higher activity in the reductive reaction than in the oxidative reaction. None of the enzyme forms catalyzed the reduction of the carbonyl group at C-3 of 5α-dihydrotestosterone.

5. Kinetic studies of the rabbit liver 3α,17β-hydroxysteroid dehydrogenase

a) Effect of substrate concentration

3α- and 17β-HSD activities of each of the enzyme forms as a function of substrate concentration was determined using testosterone as the substrate for 17β-enzyme activity and etiocholanolone as the substrate for 3α-HSD activity. The data obtained are shown in Figs. 14-19. Typical substrate saturation curves were observed for the 17β-HSD of enzyme forms I and II (Figs. 14 and 15) while enzyme III showed substrate inhibition at steroid concentration higher than 4 μM (Fig. 16). Substrate inhibition of the 3α-enzyme activity of all forms of the enzyme was observed using etiocholanolone as substrate. This inhibition occurred at steroid concentration higher than 4 μM for enzyme I and II and higher than 0.5 μM for enzyme III (Figs. 17-19).

The data shown in Figs. 14-19 were plotted according to the method of Lineweaver-Burk and the apparent K_m and V_{max} for each enzyme was determined from the plots (Figs. 14-19) and are summarized in Table 8. The only significant difference in the kinetic parameters among the three forms for both substrates is seen with enzyme III. The apparent K_m of this enzyme for etiocholanolone is approximately 20 fold lower than the K_m for testosterone.

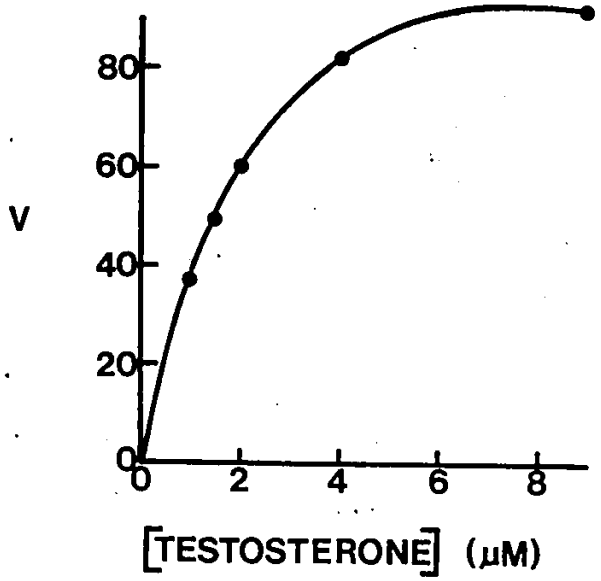
b) Inhibition of 3α- and 17β-hydroxysteroid dehydrogenase activities by testosterone and etiocholanolone

The relationships of the 3α- and 17β-enzyme activities of the multiple forms of the 17β-HSD were investigated by examining the inhibition of the

Figure 14

Lineweaver-Burk Plot for Enzyme Form I and Testosterone.

Incubations were carried out under the standard assay condition described in the General Methods (p.34) except that incubation time was 10 min. Each point on the graph is an average of three incubations. Velocity (V) has the units pmol product/min. The value obtained from each incubation was considered in the linear regression analysis. Each incubation contained 0.25 µg of enzyme and substrate oxidation did not exceed 25%. Incubations were also carried out for 20 min. and similar velocities were obtained.


80
60
V
40
20
0
0
2
4
6
8
[TESTOSTERONE] (μM)

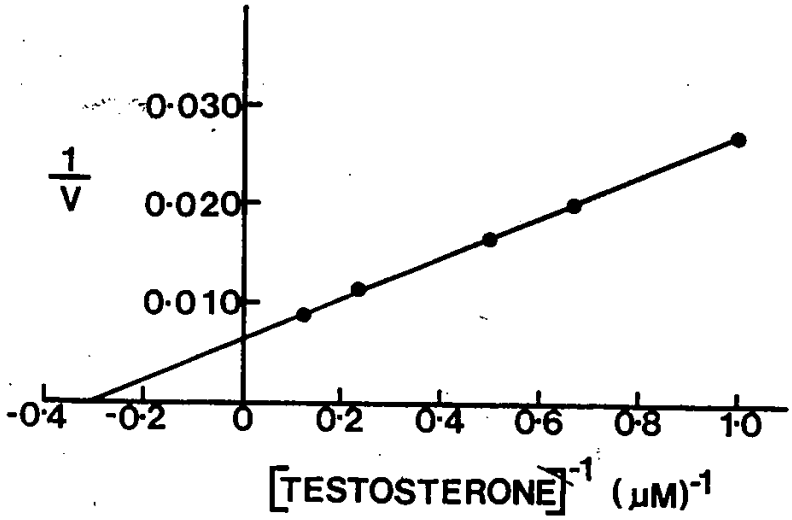
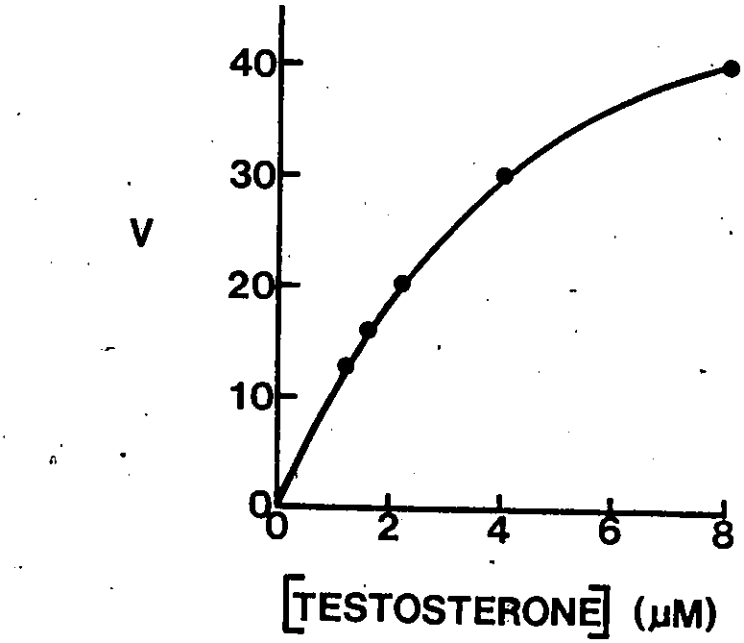

0·030
1/V
0·020
0·010
-0·4
-0·2
0
0·2
0·4
0·6
0·8
1·0
[TESTOSTERONE]-1 (μM)-1

Figure 15

Lineweaver-Burk Plot for Enzyme Form II and Testosterone

See Fig. 14 for conditions. Each incubation contained 0.12 µg of enzyme.


40
30
V
20
10
0
0
2
4
6
8
[TESTOSTERONE] (μM)

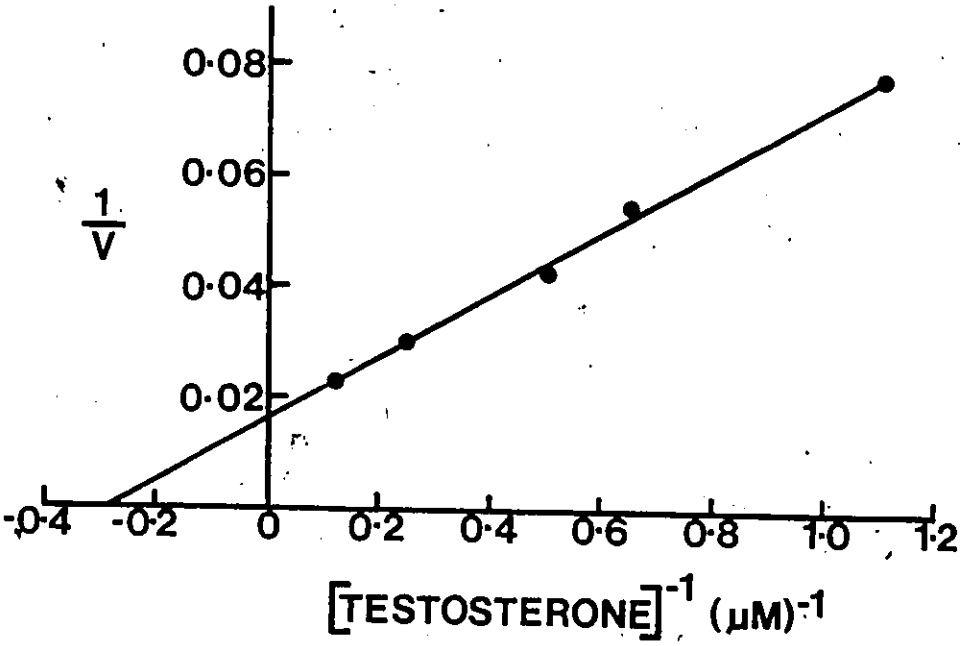
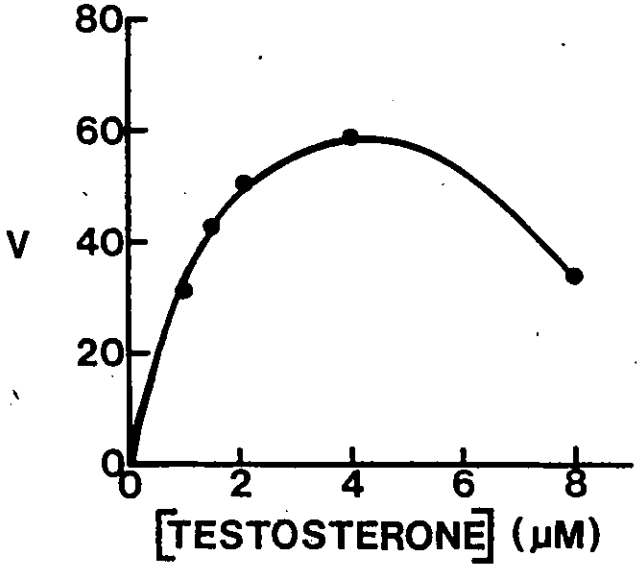

0·08
0·06
1/V
0·04
0·02
-0·4
-0·2
0
0·2
0·4
0·6
0·8
1·0
1·2
[TESTOSTERONE]$^{-1}$ (μM)$^{-1}$

Figure 16

Lineweaver-Burk Plot for Enzyme Form III and Testosterone

See Fig. 14 for conditions. Each inbubation contained 0.21 µg of enzyme.


80
60
V
40
20
0
0
2
4
6
8
[TESTOSTERONE] (μM)

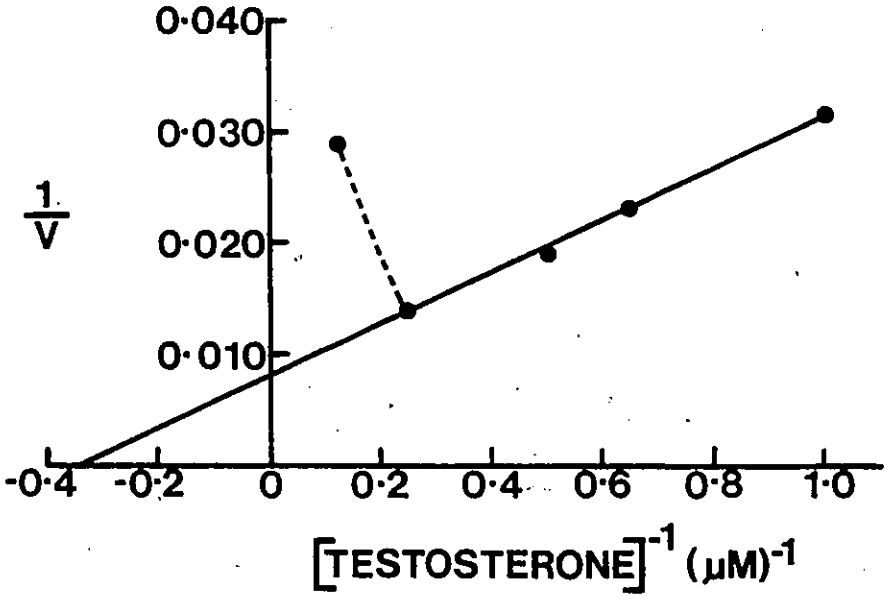
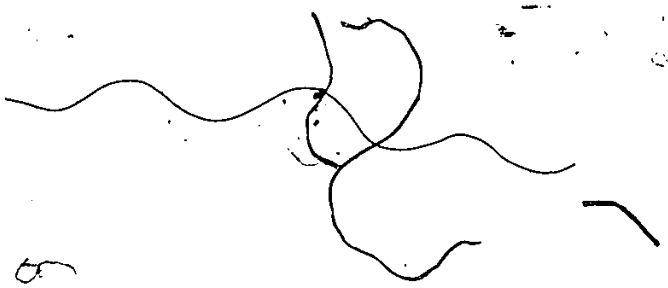
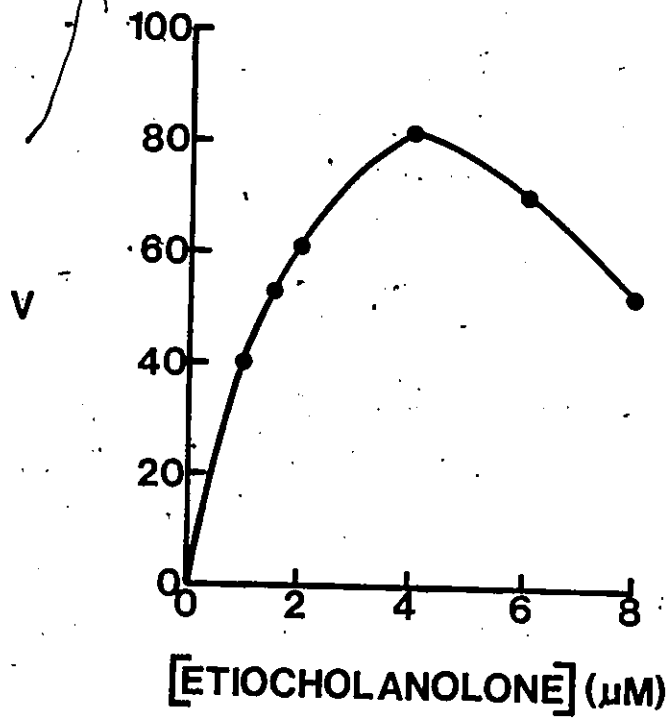

0·040
0·030
1/V
0·020
0·010
-0·4
-0·2
0
0·2
0·4
0·6
0·8
1·0
[TESTOSTERONE]$^{-1}$ (μM)$^{-1}$

Figure 17

Lineweaver-Burk Plot for Enzyme Form I and Etiocholanolone

See Fig. 14 for conditions. Each incubation contained 0.5 µg of enzyme.




100
80
60
40
20
0
V
0
2
4
6
8
[ETIOCHOLANOLONE] (μM)

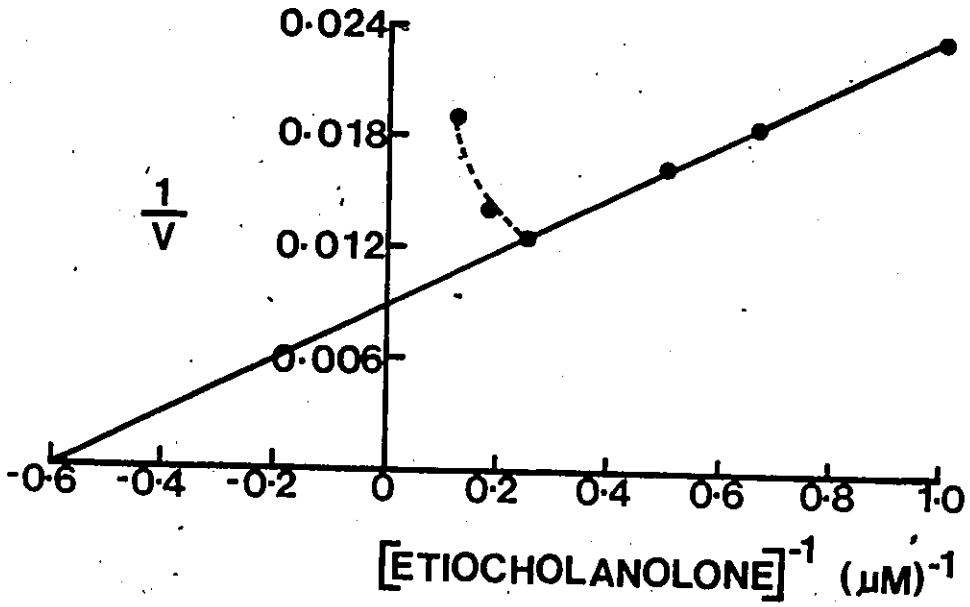
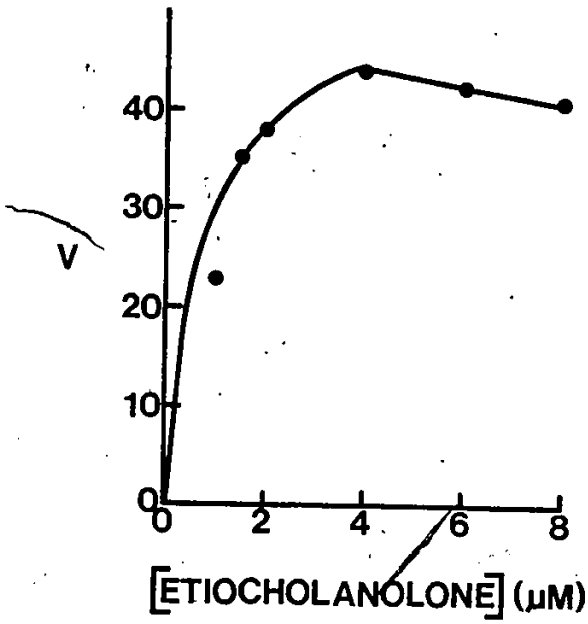

0·024
0·018
0·012
0·006
1/V
-0·6
-0·4
-0·2
0
0·2
0·4
0·6
0·8
1·0
[ETIOCHOLANOLONE]-1 (μM)-1

Figure 18

Lineweaver-Burk Plot for Enzyme Form II and Etiocholanolone

See Fig. 14 for conditions. Each incubation contained 0.24 µg of enzyme.


40
30
V
20
10
0
0
2
4
6
8
[ETIOCHOLANOLONE] (μM)

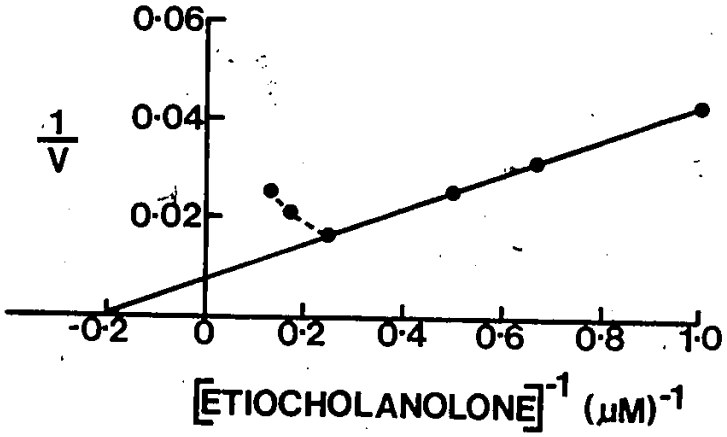
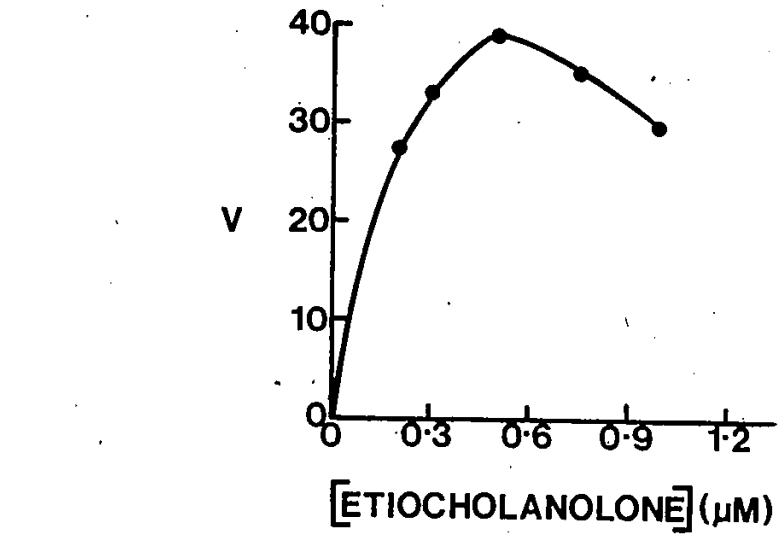

0·06
0·04
1/V
0·02
-0·2
0
0·2
0·4
0·6
0·8
1·0
[ETIOCHOLANOLONE]$^{-1}$ (μM)$^{-1}$

Figure 19

Lineweaver-Burk Plot for Enzyme Form III and Etiocholanolone

See Fig. 14 for conditions. Each incubation contained 0.42 µg of enzyme.


40
30
V
20
10
0
0
0·3
0·6
0·9
1·2
[ETIOCHOLANOLONE] (μM)

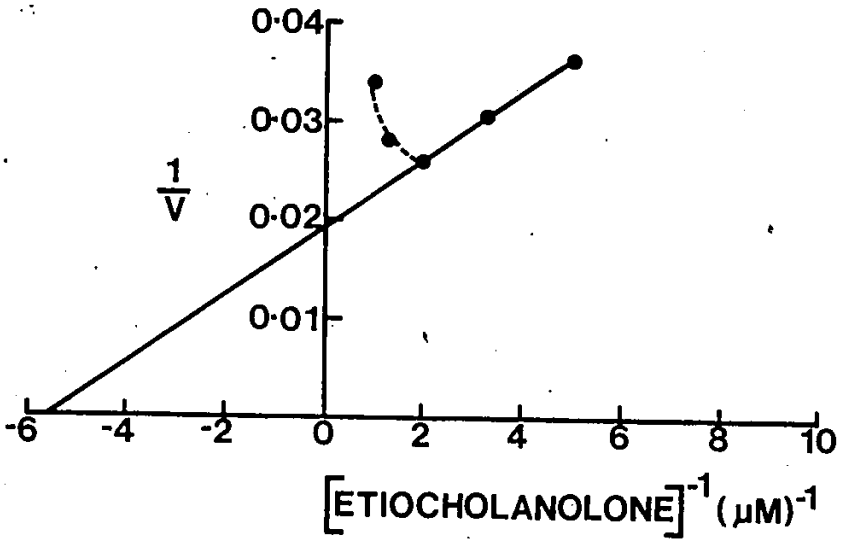

0·04
0·03
1/V
0·02
0·01
-6
-4
-2
0
2
4
6
8
10
[ETIOCHOLANOLONE]$^{-1}$ (μM)$^{-1}$

Table 8

Km and Vmax of the Multiple Forms of the Rabbit Liver 3α,17β-Hydroxysteroid Dehydrogenase

Enzyme	Testosterone		Etiocholanolone	
	Km (μM)	Vmax nmol/min/mg protein	Km (μM)	Vmax nmol/min/mg protein
I	3.2	615	1.7	217
II	3.6	505	5.0	534
III	3.0	635	0.18	107

17β-HSD activity by the 3α-substrate, etiocholanolone and 3α-HSD activity by the 17β-substrate, testosterone. The inhibition of testosterone oxidation by etiocholanolone for enzymes I, II and III is shown in Figs. 20-22. With all enzyme forms, inhibition is of the linear competitive type (Dixon, 1953). The inhibition of etiocholanolone oxidation by testosterone for enzyme forms I, II and III is shown in Figs. 23-25. Again inhibition is of the linear competitive type (Dixon, 1953) for all enzyme forms. Table 9 summarizes the inhibitory constant (Ki) determined for the multiple forms of the 3α,17β-HSD of female rabbit liver cytosol. Enzyme form III exhibits the lowest Ki with both testosterone and etiocholanolone.

c) Effect of nucleotide concentration

$NADP^+$ is a co-substrate for the multiple forms of the 17β-HSD of female rabbit liver. However the method used to establish the kinetic parameters for these enzymes with the steroid substrates can not be employed to determine these parameters for $NADP^+$ since the steroids are insoluble at the concentrations required to ensure complete saturation of the enzyme throughout the kinetic analysis. The inclusion of albumin or organic solvents in the assay mixture to solubilize the steroid substrate is not acceptable since these factors could affect the kinetics of the reaction. Dalziel (1957) has proposed a method which allows the determination of the Km for a second substrate (coenzyme) under conditions where the first substrate is not present at saturating concentrations. This method was therefore employed with one of the 17β-HSD forms (enzyme I) to obtain kinetic data on the coenzyme $NADP^+$. The enzyme was incubated with increasing concentrations of $NADP^+$ at three different concentrations of the second substrate testosterone. The reciprocals of the initial rates of oxidation were plotted against the reciprocals of the

Figure 20

Etiocholanolone Inhibition of the Oxidation of Testosterone by Enzyme Form I.

Incubations were carried out as described in the General Methods (p34) except that the incubation time was 10 min. The initial velocities (V) were measured in the presence of increasing concentrations of inhibitor (etiocholanolone) at three fixed substrate (testosterone) concentrations as indicated. Velocity (V) has the units pmol of product formed per min. Each point on the graph is an average of three incubations, but the value obtained from each incubation was considered in the linear regression analysis. Testosterone concentrations: 1 μM (▲); 2 μM (●) and 3 μM (■). Each incubation contained 0.5 μg of enzyme.

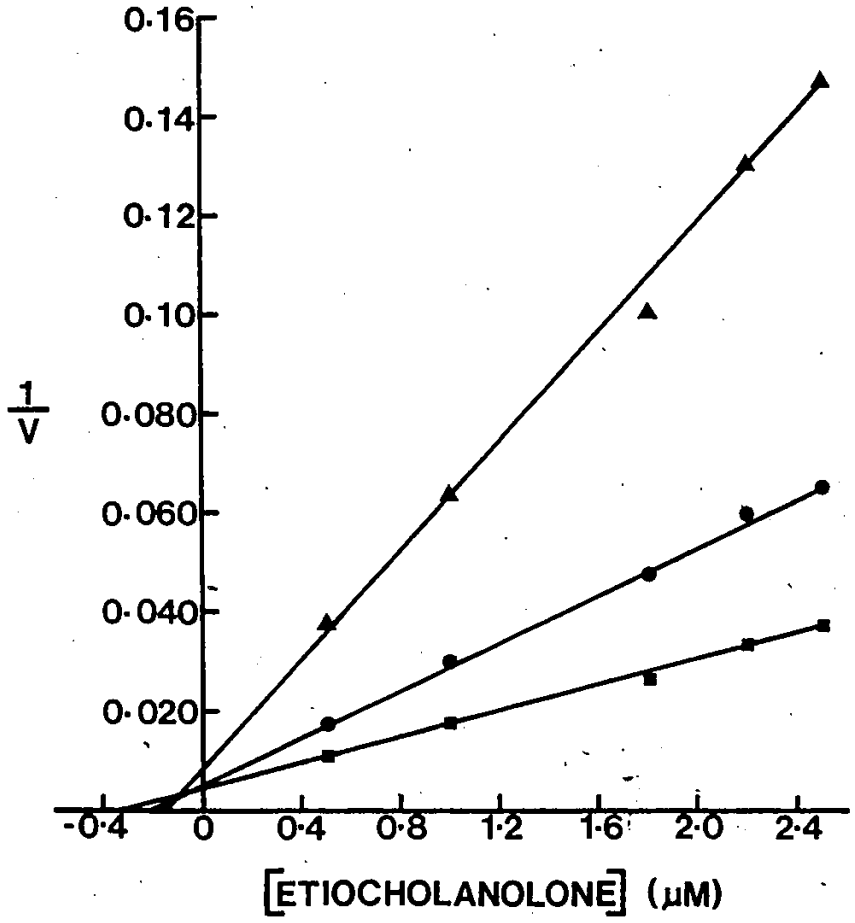

0·16
0·14
0·12
0·10
0·080
0·060
0·040
0·020
1/V
-0·4
0
0·4
0·8
1·2
1·6
2·0
2·4
[ETIOCHOLANOLONE] (μM)

Figure 21

Etiocholanolone Inhibition of the Oxidation of Testosterone by Enzyme Form II.

See legend in Fig. 20. Each incubation contained 0.24 µg of enzyme.

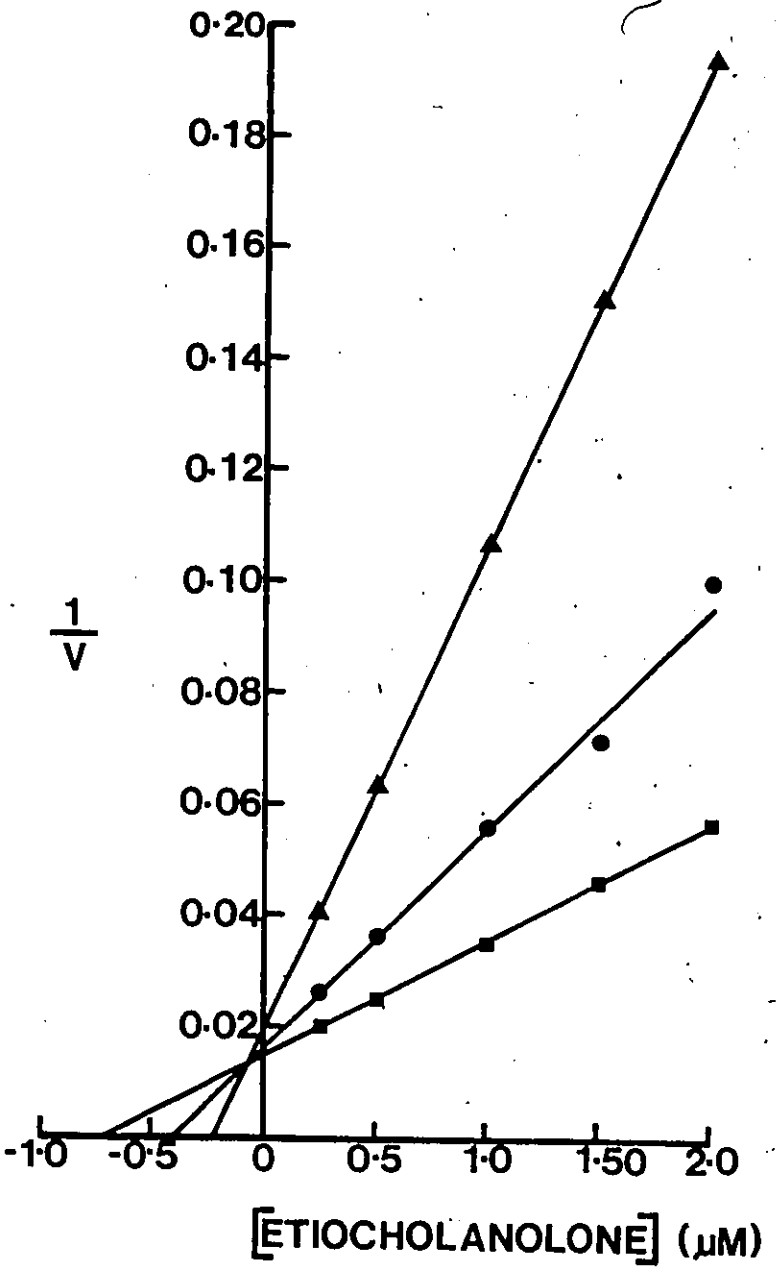

0·20
0·18
0·16
0·14
0·12
0·10
0·08
0·06
0·04
0·02
1/V
-1·0
-0·5
0
0·5
1·0
1·50
2·0
[ETIOCHOLANOLONE] (μM)

Figure 22

Etiocholanolone Inhibition of the Oxidation of Testosterone by Enzyme Form III.

See legend in Fig. 20. Each incubation contained 0.42 µg of enzyme.

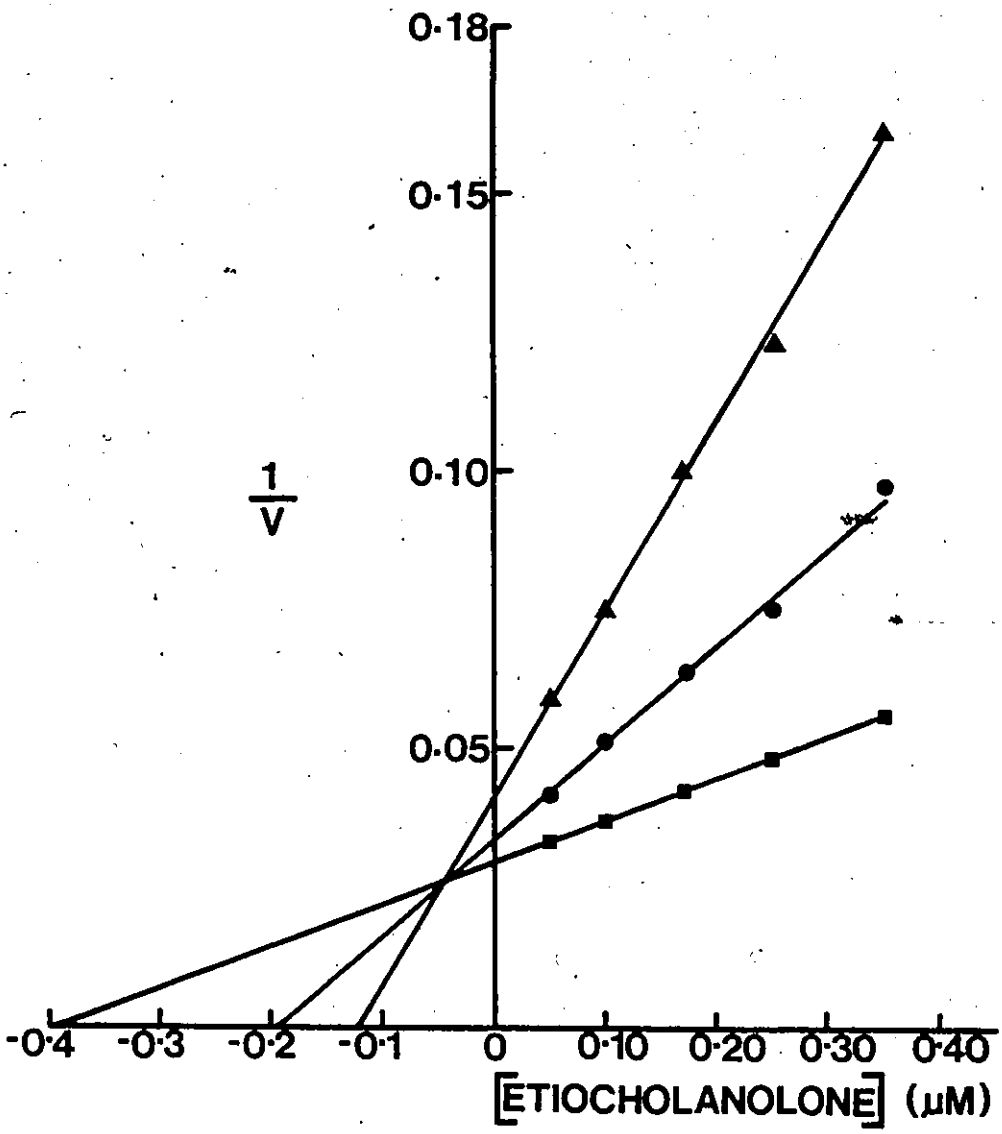

0·18
0·15
0·10
0·05
1/V
-0·4
-0·3
-0·2
-0·1
0
0·10
0·20
0·30
0·40
[ETIOCHOLANOLONE] (μM)

Figure 23

Testosterone Inhibition of the Oxidation of Etiocholanolone by Enzyme Form I.

Incubations were carried out as described in the General Methods (p.34) except the incubation time was 10 min. The initial velocities (V) were measured in the presence of increasing concentrations of inhibitor (testosterone) at three fixed substrate (etiocholanolone) concentrations as indicated. Velocity (V) has the units pmol of product formed per min. Each point on the graph was an average of three incubations, but the value obtained from each incubation was considered in the linear regression analysis. Etiocholanolone concentrations, 1 μM (▲), 2 μM (●) and 4 μM (■). Each incubation contained 2.5 μg of enzyme.

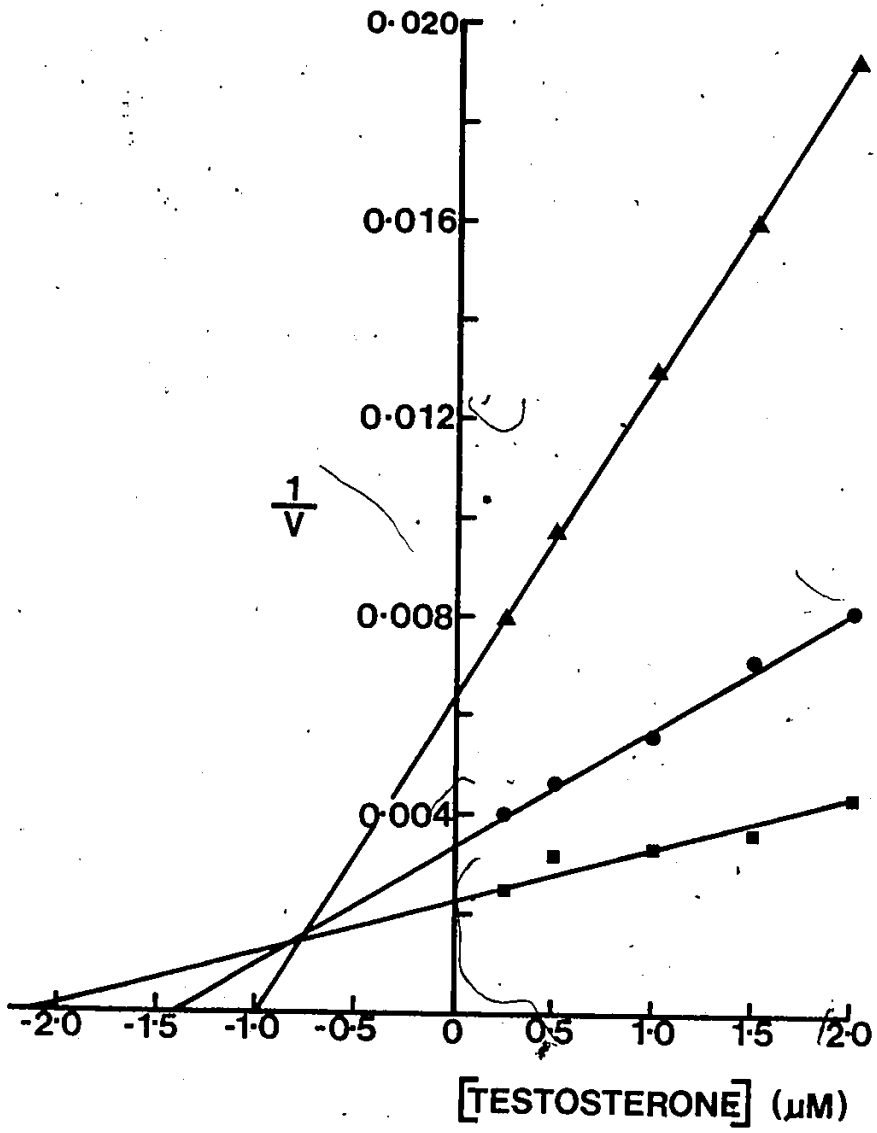
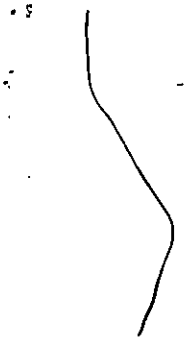

0·020
0·016
0·012
0·008
0·004
1/V
-2·0
-1·5
-1·0
-0·5
0
0·5
1·0
1·5
2·0
[TESTOSTERONE] (μM)

Figure 24

Testosterone Inhibition of the Oxidation of Etiocholanolone by Enzyme Form II.

See legend in Fig. 23. Each incubation contained 0.6 µg of enzyme.



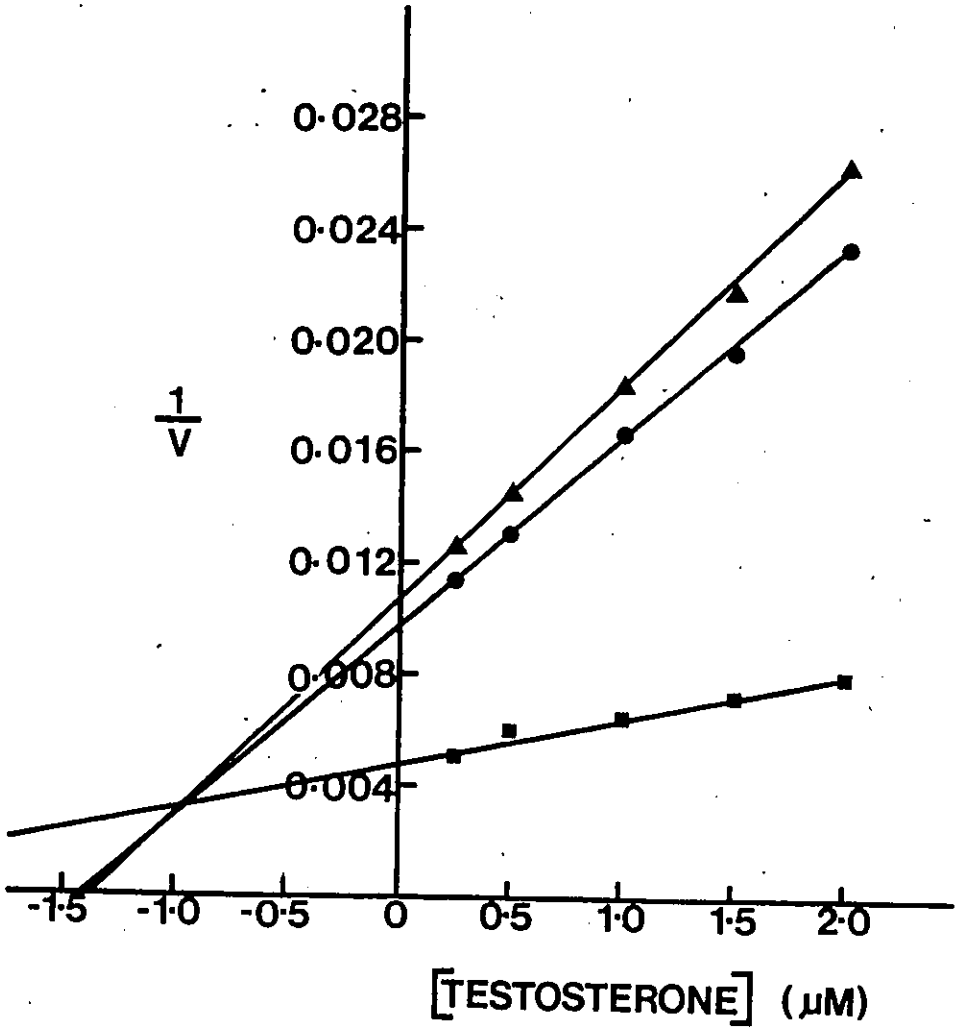

0·028
0·024
0·020
0·016
0·012
0·008
0·004
1/V
-1·5
-1·0
-0·5
0
0·5
1·0
1·5
2·0
[TESTOSTERONE] (μM)

Figure 25

Testosterone Inhibition of the Oxidation of Etiocholanolone by Enzyme Form III.

The initial velocities (V) were measured in the presence of increasing concentrations of the inhibitor testosterone at three fixed substrate (etiocholanolone) concentrations, 0.2 μM (▲), 0.3 μM (●) and 0.5 μM (■). Each incubation contained 0.63 μg of enzyme. For details see Fig. 23.

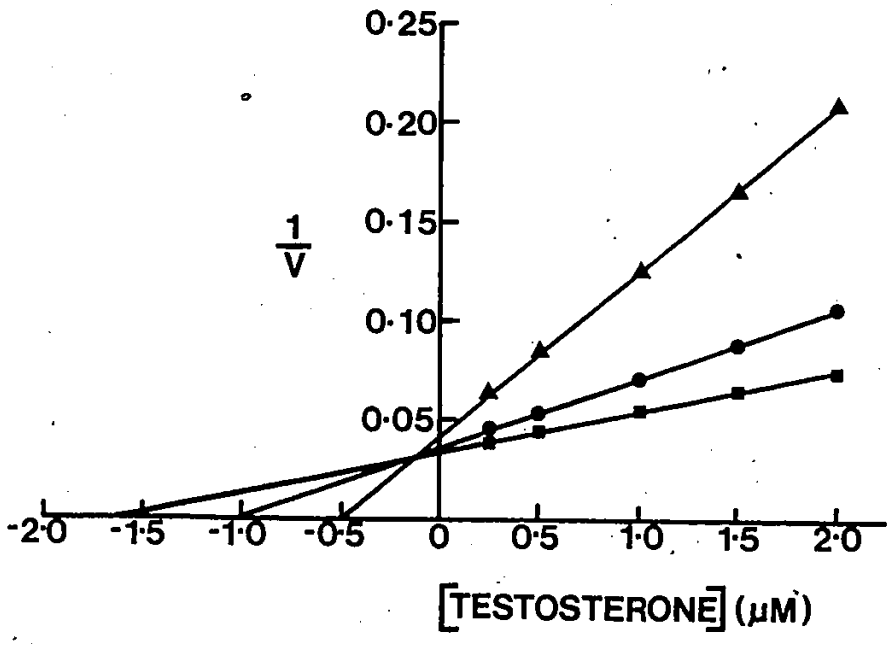

0·25
0·20
0·15
0·10
0·05
1/V
-2·0
-1·5
-1·0
-0·5
0
0·5
1·0
1·5
2·0
[TESTOSTERONE] (μM)

Table 9

The Inhibitory Constants (Ki) of the Multiple Forms of the 3α,17β-Hydroxysteroid Dehydrogenase

Enzyme	Testosterone (Ki) μM	Etiocholanolone (Ki) μM
I	0.73	0.13
II	0.97	0.09
III	0.21	0.02

$NADP^+$ concentration at each testosterone concentration (Fig. 26a). The intercepts and the slopes of primary Lineweaver-Burk graph were then plotted against the reciprocal of the testosterone concentrations (Figs. 26b and c). The Km values of testosterone and $NADP^+$ were calculated from the equations:

$$\text{Km (testosterone)} = \frac{\phi T}{\phi 0}$$

$$\text{Km } (NADP^+) = \frac{\phi N}{\phi 0}$$

whereas ϕT is the slope and $\phi 0$ is the intercept in Fig. 26b and ϕN is the intercept in Fig. 26c. T = testosterone and N = $NADP^+$. Km values of 3.3 and 2.7 μM were obtained for testosterone and $NADP^+$ respectively. The Km value for testosterone is in agreement with the value obtained by the Lineweaver-Burk plot (Fig. 14 and Table 8).

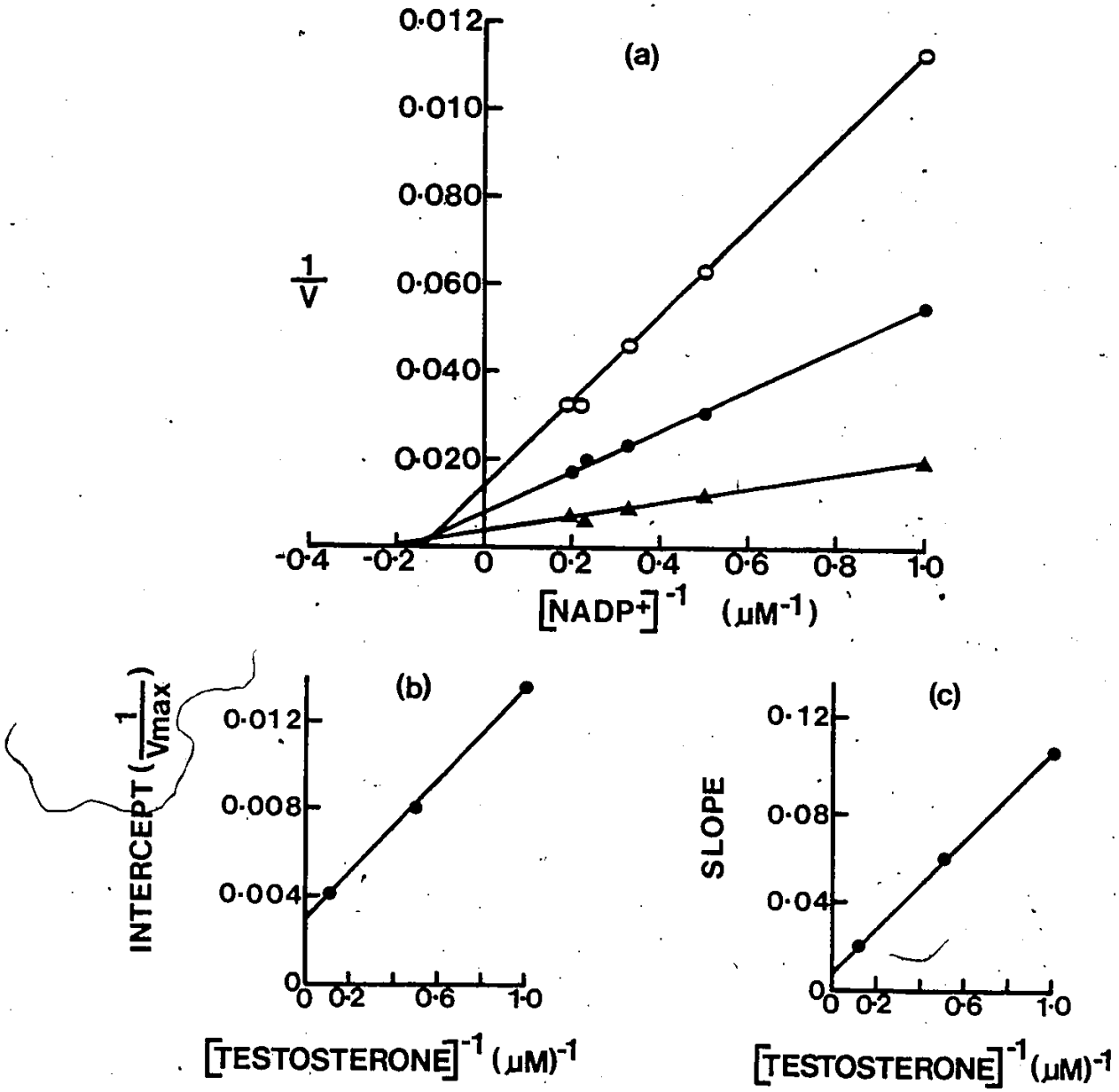
The fact that the lines in the reciprocal plots (Fig. 26a) intersect at a point implies that the reaction proceeds by an ordered mechanism with the binding of $NADP^+$ as the first substrate.

Figure 26

Dependence of 17β-Hydroxysteroid Dehydrogenase Activity of Enzyme Form I on $NADP^+$

a. Primary Plot: Double reciprocal plots of velocity of enzyme form I as a function of $NADP^+$ concentration. Incubations were carried out as described in the General Methods (p.34) except the incubation time was 10 min. The initial velocities (V) were measured in the presence of increasing concentrations of cofactor ($NADP^+$) at three fixed substrate (testosterone) concentrations as indicated. Velocity has the units pmol product per min. Each point on the graph is an average of three incubations, but the value obtained from each incubation was considered in the linear regression analysis. Each incubation contained 0.25 µg of enzyme. Testosterone concentrations: 1 µM (o), 2 µM (●) and 8 µM (▲).

b and c Secondary Plots: Variation of the intercepts (Fig. 26b) and slopes (Fig. 26c) of Fig. 26a with the reciprocal of the testosterone concentrations.


(a)
0·012
0·010
0·080
0·060
0·040
0·020
1/V
-0·4
-0·2
0
0·2
0·4
0·6
0·8
1·0
[NADP+]-1 (μM-1)
(b)
INTERCEPT (1/Vmax)
0·012
0·008
0·004
0
0 0·2 0·6 1·0
[TESTOSTERONE]-1 (μM)-1
(c)
SLOPE
0·12
0·08
0·04
0
0 0·2 0·6 1·0
[TESTOSTERONE]-1 (μM)-1

DISCUSSION

The multiple forms of the soluble 17β-HSD of rabbit liver have properties similar to those of this enzyme activity in other tissues and species. With all three forms of the rabbit liver enzyme the pH optimum of the oxidative reaction for both 3α- and 17β-substrates is 9.5 in 0.1 M glycine-NaOH buffer. A similar pH optimum has been reported for the soluble 17β-HSDs of chicken liver (Renwick et al., 1981) and human placenta (Langer and Engel, 1959). The pH optimum for the reduction of the 17-ketosteroids is 7.6 in 0.1 M Tris-maleate buffer. This difference in the pH optima for the oxidative and reductive reactions has also been observed with other HSDs. For example, Langer and Engle (1958) reported that the reduction of estrone by 17β-estradiol dehydrogenase of human placental was optimum at pH 6.2 whereas the pH optimum of the oxidative reaction was near 10. Similarly, the human ovarian 17β-estradiol dehydrogenase has a pH optimum of 6.9 and 8.1 for the reductive and oxidative reactions respectively (Pittaway et al., 1977).

It is evident from the results of the present studies that all of the multiple forms of the 17β-HSD from female rabbit liver cytosol exhibit higher activities toward androgens than toward estrogens in both the oxidative and reductive reactions. This is particularly evident with enzyme III where activity toward testosterone is 50 fold higher than toward 17β-estradiol in the oxidation direction (Table 5). Earlier studies by Thaler-Dao et al. (1972) also showed a 17β-HSD of female rabbit liver with higher activity toward testosterone than toward 17β-estradiol in the presence of $NADP^+$. Similar results have been reported for the soluble 17β-HSD of guinea pig kidney (Liu and Kochakian, 1972a). In contrast, Renwick et al. (1981) have

found that the 17β-HSD of chicken liver exhibits a high activity toward 17β-estradiol while testosterone is a very poor substrate. The soluble 17β-estradiol dehydrogenase of human placenta also exhibits a high specificity for estrogens, activity toward 17β-estradiol being 100 times higher than toward testosterone (Langer et al., 1959). Estrogen-specific 17β-HSDs have also been described in the ovary of sheep (Kautsky and Hagerman, 1970), human (Pittaway et al., 1977) and rabbit (Davenport and Mallette, 1966; Rodway and Rahman, 1978).

Differences in the relative rates of oxidation and reduction of estrogens and androgens are observed with the 17β-HSDs of female rabbit liver cytosol (Table 7). With all of the enzyme forms, activity is higher toward estrone than toward 17β-estradiol. This higher activity in the reductive direction with estrogens could be very important in vivo, increasing the levels of the active hormone, 17β-estradiol in the circulating blood or increasing the amount of 17β-estradiol available for binding to its receptors in the rabbit liver cytosol (Danzo et al., 1977; Tong et al., 1983). On the other hand, with the androgen substrates the relatively higher rate of oxidation of testosterone compared to the reduction of androstenedione suggests that in vivo the liver 17β-HSD is responsible for the inactivation of testosterone circulating in the blood. However, in vivo the relative extent of C-17 oxidation or reduction will also depend on the ratio of oxidized to reduced coenzyme present in the tissue. Therefore, the in vivo function of the liver 17β-HSD in androgen and estrogen metabolism cannot be unequivocally assigned based on in vitro observations where the rates of oxidation and reduction are determined under optimum conditions and in the presence of an excess of the oxidized or reduced form of the coenzyme.

Previous studies in our laboratory have shown that some forms of the 17α-HSD of female rabbit liver and kidney cytosol may have a specific role in estrogen metabolism. These enzyme forms exhibit a high activity toward the glucuronide derivatives of 17α-estradiol (Hasnain and Williamson, 1977; Lau et al., 1982a and b) suggesting a close relationship between conjugation with glucuronic acid at C-3 of the steroid and oxidation-reduction at C-17 in this tissue. With unconjugated estrogens the predominant interconversion is between estrone and 17β-estradiol (Hasnain and Williamson, 1974). However after the formation of the 3-glucuronide derivative, conversion to the 17α-epimer, predominates. Thus conjugation of the estrogen molecule at C-3 with glucuronic acid directs estrogen metabolism toward elimination of this molecule owing to the presence of a 17α-HSD in rabbit liver specific for the glucuronide derivative. 17α-Estradiol 3-glucuronide is the specific substrate for the enzymic transfer of N-acetylglucosamine from UDP-N-acetyl-glucosamine to the 17α-position of the steroid (Collins et al., 1968). The double conjugate formed is then excreted in the urine (Layne et al., 1964; Layne et al., 1965; Layne, 1970; Collins et al., 1968). The present studies have shown that this high activity toward estrogen glucuronide is not observed with the 17β-enzymes of female rabbit liver cytosol. Indeed, the activity toward the 3-glucuronide derivative of 17β-estradiol is one fifth of that toward 17β-estradiol for enzymes I and II and approximately equal for enzyme III. The relatively low activity of the 17β-HSD toward estrogen glucuronides when compared to the 17α-HSD activity suggests that in vivo conjugation with glucuronic acid would direct the reduction of C-17 to the 17α-derivative and subsequent excretion of the steroid.

The substrate specificity studies of the multiple forms of 17β-HSD of female rabbit liver cytosol show that these enzyme forms also catalyze

the oxidation of the 3α-hydroxysteroids. The common identity of the 3α- and 17β-activities of the rabbit liver enzymes is supported by kinetic analysis. The oxidation of the 17β-hydroxysteroid testosterone was inhibited competitively by the 3α-hydroxysteroid etiocholanolone, while the oxidation of 3α-hydroxysteroid etiocholanolone was inhibited competitively by testosterone, indicating that these steroid were competing for the same active site. A linear competitive type of inhibition was observed with all enzyme forms. The 3α-activity is relatively specific for androgen substrates of the 5β-androstane series with an A/B cis ring fusion. The activity toward etiocholanolone (5β-androstane series) is at least 10 times higher than toward androsterone (5α-androstane series). This specificity has also been reported for the 17β-HSD of female rabbit liver cytosol by Thaler-Dao et al. (1972). In contrast, 3α-enzyme activity of the 17β-HSD from guinea pig kidney cytosol (Shen and Kochakian, 1978; Liu and Kochakian, 1972a) and the 3(17)α-HSD of rabbit kidney (Lau et al., 1982a) is specific for C_{19}-steroids having an A/B trans ring fusion. This discrimination shown by the 3α-activity with regard to the A/B ring fusion of the 5α- and 5β-androstane series can be explained by the conformational differences of the steroids. Conformational analysis of 5α-steroids such as androsterone shows that it has a planar structure, while the 5β-steroids such as etiocholanolone are characterized by a marked bowing of the A-ring about the C-5, 10 bond relative to the remainder of the steroid molecule (Duax and Norton, 1975). The 3α-HSD activity in the reductive reaction toward the substrate 5α-dihydrotestosterone could not be detected with the multiple forms of 17β-HSD of female rabbit liver cytosol. This is in keeping with the lower activity of these enzyme forms toward androgens of the 5α-androstane series in the oxidative reaction. Radioactive substrates

having a 3-keto-5β-androstane configuration are not commercially available, therefore the reductive activity of the liver enzymes toward 5β-androstane substrates could not be established.

One of the multiple forms (enzyme II) of the soluble 17β-HSD of female rabbit liver exhibits significant 3β-HSD activity. The activity of this enzyme form toward the 3β-hydroxysteroid substrate dehydroepiandrosterone is approximately 40% that observed with the 3α-hydroxysteroid substrate etiocholanolone (Table 5). These results confirm the earlier studies of Thaler-Dao et al. (1972) which indicated 3β-enzyme activity for a partially purified 17β-HSD of female rabbit liver. Dehydroepiandrosterone is a Δ^5 steroid and has a planar ring structure similar to that of the 5α-androstanes (Duax and Norton, 1975). Radioactive substrates of the 5α- and 5β-androstane series having a 3β-hydroxyl group at C-3 and a ketone group at C-17 are not commercially available. Therefore it was not possible to assess the effect of the unsaturated B ring present in dehydroepiandrosterone on the 3β-enzyme activity.

Initial studies on the substrate specificity of the isolated enzymes indicated that the 17β-enzyme activity was 2-4 fold higher than the 3α-enzyme activity (Table 5). Kinetic analysis showed that enzymes I and II exhibited similar Km and Vmax values toward 17β- and 3α-hydroxysteroids (Table 8). However, for enzyme III, the kinetic data indicated that etiocholanolone was a better substrate than testosterone. The enzyme III had a significantly lower Km toward etiocholanolone than toward testosterone, a result contradictory to the initial finding that 17β-activity for enzyme III was higher than 3α-activity (Table 5). When kinetic analysis of this enzyme was carried out it was observed that substrate inhibition of enzyme III occurred at the

concentration of etiocholanolone normally used in the enzyme assays (1 μM, Fig. 19). This substrate inhibition could therefore account for the apparent lower activity of enzyme III toward the 3α-hydroxysteroid substrate than toward the 17β-hydroxysteroid substrate. Inhibition by etiocholanolone was also observed with enzymes I and II but only at much higher substrate concentrations. Only enzyme III showed substrate inhibition with testosterone and again, this occurred at relatively high substrate concentration. Substrate inhibition has been also reported for the 3(17)β-HSD of Pseudomonas testosteroni (Talalay, 1963) and for the 3(17)α-HSD from female rabbit liver and kidney cytosol (Lau et al., 1982b). The low Km of enzyme III toward etiocholanolone suggests that this enzyme could function specifically in vivo to metabolize 3α-hydroxysteroids of the 5β-androstane series which could be present at low levels in the circulation.

The bifunctional enzyme activity observed with the rabbit liver 17β-HSD has also been observed with other 17β-HSDs. Shen and Kochakian (1978) and Liu and Kochakian (1972a) found 3α-HSD activity associated with two of the purified forms of the $NADP^+$-dependent 17β-HSD of male guinea pig kidney cytosol. Battais et al. (1972) reported that 17β-HSD activity was associated with a 3α-HSD of Pseudomonas testosteroni.

HSDs other than those specific for the 3α- and 17β-hydroxysteroids also exhibit bifunctional activity. These include the 20α-activity of the 17β-estradiol dehydrogenase of the human placenta (Strickler et al., 1981) and the 20β-activity of the 3α-HSD of Streptomyces hydrogenans (Edwards and Orr, 1978). Recently, Sharaf and Sweet (1982) have purified an enzyme from calf fetal red blood cells exhibiting both 3β- and 20α-HSD activities. The bifunctional enzyme activity of the HSD from Streptomyces hydrogenans has

been examined in considerable detail. This enzyme has both 3α- and 20β-HSD activities. Edwards and Orr (1978) have presented evidence that both activities are due to a single enzyme. The two enzyme activities co-purified and exhibited the same inactivation pattern when treated with 21-iodoacetoxycortisone or 16α-bromoacetoxyprogesterone. These studies have been confirmed by Sweet and co-workers (Sweet and Samant, 1980; Sweet et al., 1980; Sweet and Samant, 1981) using 17-bromoacetoxy steroids and FSA (5'-p-fluorosulfonylbenzoyl adenosine) as substrate analogs. Sweet and Samant (1980) have proposed two models of steroid binding to account for bifunctional activity of this enzyme. One model proposes that the enzyme has a single cofactor binding region and two different steroid binding orientations at the active site. Two different enzyme-substrate complexes can be formed by a 180° rotation of the steroid at the active site. The C_3 or C_{20} carbonyl group can receive a 3β- or 20α-hydride from the same NADH molecule with the formation of the appropriate hydroxysteroid at a single catalytic region. The second model depicts the steroid binding region at the enzyme active site having two cofactor binding regions at opposite ends of the steroid molecule so that C_3 or C_{20} carbonyl group can receive a 3α- or 20β-hydride from an appropriately located NADH molecule.

CHAPTER V

THE MULTIPLE FORMS OF THE SOLUBLE 17β-HYDROXYSTEROID DEHYDROGENASE OF FEMALE RABBIT LIVER: PHYSICAL AND CHEMICAL PROPERTIES AND AGE DEPENDENT CHANGES

1. Chromatofocusing of the partially purified soluble 17β-hydroxysteroid dehydrogenase of liver from rabbit at different ages

The activities of many steroid-metabolizing enzymes exhibit age-related variations. Most of these changes can be directly related to the increased production of gonadal hormones that occurs during animal development. Bongiovanni and Marino (1972) studied the 17β-HSD activity in the liver of rabbits at various developmental stages. They found that the fetal liver (30-33 days of gestation) homogenates had no apparent 17β-HSD activity. Homogenate prepared from liver of the 20-day-old rabbit was better able to reduce androstenedione to testosterone than the liver homogenate from the mature (17-19 months) rabbit.

In our studies soluble 17β-HSD activity has been examined in the liver of female rabbits at various ages. Table 10 shows the specific activity of the soluble 17β-HSD when testosterone is used as substrate and $NADP^+$ as coenzyme. The specific activity of the soluble 17β-HSD is lowest in the liver of rabbits 28-days-old and increases with the age of the animal.

In order to investigate the relationship between the age of the animal and the 17β-HSD activity the cytosol fraction obtained from rabbits 28-, 35-, 42-, 49- and 56-days-old were concentrated by ultrafiltration and each separate preparation was subjected to gel filtration on Sephadex G-75 (superfine) as described in General Methods (p. 37). The fractions containing

Table 10

17β-Hydroxysteroid Dehydrogenase Activity of the Liver Cytosol from Female Rabbits at Different Ages

Age (days)	Specific Activity (μunits/mg protein)
28	215 ± 30
35	240 ± 20
42	440 ± 40
49	485 ± 50
56	635 ± 60

For the ages 28 and 56 days, the specific activities are the average from 4 animals, while for the other ages the values are the average from 2 animals. Steroid concentration was 1 μM and the assay was carried out in 67 mM glycine-NaOH buffer pH 9.5.

the 17β-HSD activity were pooled, concentrated by ultrafiltration and subjected to chromatofocusing chromatography under the conditions described in the General Methods (p. 37). Fractions obtained by chromatofocusing were assayed for 17β-HSD activity using the substrate testosterone. Fig. 27 shows the 17β-HSD activity patterns obtained after chromatofocusing chromatography. The enzyme activity profile differs with the age of the animal. In 28-day-old animals, the 17β-enzyme profile shows one major peak of activity (enzyme IIIY) and two minor peaks. In 35-day-old rabbit, two peaks of 17β-HSD activity are present. The enzyme pattern of the 42-day-old rabbit differs from that obtained from the 28- and 35-day-old animals, one major activity is observed with a shoulder likely due to a more acidic enzyme form. The profile of the 17β-HSD activity obtained from 49- and 56-day-old rabbits are similar, two peaks of activity being present. This pattern was also obtained from rabbits at ages of 70- and 84-days and also from a 42-month-old animal (data not shown).

2. Purification and properties of the soluble 17β-hydroxysteroid dehydrogenase of liver from the female rabbit 28-days-old

In order to compare the 17β-HSD from rabbits at different ages, the 17β-HSD of the 28-day-old rabbit (enzyme IIIY) was purified. The 17β-HSD fraction obtained by gel filtration was subjected to affinity chromatography as described in the General Methods (p. 37). The fractions of the affinity chromatography column with 17β-HSD activities were concentrated in the presence of 20% glycerol (v/v), dialyzed against 10 mM Tris-HCl, pH 8.0, 0.5 mM DTT, 20% glycerol (v/v) and subjected to chromatofocusing chromatography.

Figure 27

Chromatofocusing Chromatography of the Partially Purified 17β-Hydroxysteroid Dehydrogenase of Female Rabbit Liver Cytosol at Different Ages.

Each 17β-HSD was partially purified by gel filtration on Sephadex G-75, concentrated by ultrafiltration and subjected separately to chromatofocusing chromatography as described in General Methods (p. 38). 17β-HSD activity was measured using testosterone as substrate. The amount of each column fraction assayed was 10, 10, 5, 3 and 2 µl for the ages 28-(A), 35-(B), 42-(C), 49-(D) and 56-(E) day-old rabbits respectively.

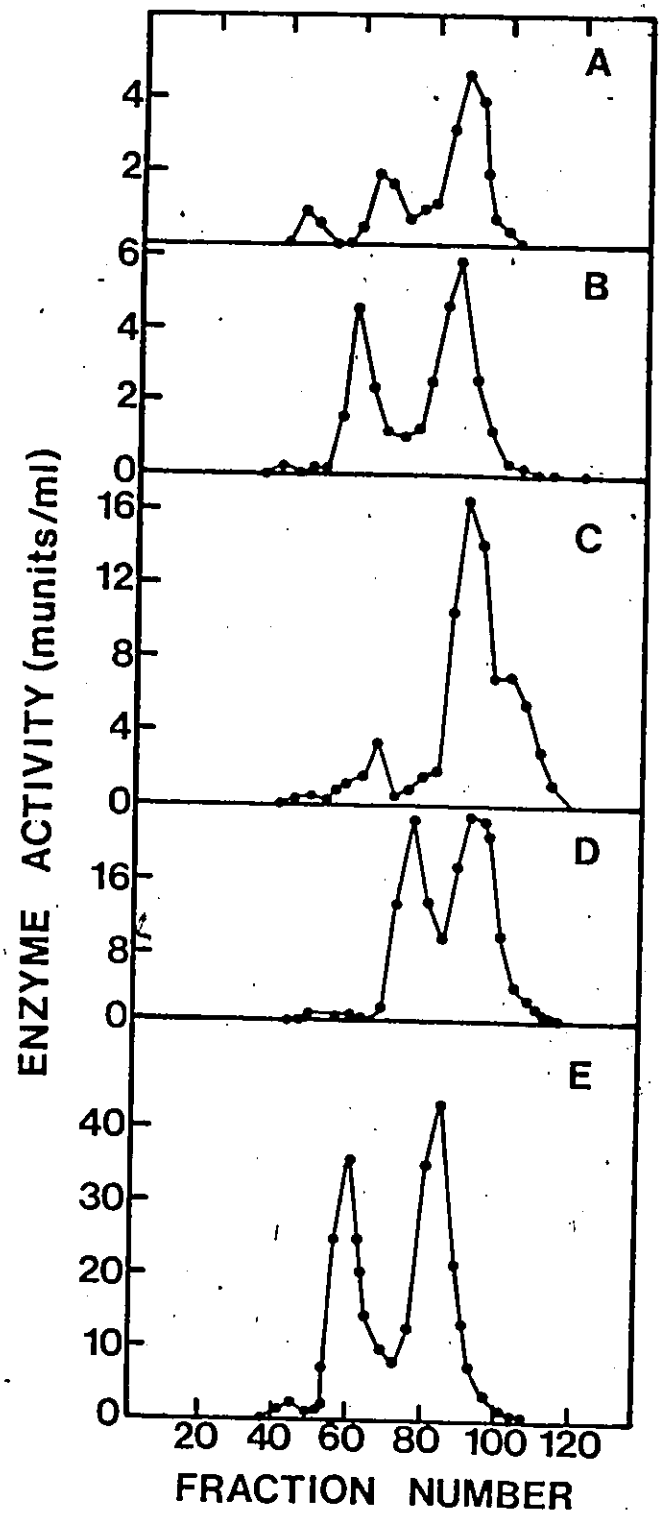

A
B
C
D
E
ENZYME ACTIVITY (munits/ml)
FRACTION NUMBER
20 40 60 80 100 120

Fig. 28 shows a typical profile of the soluble 17β-HSD obtained by chromatofocusing chromatography. The fractions comprising the major 17β-HSD activity (peak IIIY) were concentrated and again subjected to chromatofocusing in a small column as described previously to yield a 17β-HSD that was homogeneous as established by polyacrylamide gel disc electrophoresis (Fig. 29). Substrate staining toward both testosterone and etiocholanolone showed that the single protein band has activity toward both steroid substrates. The properties of the purified 17β-HSD were determined in order to compare these properties to those established for the enzyme forms isolated from the 8-week-old rabbit. The molecular weight of the enzyme (IIIY) determined by SDS-polyacrylamide gel disc electrophoresis was approximately 40,500. The enzyme had the same pH optimum toward testosterone and etiocholanolone as the enzyme from the 8-week-old rabbit (Figs 9 and 10). The activity of the enzyme IIIY toward a number of steroid substrates was examined and the results are summarized in Table 11. In general, activity toward androgens is higher than toward estrogens. Enzyme IIIY also has 3α-HSD activity relatively specific for substrates of the 5β-androstane series, the activity toward etiocholanolone being approximately 3 times higher than toward androsterone (5α-androstane series).

Fig. 30 shows the Lineweaver-Burk plot, for the 17β-activity of enzyme IIIY when testosterone is used as a substrate. The apparent Km and Vmax values for the enzyme were determined to be 1.35 μM and 270 nmol/min/mg protein respectively. There was no substrate inhibition at concentrations up to 8 μM.

Figure 28

Chromatofocusing Chromatography of the Soluble 17β-Hydroxysteroid Dehydrogenase of Female Rabbit Liver, 28-Days-Old.

Chromatofocusing Chromatography was carried out as described in the General Methods (p. 38). Aliquots (10 µl) of the column fractions (1.5 ml) were assayed for 17β-HSD activity toward the substrate testosterone.

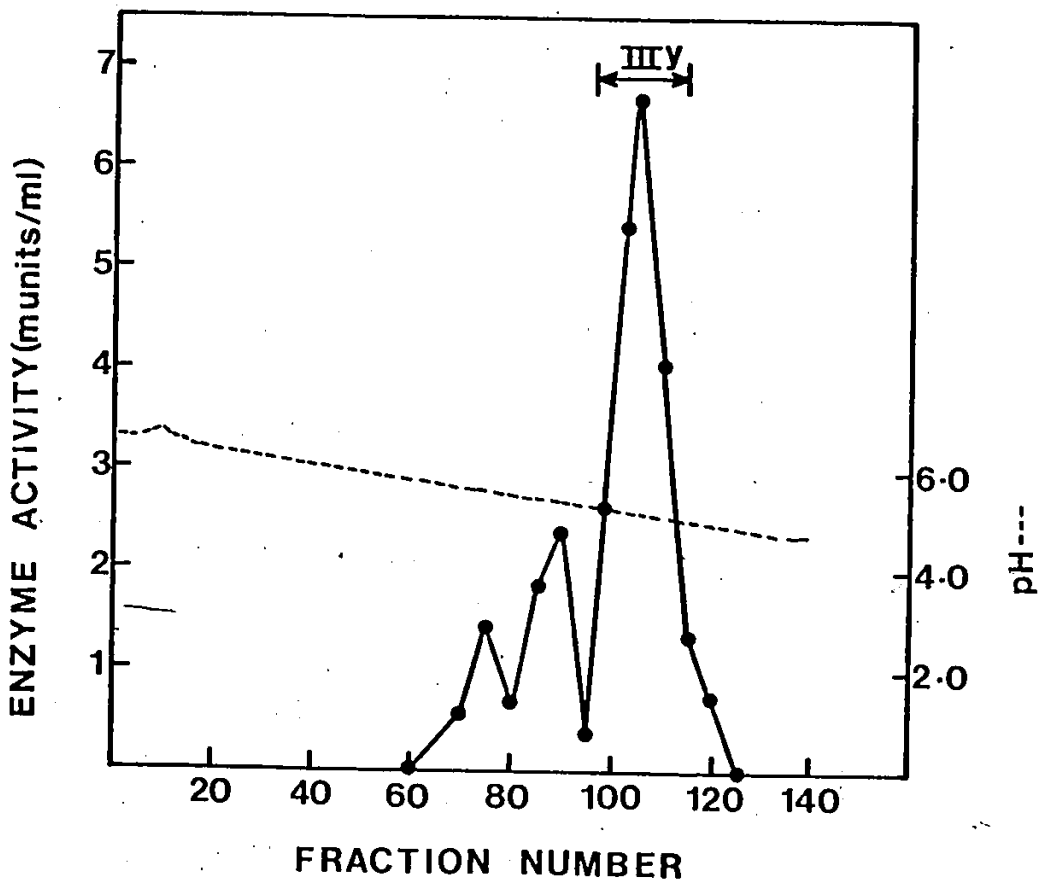

IIIY
ENZYME ACTIVITY(munits/ml)
7
6
5
4
3
2
1
6·0
4·0
2·0
pH---
20
40
60
80
100
120
140
FRACTION NUMBER

Figure 29

Polyacrylamide Gel Electrophoresis of Enzyme Form IIIY.

Gel a: polyacrylamide gel electrophoresis according to the method of Davis (1964).

Gel b: SDS-polyacrylamide gel electrophoresis according to the method of Weber et al. (1972).

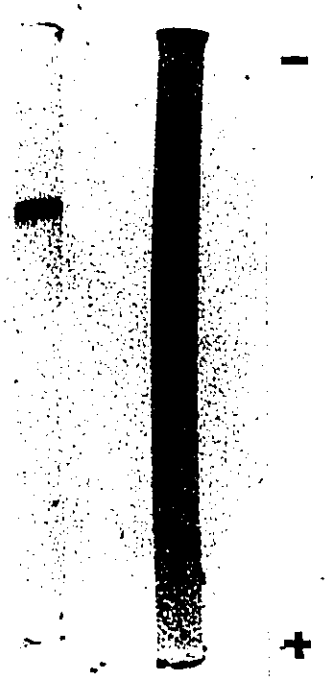

−
+
a
b

Table 11

Substrate Specificity of the Purified 17β-Hydroxysteroid Dehydrogenase of Female Rabbit Liver (28-days-old)

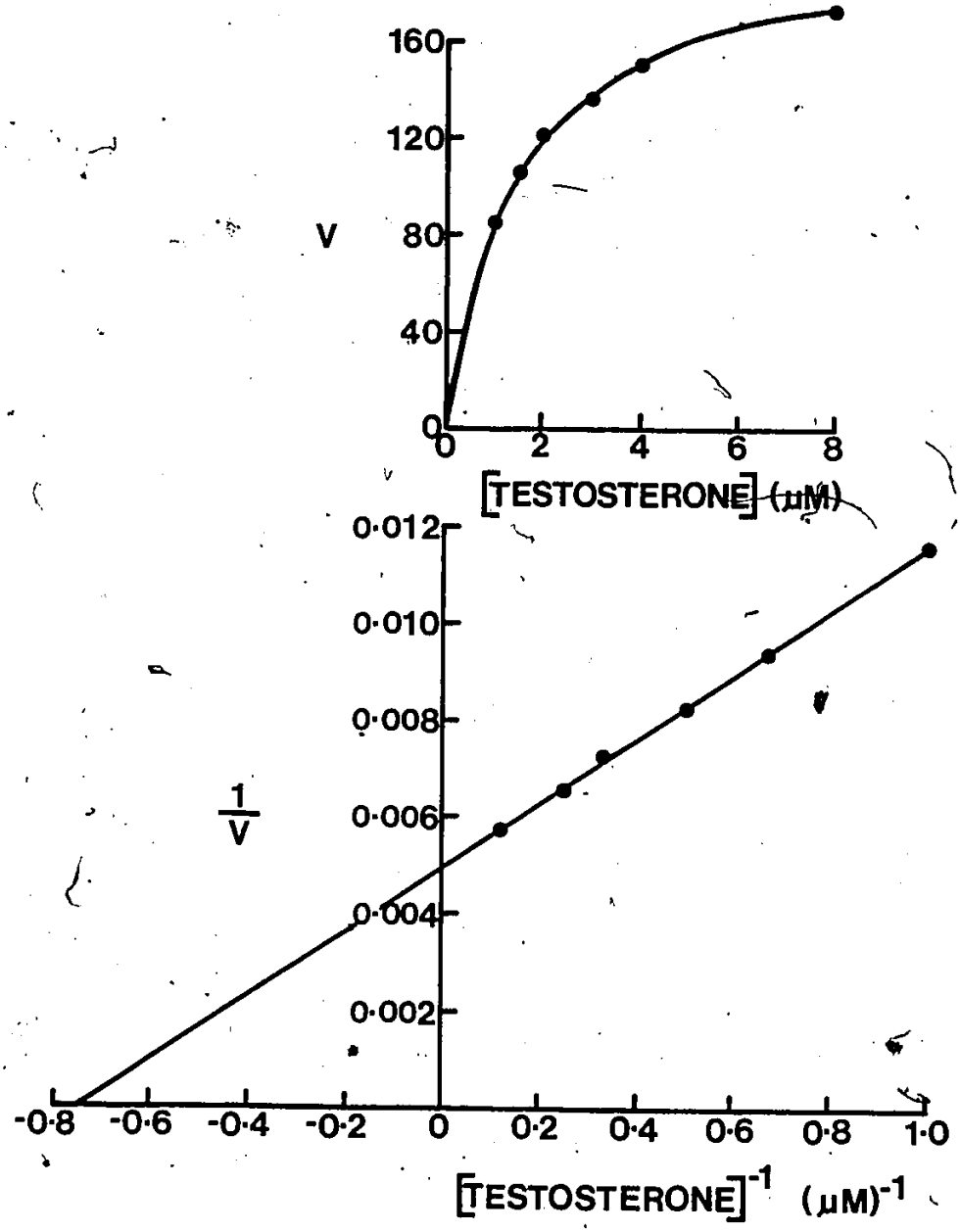
Substrate	Specific Activity (munits/mg protein)
Testosterone	19.5
17β-Estradiol	0.62
17β-Estradiol-3-glucuronide	0.15
Etiocholanolone	4.65
Androsterone	1.70
Dehydroepiandrosterone	0.46
Epitestosterone	0.50
17α-Estradiol	0.60

Incubations were carried out as described in the General Methods. The amount of enzyme assayed with testosterone was 2 µg (final concentration 16 nM) and the amount assayed with other substrates were adjusted accordingly. Steroid concentration was 1 µM and the assay was carried out in 67 mM glycine-NaOH buffer pH 9.5.

Figure 30

Lineweaver-Burk Plot for Enzyme Form IIIY and Testosterone.

Incubations were carried out under the conditions described in the General Methods (p. 34) except that the incubation time was 10 min. Each point on the graph is an average of three incubations. Velocity (V) has the units pmol product/min. The value obtained from each incubation was considered in the linear regression analysis. 0.75 µg of protein was used per incubation.


160
120
V
80
40
0
0
2
4
6
8
[TESTOSTERONE] (μM)
0·012
0·010
0·008
1/V
0·006
0·004
0·002
-0·8
-0·6
-0·4
-0·2
0
0·2
0·4
0·6
0·8
1·0
[TESTOSTERONE]-1 (μM)-1

3. Comparative studies on the properties of the multiple forms of the 17β-hydroxysteroid dehydrogenase

Comparative studies on the properties of the soluble 17β-HSDs purified from the liver of 28- and 56-day-old rabbits were carried out in order to establish the similarities and the differences among these enzyme forms.

a) Substrate specificity

The rates of oxidation of various substrates, as compared to the oxidation of 17β-estradiol, by enzyme forms I, II and III from the liver of the 56-day-old rabbit and enzyme form IIIY from the liver of the 28-day-old rabbit are shown in Table 12. The data for enzyme forms I, II and III has been shown previously in Table 5 (p. 92). Table 12 shows that the general substrate specificity profile of enzyme form IIIY differs from those obtained with the enzyme forms from the older rabbit. However some similarities are observed when activities of the enzyme forms toward individual substrates are compared. The activity of enzyme IIIY toward 17β-estradiol 3-glucuronide is similar to that shown by enzymes I and II. The activity of enzyme IIIY toward testosterone and androsterone is intermediate between enzymes I and III and the activity of enzyme IIIY toward etiocholanolone is most similar to enzyme I.

b) Isoelectric Focusing Polyacrylamide Gel Electrophoresis

The isoelectric points for enzyme forms I, II, III and IIIY determined by chromatofocusing chromatography were 5.60, 5.45, 5.35 and 5.20 respectively. Enzyme forms III and IIIY were further compared by isoelectric focusing polyacrylamide gel electrophoresis (General Methods p. 40). Fig. 31 confirms that the isoelectric point of enzyme IIIY is more acidic than

Table 12

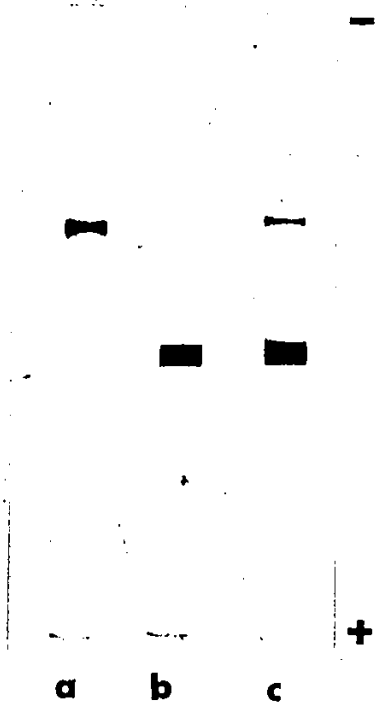
Rate of Oxidation of Substrates as Compared to Oxidation of 17β-Estradiol by the Soluble 17β-Hydroxysteroid Dehydrogenase of Female Rabbit Liver

Substrates	Enzyme			
	I	II	III	IIIY
17β-Estradiol	100	100	100	100
17β-Estradiol-3-glucuronide	20	20	110	24
Testosterone	2573	1100	4757	3150
Androsterone	80	18	471	274
Etiocholanolone	673	436	3142	750

Figure 31

Isoelectric Focusing Polyacrylamide Gel Electrophoresis of Enzyme Forms III and IIIY.

Electrophoresis and substrate staining were carried out as described in General Methods (p. 40). Approximately 2000 μunits of enzyme activity toward the substrate testosterone was applied to the gel. Gel a, enzyme III; gel b, enzyme IIIY and gel c, enzyme III plus IIIY.


a
b
c
+

that of enzyme III. Similar results were obtained when enzyme IIIY was compared to enzymes I or II (data not shown).

c) Amino acid composition

The majority of the data accumulated on the properties of the different forms of the 17β-HSD indicates that these enzyme forms are distinct entities. Therefore studies were undertaken to investigate whether these differences were a result of differences in the primary structure of the enzyme forms.

The enzymes were subjected to amino acid analysis and the data obtained are presented in Table 13. No major differences in amino acid composition among the enzyme forms were observed. Although the data presented in Table 13 are not corrected for incomplete recovery and present a single set of determinations, there are possible differences in amino acid composition among the multiple forms of 17β-HSD. Enzyme form IIIY has a higher glycine, alanine and valine content and lower in the tyrosine residues than the other multiple forms. Moreover, enzyme form III is lower than others in the glycine content (Table 13).

Since heterogeneity in proteins can be caused by the presence of carbohydrates, we examined the multiple forms of 17β-HSD for the presence of amino sugars, common components of glycoproteins. This analysis was initially carried out on the enzyme fraction obtained after affinity chromatography. Glucosamine or galactosamine was not detected in this fraction which contains all forms of the enzyme. This result was confirmed when individual enzyme forms I, II, III and IIIY were analyzed for these two amino sugars.

Table 13

Amino Acid Composition of the Multiple Forms of the 17β-Hydroxysteroid Dehydrogenase of Female Rabbit Liver.

Proteins (100 µg) were hydrolyzed as described in the General Methods (p. 42) for 16 h and analysis were performed with a Durrum D-500 amino acid analyzer. Cysteine was determined as cysteic acid by the method of Hirs (1956). Tryptophan and amide content of the proteins were not measured. Values are not corrected for incomplete recovery and represent a single set of determinations. The residues/mol protein were estimated on the basis of the molecular weight of each protein determined by SDS-polyacrylamide gel electrophoresis. The residue number was calculated by multiplying the nmol percent of each amino acid by a number until the molecular weight of the sum of the amino acid residues approximated the molecular weight determined by SDS-polyacrylamide gel electrophoresis. The molecular weights listed in the table are the sums of the molecular weights of the amino acid residues of each protein. Asx = aspartic acid + asparagine; Glx = glutamic acid + glutamine.

Table 13

Amino Acid Composition of the Multiple Forms of 17β-Hydroxysteroid Dehydrogenase of Female Liver

Amino Acid Residue	Nearest Integral Number of Residues (nmol residues/nmol protein) ENZYME I	II	III	III Y
Cys	8	7	9	11
Asx	37	40	38	35
Thr	17	18	16	23
Ser	24	24	17	25
Glx	40	43	40	40
Pro	8	14	8	12
Gly	28	30	20	37
Ala	29	31	25	35
Val	18	20	16	27
Met	12	12	10	10
Ile	16	17	17	14
Leu	39	37	38	39
Tyr	12	12	15	9
Phe	13	14	14	13
His	10	9	10	10
Lys	24	22	23	19
Arg	12	9	7	12
Number of residues	347	359	323	371
Molecular weight	37,900	39,682	36,959	40,273

d) Peptide mapping

The primary structures of the multiple forms of the 17β-HSD were further compared by peptide mapping. Because of the small quantities of the purified enzymes available, the usual method for peptide mapping was not practical. The sensitivity of the method was increased by using a radioactive reagent which would generate radioactive peptides upon proteolytic digestion. Acetylation of amino groups in the protein sample (the ε-amino groups of lysine residues and the α-amino group of the amino terminus) with [^{14}C] acetic anhydride was selected for the following reasons: a) lysine residues usually occur in sufficient abundance in proteins to ensure that an adequate number of radioactive spots would arise to facilitate the detection of differences between the proteins; b) acetylation of amino groups is rapid, mild and relatively group specific and c) lysine residues are usually exposed on the surface of the molecule and therefore readily accessible to chemical reagents. Since it is known that changes which occur in the primary sequence of closely related enzymes are usually associated with the outer core of the protein, this method would then best illustrate any differences occurring among the multiple forms of the enzymes.

As described in the General Methods (p. 43) the papain digest of each acetylated protein was first electrophoresed at pH 6.5. The autoradiogram of the acetylated proteins obtained after this procedure showed no distinct differences among these enzyme forms (data not shown). Each strip of radioactive material was then subjected to electrophoresis in the second dimension at pH 2.1 and autoradiographs of the peptide maps obtained are

shown in Figs. 32-35. It is evident from the peptide maps that there is considerable homology among the enzyme forms (Figs. 32-35). The most apparent differences are found in enzyme III which has 3 peptides marked a, b and c (Fig. 34) that are not present in the other forms. In addition enzymes III and IIIY have a peptide (d) not present in form I and II. Although close examination of the peptide maps reveals other minor differences it is not possibily to establish from the autoradiographs where these differences are due to different peptides or just quantitative differences.

When the peptides that were neutral on electrophoresis at pH 6.5 were subjected to electrophoresis at pH 2.1, no distinct bands were observed. This band was therefore subjected to electrophoresis in the second dimension at pH 3.5. The peptide maps obtained for the enzyme forms I, II, III and IIIY are shown in Figs. 36-39. The peptide maps again reveal considerable homologies among these enzyme forms. However, unique peptides (marked 1-8) are apparent in enzyme forms I, II and III.

e) Amino-terminal identification

Identification of the amino-termini of the multiple forms of 17β-HSD of female rabbit liver cytosol was carried out according to the method of (Kaplan et al., 1983) described in the General Methods (p. 46). According to this method, the Dnp-amino acid with the highest $[^3H]/[^{14}C]$ ratio is the amino-terminal residue. Table 14 shows an analysis of the amino-termini of the multiple forms of 17β-HSD of female rabbit liver cytosol. For enzyme form I, the amino-terminus is serine. For enzyme form II serine is the major amino-terminus;however, alanine may be a significant component. Enzyme form III has either aspartic acid or asparagine and serine as the amino terminus. The analysis suggests that enzyme form III may be contaminated with enzyme II. This is likely since enzyme forms I, II and III are eluted

Figure 32

Autoradiogram of the Peptide Map of ^{14}C-acetylated Enzyme I.

The papain digest of the ^{14}C-acetylated enzyme I was electrophoresed in the first dimension at pH 6.5 for 45 min at 3000 volts and then in the second dimension at pH 2.1 for approximately 75 min at 60 volts/cm.

(-)
pH 6.5
(+)
(-)
pH 2.1
(+)

Figure 33

Autoradiogram of the Peptide Map of ^{14}C-acetylated Enzyme II.

Electrophoresis was carried out as described in Fig. 32.

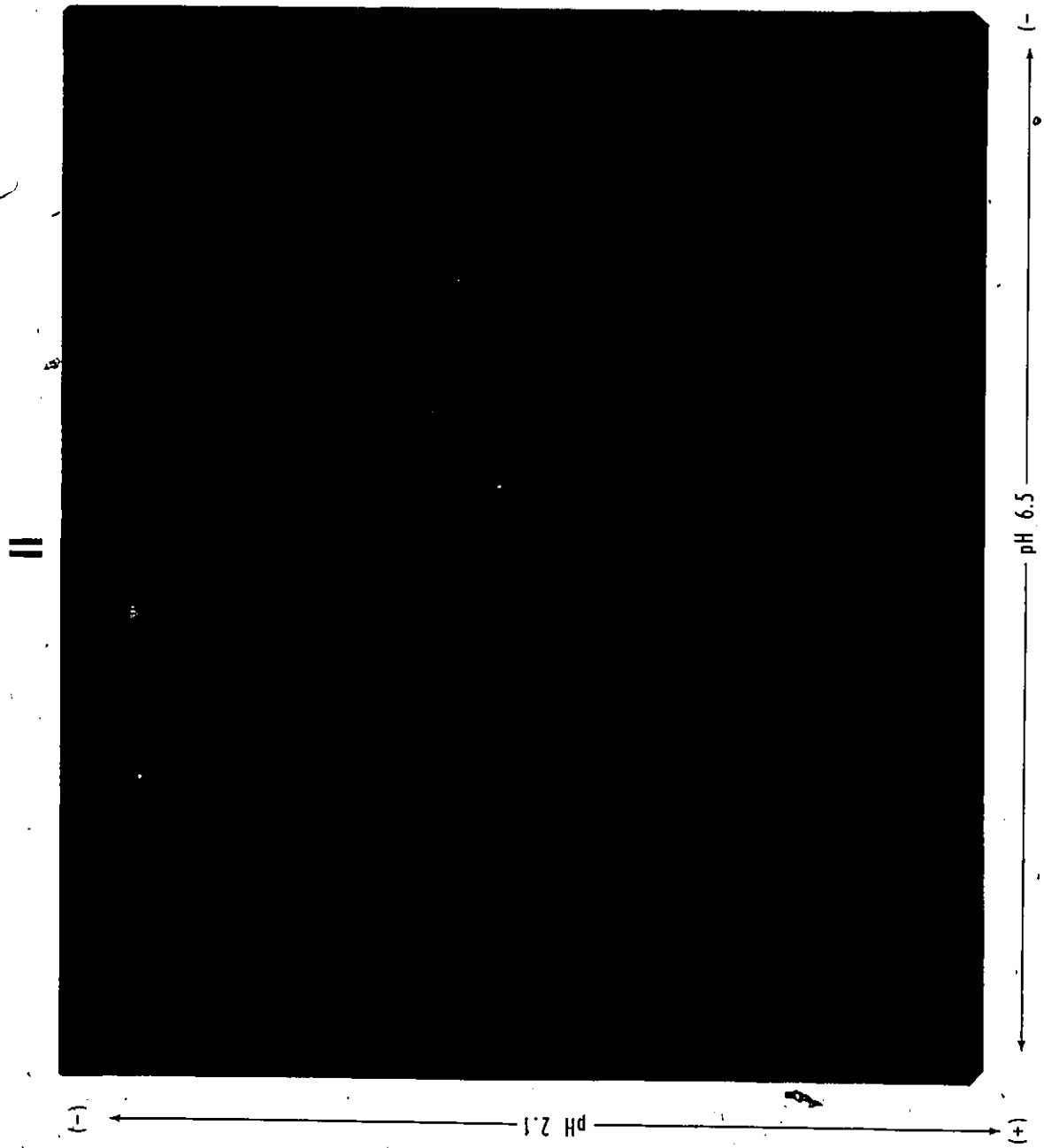

pH 6.5
(-)
(+)
pH 2.1
(-)
(+)

Figure 34

Autoradiogram of the Peptide Map of ^{14}C-acetylated Enzyme III.

Electrophoresis was carried out as described in Fig. 32.


(+)
pH 2.1
(-)
pH 6.5

Figure 35

Autoradiogram of the Peptide Map of ^{14}C-acetylated Enzyme III Y.

Electrophoresis was carried out as described in Fig. 32.

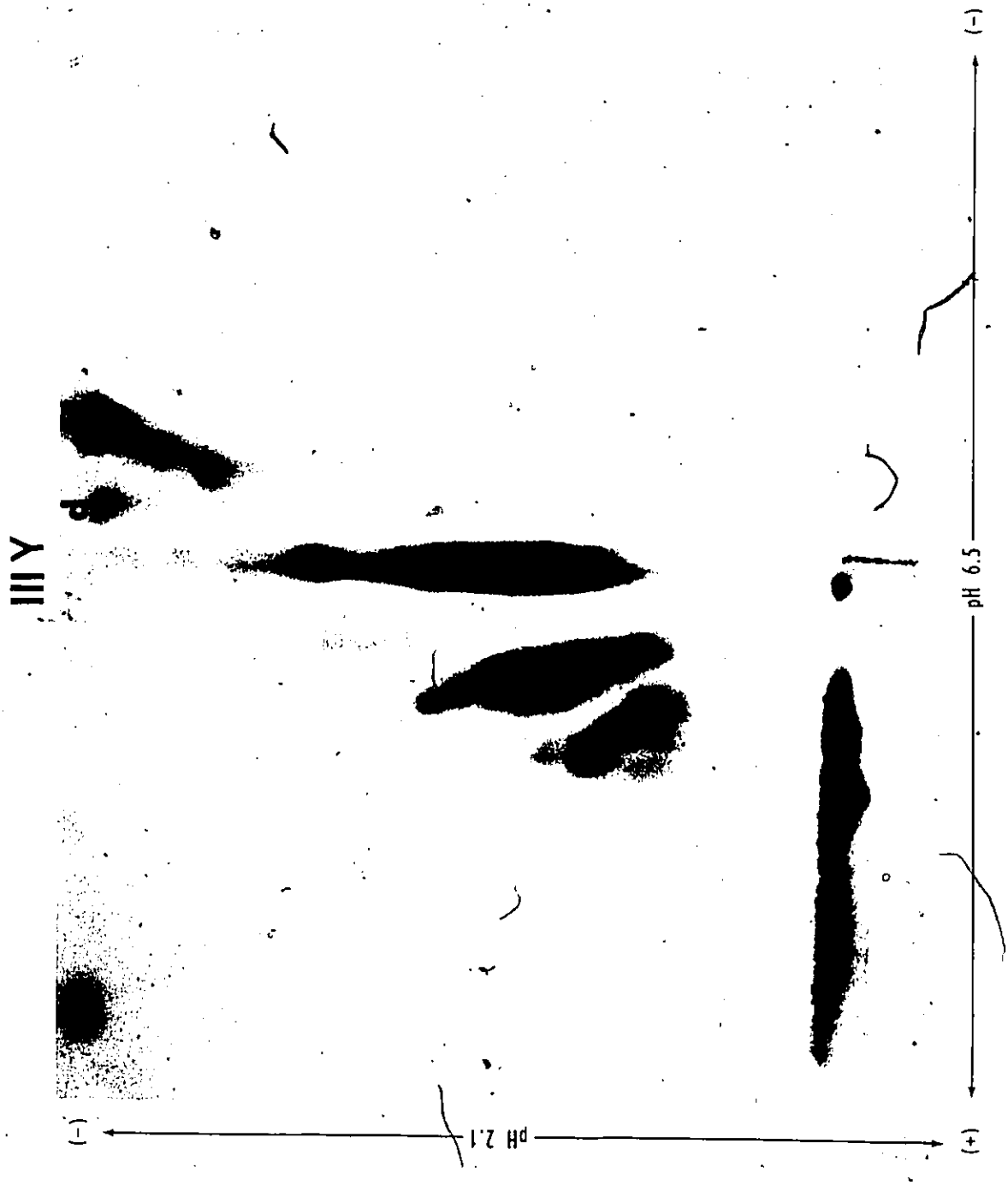

III Y
pH 2.1
pH 6.5
(-)
(+)
(-)

Figure 36

Autoradiogram of the Peptide Map of the Neutral Peptides of Enzyme I.

The neutral peptides of enzyme I at pH 6.5 were subjected to electrophoresis in the second dimension at pH 2.1. The band of peptides was cut out and electrophoresed at pH 3.5 for 90 min at 60 volts/cm. The spot located at the lower right corner of the autoradiogram is due to the radioactive identification marker.

pH 2.1
(-)
(+)
pH 3.5

Figure 37

Autoradiogram of the Peptide Map of the Neutral Peptides of Enzyme II.

Electrophoresis was carried out as described in Fig. 36.

pH 3.5
pH 2.1
(+)
(−)

Figure 38

Autoradiogram of the Peptide Map of the Neutral Peptides of Enzyme III.

Electrophoresis was carried out as described in Fig. 36.


pH 3.5
(-)
(+)
pH 2.1

Figure 39

Autoradiogram of the Peptide Map of the Neutral Peptides of Enzyme III Y.

Electrophoresis was carried out as described in Fig. 36.

AIII
pH 2.1
(-)
(+)
pH 3.5
(-)

Table 14

Analysis of Amino-Termini of the 17β-Hydroxysteroid Dehydrogenase Multiple Forms ($[^3H]/[^{14}C]$ ratios)

	Enzyme			
Amino Acid	I	II	III	IIIY
Asx	2.2	0.9	5.6	4.2
Thr	1.2	0.8	0.8	0.9
Ser	10.4	3.9	3.7	1.3
Glx	2.3	1.0	1.2	1.2
*Pro	0.0	0.0	0.0	0.0
Gly	1.4	1.5	2.1	0.0
Ala	1.6	2.1	1.3	0.0
Val	0.4	0.7	0.6	0.0
Met	0.7	1.6	1.5	0.0
Ile	0.5	0.2	0.7	0.0
Leu	0.5	0.3	0.6	0.0
Tyr	0.0	0.7	0.8	0.0
Phe	1.5	0.0	0.0	0.0
His	0.2	0.1	0.3	0.1
Lys	0.5	0.0	1.2	0.0
Arg	0.0	0.5	0.2	0.2

*The proline derivative of Dnp was completely destroyed under the digestion conditions.

sequentially from a chromatofocusing chromatography column in the final step of their purification. Aspartic acid or asparagine is the only amino-terminus obtained for enzyme form IIIY.

f) Carboxyl-terminal peptides

The carboxyl-terminal peptides of the multiple forms of the soluble 17β-HSD of female rabbit liver were compared by the method of Duggleby and Kaplan (1974). This method is based on the fact that when an enzymatic digestion is performed on a protein in which all the free carboxyl groups have been modified by coupling with [^{14}C] ethanolamine, the carboxyl-terminal peptides will be unique in that they lack free carboxyl groups. A carboxyl-blocked peptide will have the same electrophoretic mobility at pH 4.4 and at pH 2.1, whereas, a peptide with a free carboxyl group will migrate more rapidly toward the cathode at pH 2.1 than at pH 4.4. Using a diagonal electrophoretic procedure based on this property, carboxyl-terminal peptides can be identified and compared. The results showing the two dimensional electrophoretogram obtained from a papain digestion of enzyme forms I and II are shown in Figs. 40 and 41 (data for enzyme forms III and IIIY are not shown). No observable differences are apparent in the carboxyl-terminal peptides of enzyme forms I and II. Enzyme forms III and IIIY gave a similar pattern (data not shown).

Table 15 shows the relative mobilities of carboxyl-terminal peptides of enzyme forms I and II compared to the mobility of the marker glycinamide at pH 4.4 and 2.1 calculated from Figs. 40 and 41. Again, no major differences are observed between the carboxyl-terminal peptides of enzyme forms I and II.

Figure 40

Autoradiogram of the Two-Dimensional Electrophotogram of Carboxyl-Terminal Peptides of Enzyme I.

The papain digest of the ^{14}C-ethanolamine enzyme I was electrophoresed first at pH 4.4 and then at pH 2.1 as described in the General Methods (p. 52). The peptides lying on the diagonal line between the two markers taurine (T) and glycinamide (G) are the carboxyl terminal peptides.

(+)
2.1 m
(-)
(+)
4.4 m
(-)

Figure 41

Autoradiogram of the Two-Dimensional Electrophoretogram of Carboxyl-Terminal Peptides of Enzyme II.

Electrophoresis was carried out as described in Fig. 40.

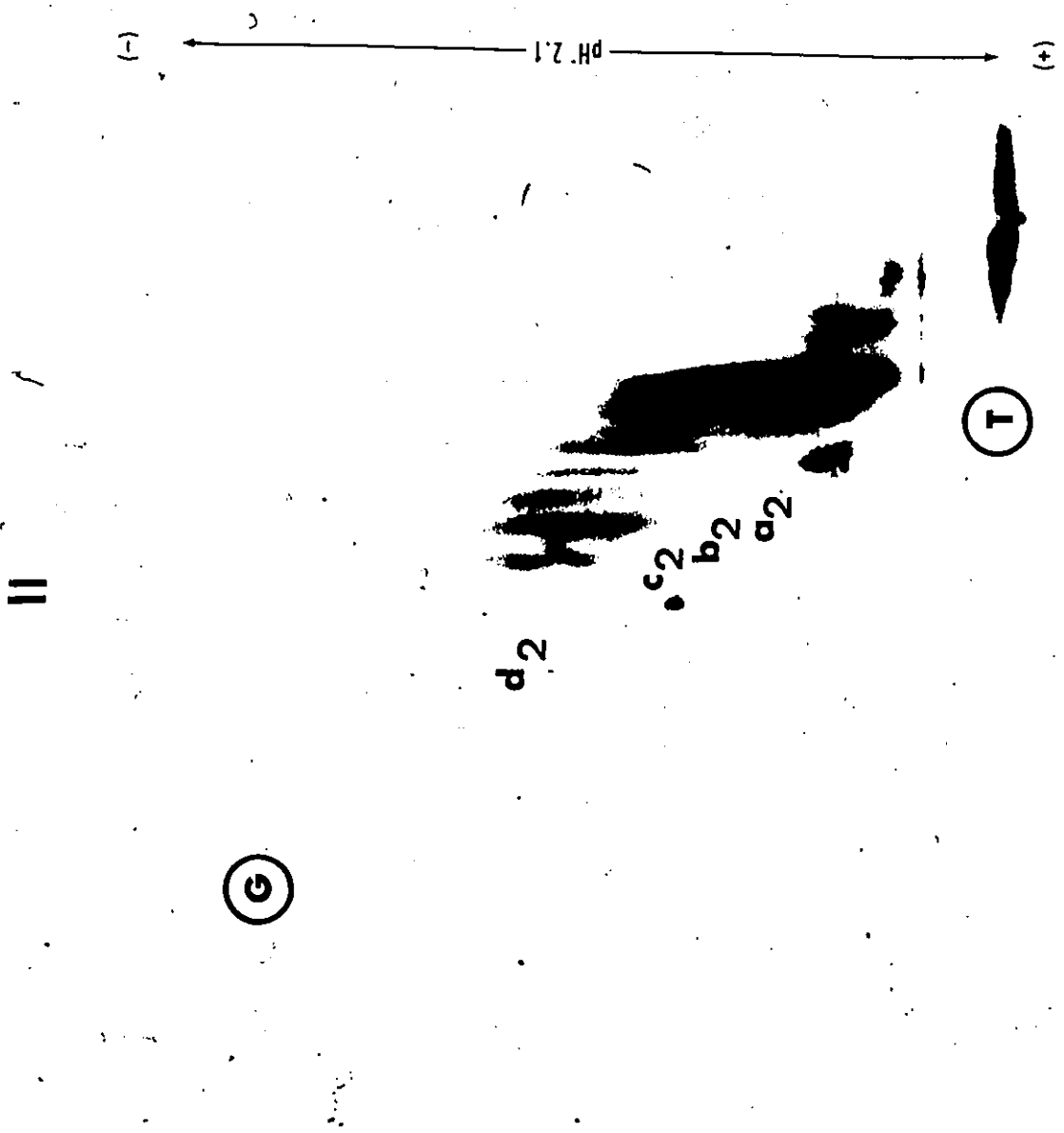

pH 2.1
(+)
(-)
pH 4.4
(+)
(-)
T
O
II
d_2
c_2
b_2
a_2

Table 15

Relative Mobility of Carboxyl-Terminal Peptides of Enzyme Forms I and II Compared to the Mobility of Glycinamide at pH 4.4 and 2.1

pH	Relative Mobility of Carboxyl-Terminal Peptides of Enzyme							
	I				II			
	a_1	b_1	c_1	d_1	a_2	b_2	c_2	d_2
4.4	0.29	0.38	0.43	0.74	0.28	0.35	0.38	0.66
2.1	0.28	0.39	0.45	0.73	0.30	0.39	0.44	0.69

DISCUSSION

17β-HSD activity has been shown to be age-dependent in some mammalian tissues. Inano et al. (1967) reported that the 17β-HSD activity of rat testis increased with age reaching a maximal level in 60-day-old animals. More recently, Furubayashi et al. (1982) stated that the 17β-HSD in the testis of the adult golden hamster was approximately 10 times that in this tissue from immature animals. Ghraf et al. (1975c) carried out an extensive examination of the 17β-HSD in the cytosol and microsomal fractions from a variety of tissues including the kidney, liver, adrenal and gonads of male and female rats 15- to 120-days-old. The enzyme activity in at least one of the subcellular fractions of each tissue displayed an age-dependent development, maximal levels of activity being attained in animals 60-days-old. Bongiovanni and Marrino (1972) measured 17β-HSD activity in rabbit liver homogenates during different stages of development. No 17β-HSD activity could be detected in the fetal liver homogenates while activity was present in the liver homogenates of the young rabbit (20-days-old) and mature rabbit liver homogenates (17-19-months-old).

Data presented in this thesis have demonstrated that the soluble 17β-HSD activity of female rabbit liver increases with the age of the animal. The 17β-HSD pattern obtained by chromatofocusing chromatography of the partially purified 17β-HSD from rabbits at the age of 28- and 56-days suggests that the increases in total 17β-HSD activity between the 28- and 56-days-old rabbit could be due to a relative increase in one of the enzyme forms (peak I, Fig. 27). However, the pattern of the multiple forms of the 17β-HSD observed with rabbits of intermediate age suggests a more complex modification of the enzyme activity could be occurring during development (Fig. 27).

Conclusions with regard to the developmental aspects of this enzyme must await a structural comparison of the enzyme forms in rabbits of intermediate age (e.g. 5 to 6 weeks) with the forms present in the 4- and 8-week-old animal.

The major enzyme form (IIIY) of the 28-day-old rabbit liver and the enzyme form III of the 56-day-old rabbit liver are the most acidic forms of each age in terms of their elution on chromatofocusing and their isoelectric points. Both of these enzyme forms have aspartic acid or asparagine as amino-termini and have similar carboxy-terminal peptide patterns. However enzyme forms III and IIIY do differ in their primary structure to some extent and also differ in substrate specificity. Only enzyme form III exhibits the high 3α-HSD activity and low Km toward the 5β-androstane substrate etiocholanolone (Table 8). Although enzyme IIIY is similar to enzyme I and II in the substrate specificity, it differs from these enzyme forms at its amino-terminal and has minor differences in primary structure as revealed by peptide mapping. Thus from these studies it can be concluded that the major form of the 17β-HSD present in the 4-week-old animal (enzyme IIIY) is not present in animals 8 weeks of age. If enzyme III is the major activity responsible for the oxidation/reduction of 3α-hydroxysteroids of the 5β-androstane series as suggested by the in vitro kinetic data, then at 4 weeks of age the rabbit would have a relatively lower ability to metabolize androgens due to the absence of this enzyme form. Enzyme III is also the enzyme form which, in vitro exhibits a higher 17β-enzyme activity toward androgens in the reductive direction and this enzyme could be important in vivo for the inactivation of testosterone. Again, the absence of this activity in the liver of 28-day-old animals could

indicate a reduced capability of animals at this age to carry out this inactivation. Alternatively the absence of this enzyme could reflect the possibility that circulating levels of testosterone are lower in animals at this age.

With regard to the forms of the 17β-HSD present in the 8-week-old rabbit, the results obtained by peptide mapping and amino- and carboxyl-terminal studies support the conclusion that the three forms (I, II and III) are distinct enzymes, differing in primary structure. Enzyme I and II are the most similar, having the same amino-terminal amino acid residue, the same carboxyl-terminal peptide patterns and the greatest degree of structural homology as revealed by comparison of their peptide maps. These structural similarities are in agreement with the similar substrate specificities and kinetic properties of the two enzymes. In contrast, there are more distinct differences in enzyme III both in terms of structure (amino-terminal residue and peptide maps) and substrate specificity.

The similarities among the multiple forms of the 17β-HSD of female rabbit liver cytosol as shown by peptide mapping, amino-terminal and carboxyl terminal peptides data are consistent with the hypothesis that the enzyme forms could arise from multiple but similar genes. Alternatively, the different forms could be a result of postsynthetic modification of the proteins by deamination of asparagine and glutamine residues. Deamination has been shown to be a significant *in vivo* modification step. For example, in rabbit muscle aldolase, deamination of an asparaginyl residue of each subunit gave rise to five enzyme forms (Midelfort and Mehler, 1972). Williams and John (1979) were able to generate multiple forms of aspartate transaminase from pig heart cytosol *in vitro* by deamination and these forms

were fully active. Successive deaminations of the rabbit liver 17β-HSD would endow the proteins with a progressively more negative charge producing a series of charge isomers of the enzyme.

The presence of carbohydrate can also produce heterogeneity in proteins. For example, the heterogeneity of aspartate transaminase of pig heart cytosol (Denisova and Polyanovasky, 1973) and adenosine deaminase of human liver (Swallow et al., 1977) have been shown to be due in part to carbohydrate. Our studies have shown that the 17β-HSD of rabbit liver is devoid of glucosamine or galactosamine. These amino sugars are usually found directly attached to the peptide chain of glycoproteins (Spiro, 1973). It is therefore unlikely that the 17β-HSDs are glycoproteins and that the heterogeneity results from differences in carbohydrate composition. Indeed, no 17β-HSD of mammalian (Burns et al., 1971; Pons et al., 1977; Kobayashi and Kochakian, 1978) or bacterial (Schultz et al., 1977) origin has been shown to be glycoprotein. The absence of the amino sugars glucosamine and galactosamine does not exclude the possibility that other carbohydrate attached to the 17β-HSD of female rabbit liver cytosol contribute to the heterogeneity of this enzyme.

An alternate explanation for the multiple forms of the soluble 17β-HSD of rabbit liver is that they are the products of protease action on the 17β-HSD. Although, as described previously it is unlikely that this occurred during the purification procedure it is possible that such a process could occur in vivo or immediately upon isolation of the liver. A comparison of enzyme forms I and II reveals that these forms have the same amino-termini, serine, and very similar carboxyl-terminal peptides. However, these enzyme forms do exhibit differences in their primary structure as revealed by peptide

mapping. Together these results suggest that these differences occur within the interior of the enzyme structures and that one enzyme form does not arise from proteolysis of the other form unless this proteolytic cleavage generates a new amino-terminal serine residue. The data obtained in our studies do not rule out the possibility that enzyme III is produced by proteolysis of enzyme I or II *in vivo* since enzyme form III has a slightly lower molecular weight than enzyme forms I and II had has a different amino-terminal residue.

Forms of the 17β-HSD more acidic than those purified by chromatofocusing have also been identified in the present studies. These acidic forms were not retained by affinity chromatography and could utilize NAD^+ as cofactor. It is unlikely that these acidic enzyme forms are converted by proteolysis to the less acidic forms of the enzyme since an alternation would have to be accompanied by a loss of the enzyme's ability to utilize NAD^+ as a cofactor. The structural relationship of the acidic enzymes to the less acidic forms must await purification and structural analysis of these acidic enzymes.

Postsynthetic modification could be also responsible for the changes in the multiple forms of the 17β-HSD observed during development in the rabbit. Thus enzyme form IIIY, the major form present in the 28-day-old rabbit could be a precursor of the enzyme forms in the 56-day-old rabbit and the enzyme forms observed in animals between these two ages could represent different stages of this modification process. Enzyme form IIIY shows more similarity to enzyme forms I and II in terms of substrate specificity and peptide mapping than to enzyme form III. That enzyme I or II is formed during development in the rabbit by removal of a peptide fragment from the amino-terminal region of enzyme IIIY is a possibility. This hypothesis is

also supported by the fact that enzyme form IIIY has a different amino-terminal residue (aspartic acid or asparagine) than enzyme forms I and II (serine) and by the higher molecular weight of enzyme form IIIY than enzyme forms I and II. It is less likely that enzyme IIIY is the precursor of enzyme III. There is considerable difference in the peptide maps and substrate specificities of the two enzymes even though they have the same amino-terminal amino acid residue. It is unlikely that the enzyme forms present in the 56-day-old rabbit are produced by a modification of the enzyme of the 28-day-old animal through deamination since the enzyme form present at 28 days is more acidic than any of the forms present at 56 days.

The soluble 17β-HSD of rabbit liver could be regulated by steroid hormones and the changes in 17β-HSD multiple forms during development could result from increases in steroid (testosterone and estrogens) levels in the blood due to maturation of gonadal tissue. Kochakian et al. (1973) have reported that the activity of the soluble 17β-HSD of the female guinea pig kidney was decreased after castration and restored by testosterone treatment. The difference in activity was attributed to the disappearance and appearance of one of the multiple forms of the enzyme. However, in more recent studies, Shen and Kochakian (1978) were not able to confirm this result although they did observe a decrease in the 17β-HSD activity after castration. These workers also showed that the soluble and microsomal 17β-HSD activities of female guinea pig kidney were low toward several hydroxy-C_{19}-steroids. The administration of testosterone induced an increase in the 17β-HSD activity of the cytosol but not the microsomes to the normal male kidney level. In contrast, the liver enzyme activity did not respond to the testosterone indicating that the regulation of the liver enzyme in the female guinea pig is under different regulatory mechanism than that of the kidney enzyme.

It has been shown that the 17β-HSD activity is different in the liver of male and female rats (Thaler-Dao and Breuer, 1974; Ghraf et al., 1975b; Breuer et al., 1979). Ovariectomy of the female rat resulted in increase in the soluble liver 17β-HSD activity to that observed in the male rat (Breuer et al., 1979). On the basis of this finding it was speculated that estrogens appeared to exert a repressing effect on the activity of the 17β-HSD in adult rats. Further evidence for the repressing effect of 17β-estradiol has been provided by the variation in 17β-HSD activity during the estrus cycle; high enzyme activities were observed during metoestrus and dioestrus and low enzyme activity during pro-estrons. Breuer et al. (1979) have proposed the following possible mechanisms by which estrogens may act as repressors of the 17β-HSD multiple forms activity in male and female rat liver: (1) estrogens alter the affinities of the enzyme for substrates, e.g. testosterone, possibly through a direct modification of the enzyme molecule, (2) estrogens might induce a protein which interferes with the formation of enzyme substrate complexes and (3) the difference between the specific activities on one hand and the similarities of the Km values on the other makes it likely that estrogens have a repressing effect on enzyme synthesis.

To date no studies have been carried out in the rabbit to establish the relationship between the age of the animal and the circulating levels of steroid hormones. Such studies must be carried out to determine whether the alternation in the enzyme forms of the 17β-HSD of rabbit liver are hormone-dependent.

CONCLUSION

Three forms of the soluble 17β-HSD of female rabbit liver have been purified and characterized for the first time, in this thesis, by a combination of affinity chromatography and chromatofocusing. These forms are not artifacts produced during the purification procedures. With the studies presented in Chapter III, we were able to show that the forms of the 17β-HSD also exhibit 3α-HSD toward C_{19}-steroids, with a preference for the 5β-androstane substrates. Furthermore, kinetic analysis revealed that one of these forms has higher 3α-HSD activity than 17β-activity at low substrate concentrations. We have also provided evidence of the common identity of the 3α- and 17β-HSD activities of the rabbit liver cytosol enzyme by the criteria of their co-migration on polyacrylamide gel electrophoresis. Kinetic analysis with the steroid substrates testosterone and etiocholanolone indicated a competitive inhibition pattern, supporting the hypothesis of a common active site in the enzyme for these two substrates.

The enzyme forms of the 17β-HSD of female rabbit liver cytosol all exhibit activity toward both androgen and estrogen substrates, however activity toward the former is higher than that toward the latter. In vitro the enzymes have activity in both the oxidative and reductive directions, however, there are differences in this respect among the enzyme forms. In particular, one enzyme form shows a distinct preference for the oxidative direction with androgen substrates and for the reductive direction with estrogen substrate. Thus in vivo this enzyme form may function in the liver to inactivate androgens and activate estrogens.

Amino acid composition, peptide maps, amino-terminal identification

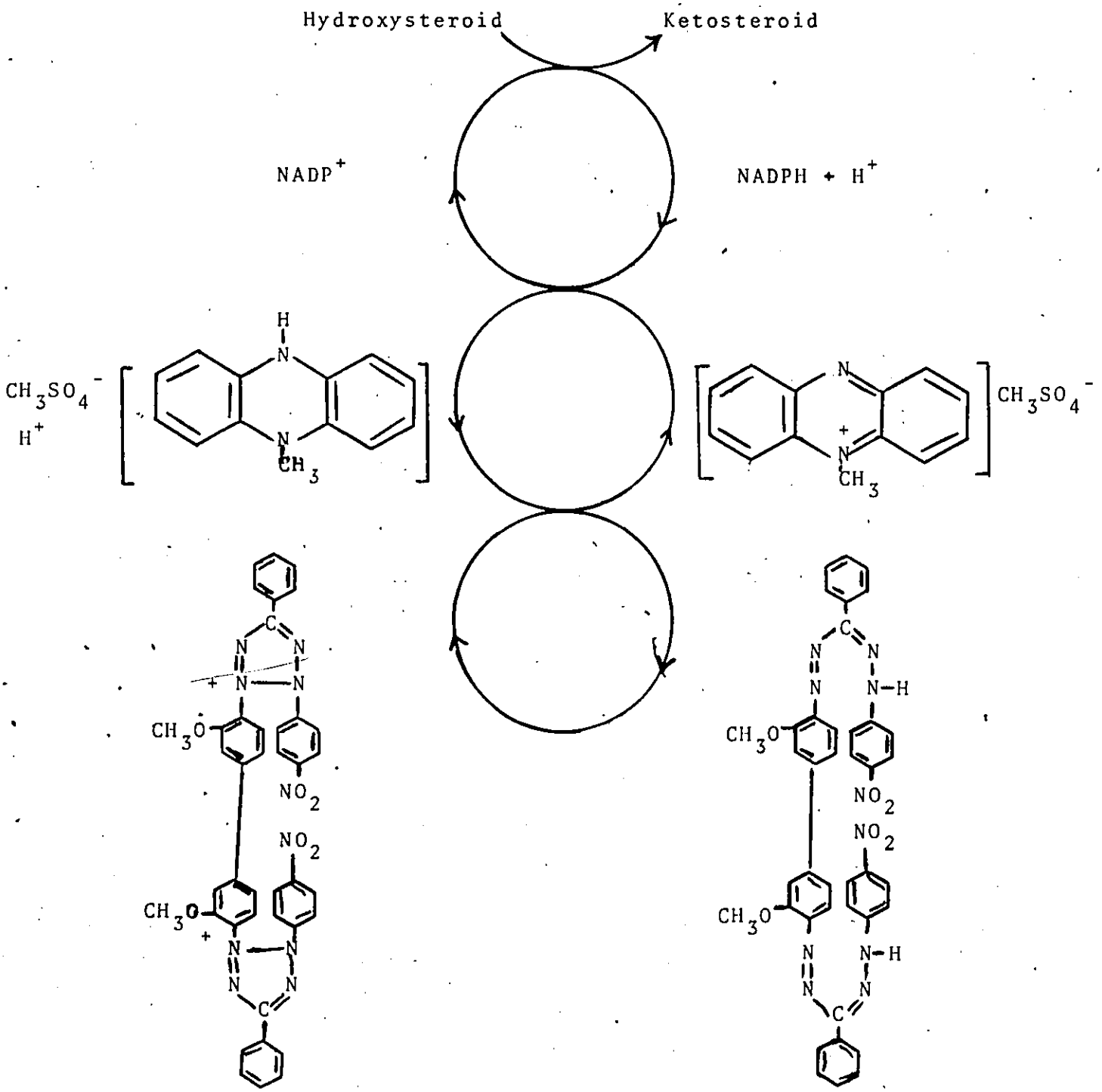
and carboxyl-terminal peptide maps show that enzyme forms I and II have the most structural homology while enzyme III is distinct from both of these forms. This structural homology is in agreement with the comparative substrate specificities of the three enzyme forms.

Our results also show for the first time that there are age-related changes in the multiple forms of the soluble 17β-HSD of female rabbit liver. One major form of the soluble 17β-HSD is present in the liver of the 28-day-old rabbit and this form (IIIY) has been purified. The enzyme form (IIIY) is similar to enzyme form III of the 56-day-old rabbit in terms of its elution on chromatofocusing, amino acid composition, amino-terminal residue and carboxyl-terminal peptides. However, isoelectric focusing polyacrylamide gel electrophoresis reveals that these enzyme forms are distinct and exhibit differences in substrate specificity and primary structure. Indeed, enzyme form IIIY is more similar in these respects to enzyme forms I and II. These data led us to postulate that enzyme form IIIY is converted to enzyme form(s) I and/or II by the removal of a peptide fragment from the amino-terminal during development in the rabbit.

Further experiments could be directed to: (1) purification and structural comparison of the multiple forms of 17β-HSD of rabbit liver cytosol including the most acidic forms during the stage of development, (2) study the relationship between the age of the animal and the circulatory levels of steroid hormones and (3) the relationship between ovariectomy and the multiple forms pattern of 17β-HSD of female rabbit liver.

APPENDIX I

Reduction of NBT by Hydroxysteroid Dehydrogenase

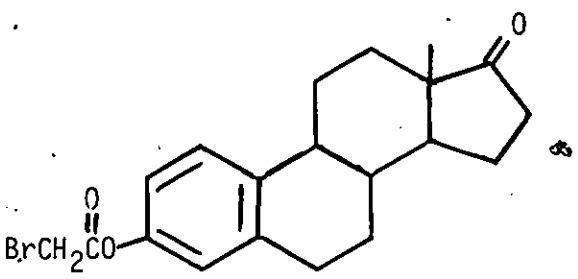


NBT (2 Cl^-)

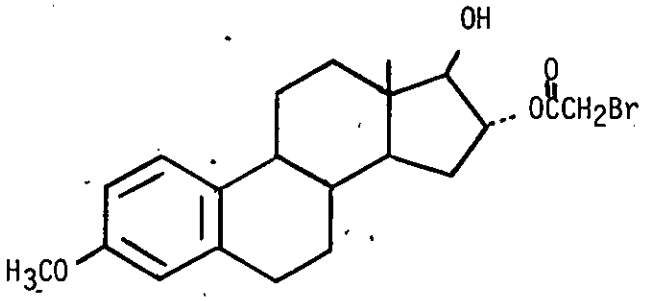
Diformazan

APPENDIX II

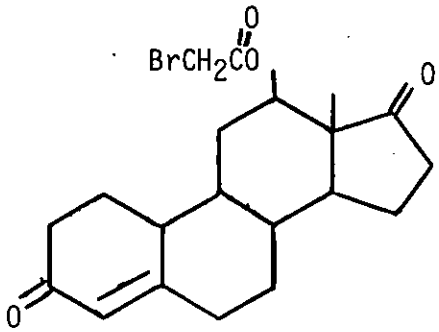
Structures of Modified Steroids used in Affinity Labeling



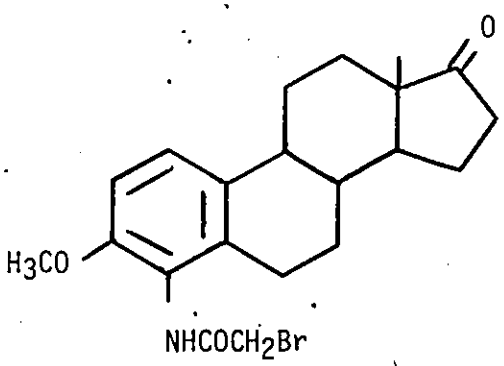
3-Bromoacetoxyestrone



16α-Bromoacetoxyestradiol
3-methyl ether



12β-Bromoacetoxy 4-estrene
3,17-dione



4-Bromoacetamidoestrone
3-methyl ether

(Refer to Pons et al., 1977 for other affinity labels)

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