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**LA THÈSE A ÉTÉ  
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MULTIPLE FORMS OF THE  
3(17) $\alpha$ -HYDROXYSTEROID DEHYDROGENASES OF  
RABBIT KIDNEY CYTOSOL: COMPARISON WITH THE CORRESPONDING LIVER ENZYMES

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Dedicated  
to those who care  
though not necessarily understand

SUMMARY

Both the NAD(P)<sup>+</sup>-linked 17 $\alpha$ -hydroxysteroid dehydrogenase (HSD) and 17 $\beta$ -HSD activities were present in the soluble fraction of the female rabbit kidney. The NADP<sup>+</sup>-dependent 17 $\alpha$ -enzyme was present in considerably greater amounts than the 17 $\beta$ -enzyme. These two activities were separable by DEAE-cellulose column chromatography and isoelectric focusing in granulated gel. Multiple molecular forms of the 17 $\alpha$ -enzyme were evident by electrophoresis of the crude cytosol in polyacrylamide gels stained for enzyme activities, by DEAE-cellulose column chromatography and by isoelectric focusing. At least four forms of the 17 $\alpha$ -enzyme and a very acidic species were resolved by these techniques. Three of the enzymes were obtained in a homogeneous state judged by the criteria of protein staining in polyacrylamide gels and molecular weight determination. These enzyme forms were charge isomers with a mean molecular weight of 40,700. Substrate specificity studies revealed that these enzyme forms in addition to catalyzing dehydrogenation of 17 $\alpha$ -hydroxyl groups of both C<sub>18</sub>- and C<sub>19</sub>-steroids, also have 3 $\alpha$ -HSD activities toward C<sub>19</sub>-steroids having an A/B trans configuration. In general, the order of reactivity toward various substrates is: androsterone > 5 $\alpha$ -androstan-3 $\alpha$ ,17 $\beta$ -diol > epitestosterone > 17 $\alpha$ -estradiol 3-glucuronide > 17 $\alpha$ -estradiol. Etiocholanolone having an A/B cis configuration was a poor substrate. 5 $\alpha$ -Androstan-3 $\beta$ ,17 $\beta$ -diol was also negligibly oxidized. Two of the enzyme forms were distinctive - with the most basic species, the relative activity toward epitestosterone and 17 $\alpha$ -estradiol 3-glucuronide was reversed, contrary to the above order of substrate reactivity; on the other hand, the most acidic species displayed a high

specificity toward  $3\alpha$ -hydroxysteroids relative to  $17\alpha$ -hydroxysteroids. However, the  $17\beta$ -HSD activity was associated with the latter impure fraction.

Inhibition studies with non-radioactive steroids revealed that the  $C_{21}$ -steroids possessing a  $3\alpha$ -hydroxyl group and possibly the  $20\alpha$ - or  $20\beta$ -hydroxyl group are potential substrates for the rabbit kidney  $3(17)\alpha$ -HSDs. This wide spectrum of substrate specificity was also shown in this study to be exhibited by two corresponding enzyme forms of the  $17\alpha$ -HSDs from the female rabbit liver cytosol previously purified by Hasnain and Williamson, (1975, 1977).

The reductions of various steroids having a 3-ketone or 17-ketone group by two purified  $3(17)\alpha$ -enzyme forms from the liver and kidney, in the presence of NADPH, showed the following order of reactivity:  
 $5\alpha$ -dihydrotestosterone > androsterone > estrone 3-glucuronide > androstenedione > estrone 3-sulfate > estrone. Like the forward oxidative reactions, the reductive reaction at C-3 was higher than that at C-17. The presence of a double bond at C-4 impeded the reduction of the 3-ketone group. These results together with the oxidative reactions point out the importance of a saturated steroid A-ring in the reduction of the functional groups at C-3 or C-17. Pregnenolone also acted as a poor substrate for these purified enzymes. However, the 20-reduced metabolites formed were not fully resolved.

Both the liver and kidney possessed a  $17\alpha$ -HSD enzyme form that exhibited a higher specificity toward the 3-glucuronide derivatives of  $17\alpha$ -estradiol or estrone than the corresponding free steroids. The specific activity of the kidney enzyme toward  $17\alpha$ -estradiol 3-glucuronide was higher than that of the liver enzyme. This group of enzymes (LIB and KIB) is particularly important in the inactivation of estrogens in the rabbit. Enzyme forms LIB and KIB also shared a common apparent isoelectric point, molecular weight and  $K_m$  values

determined for the substrates androsterone and epitestosterone. In contrast to the above group of enzymes were the enzyme forms designated LIA and KII from the two tissues which were characterized by a low  $17\alpha$ -estradiol 3-glucuronide dehydrogenase activity and a lower isoelectric point. Like the liver and kidney IB enzyme, LIA and KII enzymes were very similar to each other with respect to their substrate specificities and ionic properties. All of the enzymes purified have similar molecular weight. In addition, the activities of all the enzymes were inhibited by low concentrations of PCMB,  $\text{Hg}^{2+}$  and  $\text{Cu}^{2+}$  ions.

Acetylation of enzymes LIA, LIB and KII by radioactive acetic anhydride and generation of peptide maps by subsequent papain digestion and high voltage electrophoreses at various pH's revealed extreme structural homologies between these enzyme forms. However, differences were also apparent among these peptide maps. It appeared that the kidney II and liver IA proteins have a similar gene distinct from the gene for the liver IB protein. The homologous structures of these proteins offered an explanation for the similarities and differences in properties shown by the two groups of enzymes isolated from the two tissues.

The common identity of the  $3\alpha$ - and  $17\alpha$ -HSD activities of the rabbit kidney cytosol was established by the criteria of their co-purification throughout the stages of the purification procedures and their co-migration in polyacrylamide gel electrophoresis stained with both  $3\alpha$ - and  $17\alpha$ -hydroxysteroids. Kinetic analysis with the steroids androsterone and epitestosterone indicated a competitive inhibition pattern supporting the notion of a common active site in the enzyme for the two substrates. The two liver enzyme forms displayed similar kinetic behaviour. The relatively higher activity of these enzymes toward androsterone when compared to epitestosterone was attributed to a higher

maximum velocity value rather than to their Michaelis constants.

The heterogeneity of the 3(17) $\alpha$ -HSDs of rabbit kidney cytosol was attributed to a number of factors. The formation of artefacts during purification was excluded. A genetic difference due to gene duplication is speculated. In vivo deamidation is another plausible explanation.

The identification of multiple forms of the 3(17) $\alpha$ -HSDs exhibiting differences in substrate specificity suggests that these enzymes may have distinct roles in the metabolism of androgens as well as of estrogens in the rabbit. The role of the kidneys in the extrahepatic metabolism and/or conjugation of estrogens in the rabbit is also exemplified in this study.

During the electrophoretic analyses of the multiple forms of the rabbit kidney 3(17) $\alpha$ -HSDs in polyacrylamide gels we encountered two non-steroidal activities. These are the superoxide dismutase (SOD) and the so-called "nothing dehydrogenase" activities. Multiple forms of the SOD activity interfered with the staining of crude fractions of the hydroxysteroid dehydrogenase activities in the gels by the nitro-blue tetrazolium reduction method. The addition of 2 mM cyanide abolished the SOD activities but not the steroidal activities. However, a cyanide-insensitive SOD species was also present but this activity on the polyacrylamide gel was far removed from the sites of the steroid dehydrogenase activities. The "nothing dehydrogenase" activity also located on gels was found to be particularly associated with one of the kidney 3(17) $\alpha$ -HSD forms when this latter activity was stained in the presence or absence of added steroid. Some of the properties of this non-specific staining in the apparent absence of added substrate are discussed.

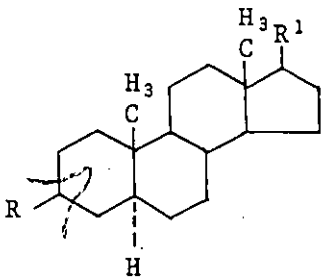
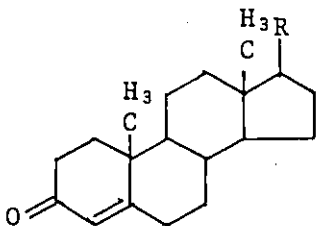
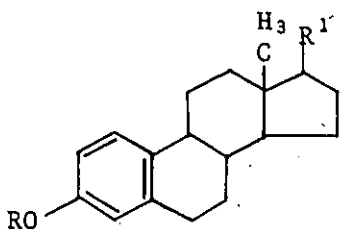
GLOSSARY

1) Trivial and systematic names of steroids:

Androstanedione	5 $\alpha$ -Androstan-3,17-dione
Androstenedione	4-Androsten-3,17-dione
Androsterone	5 $\alpha$ -Androstan-3 $\alpha$ -ol-17-one
Cortisol	4-Pregnen-11 $\beta$ ,17 $\alpha$ ,21-triol-3,20-dione
Cortisone	4-Pregnen-17 $\alpha$ ,21-diol-3,11,20-trione
Dehydroepiandrosterone	5-Androsten-3 $\beta$ -ol-17-one
17 $\beta$ -Dihydroequilenin	1,3,5(10),6,8-Estrapentaen-3,17 $\beta$ -diol
17 $\beta$ -Dihydroequilin	1,3,5(10),7-Estratetraen-3,17 $\beta$ -diol
5 $\alpha$ -Dihydrotestosterone	5 $\alpha$ -Androstan-17 $\beta$ -ol-3-one
16,17-Epiestriol	1,3,5(10)-Estratrien-3,16 $\beta$ ,17 $\alpha$ -triol
17-Epiestriol	1,3,5(10)-Estratrien-3,16 $\alpha$ ,17 $\alpha$ -triol
Epitestosterone	4-Androsten-17 $\alpha$ -ol-3-one
Equilenin	1,3,5(10),6,8-Estrapentaen-3-ol-17-one
Equilin	1,3,5(10),7-Estratetraen-3-ol-17-one
17 $\alpha$ -Estradiol	1,3,5(10)-Estratrien-3,17 $\alpha$ -diol
17 $\alpha$ -Estradiol 3-acetate	1,3,5(10)-Estratrien-3,17 $\alpha$ -diol 3-acetate
17 $\alpha$ -Estradiol 3-glucuronide	17 $\alpha$ -Hydroxyestra-1,3,5(10)-trien- 3yl- $\beta$ -D-glucopyranosiduronic acid
17 $\alpha$ -Estradiol 3-glucuronide- 17-N-Acetylglucosaminide	Estra-1,3,5(10)-trien-3,17 $\alpha$ -di-yl- 3- $\beta$ -D-glucopyranosiduronic acid-17- 2 <sup>1</sup> -acetamido-2 <sup>1</sup> -deoxy- $\beta$ -D- glucopyranoside

17 $\alpha$ -Estradiol trimethylacetate	1,3,5(10)-Estratrien-3,17 $\alpha$ -diol-3-trimethylacetate
17 $\beta$ -Estradiol	1,3,5(10)-Estratrien-3,17 $\beta$ -diol
17 $\beta$ -Estradiol 3-glucuronide	17 $\beta$ -Hydroxyestra-1,3,5(10)-trien-3yl- $\beta$ -D-glucopyranosiduronic acid
Estriol	1,3,5(10)-Estratrien-3,16 $\alpha$ ,17 $\beta$ -triol
Estrone	1,3,5(10)-Estratrien-3-ol-17-one
Estrone glucuronide	17-Oxo-1,3,5(10)-Estratrien-3yl- $\beta$ -D-glucopyranosiduronic acid
Estrone sulfate	17-Oxo-1,3,5(10)-Estratrien-3yl-sulfate
Etiocholan-3,17-dione	5 $\beta$ -Androstan-3,17-dione
Etiocholanolone	5 $\beta$ -Androstan-3 $\alpha$ -ol-17-one
17 $\alpha$ -Hydroxyprogesterone	4-Pregnen-17 $\alpha$ -ol-3,20-dione
6-Keto-17 $\alpha$ -estradiol	1,3,5(10)-Estratrien-3,17 $\alpha$ -diol-6-one
Pregnanediol	5 $\beta$ -Pregnan-3 $\alpha$ ,20 $\alpha$ -diol
Pregnenolone	5-Pregnen-3 $\beta$ -ol-20-one
Progesterone	4-Pregnen-3,20-dione
Testosterone	4-Androsten-17 $\beta$ -ol-3-one

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



	<u>R</u>	<u>R<sup>1</sup></u>
17β-Estradiol	H	—OH
17α-Estradiol	H	---OH
Estrone	H	=O

Estrone 3-sulfate		=O
-------------------	--	----

17α-Estradiol 3-glucuronide		---OH
-----------------------------	--	-------

Estradiol-3β-glucuronide-17α-β-N-acetylglucosaminide		
--	--	--

Epitestosterone	<u>R</u> ---OH	
-----------------	-------------------	--

Testosterone	—OH	
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Androstenedione	=O	
-----------------	----	--

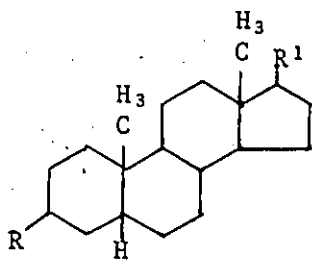
	<u>R</u>	<u>R<sup>1</sup></u>
Androsterone	---OH	=O

Androstenedione	=O	=O
-----------------	----	----

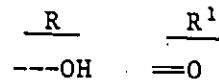
5α-Androstan-3α,17β-diol	---OH	—OH
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5α-Androstan-3β,17β-diol	—OH	—OH
--------------------------	-----	-----

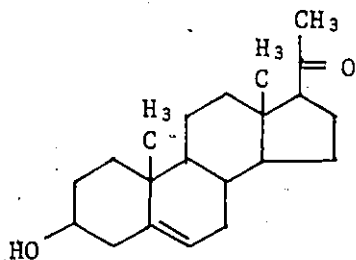
5α-Dihydrotestosterone	=O	—OH
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Etiocholanolone




Etiocholan-3,17-dione



Pregnenolone

### 3) Abbreviations and Symbols

Bis	N,N <sup>1</sup> -methylenebisacrylamide
Ci	curie (2.22 x 10 <sup>12</sup> d.p.m.)
DEAE-	diethylaminoethyl-
d.p.m.	disintegrations per minute
DTT	dithiothreitol
g	acceleration due to gravity
Ki	inhibitor constant
Km	Michaelis constant
μ	micro (10 <sup>-6</sup> ) as in microgram, micromole, micromolar, microliter, etc.
m	milli (10 <sup>-3</sup> )
mA	milliamperere
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 ( $10^{-12}$ )
PCMB	para-chloromercuribenzoate
pI	isoelectric point
PMS	phenazine methosulfate
PPO	2,5-diphenyloxazole
	relative distance of migration with respect to the solvent front
SA	specific activity
SDS	sodium dodecyl sulfate
TEMED	N,N,N <sup>1</sup> ,N <sup>1</sup> -tetramethylenediamine
t.l.c.	thin-layer chromatography
Tris	2-amino-2-hydroxymethylpropane- 1,3-diol
UDP-	uridine-5 <sup>1</sup> -diphospho-
UV	ultraviolet
Vmax	maximum velocity
v/v	by volume
w/v	weight in volume

Addendum

EDH	17 $\beta$ -Estradiol dehydrogenase
K	Kidney
L	Liver

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## CHAPTER 1

### INTRODUCTION

#### A) BACKGROUND

An understanding of the biochemistry of steroid hormone metabolism is important in view of the profound trophic or pleiotypic effects steroid hormones have in target tissues on a variety of biological phenomena such as sex differentiation, maintenance of pregnancy, or the regulation of energy utilization and electrolyte balance. Steroid hormones also play a role in alleviating certain tumors of the breast and prostate gland. Once secreted by an endocrine gland, the hormones travel in the blood stream to reach their target organs. At the same time, these active hormones are being metabolized by both the liver and kidney to inactive products which are then excreted. Clearly, the metabolic processes for the inactivation and removal of steroid hormones are important in regulating the hormonal status of the body and therefore affect hormone action on target tissues.

The principal metabolic changes undergone by steroids are oxidation and reduction, conjugation and hydrolysis. Oxidation and reduction include alcohol-ketone interconversions, reduction of double bonds, hydroxylations or oxidative scissions of the side chains. The process of conjugation usually involves glucuronidation or sulfation of the steroid into a form that is readily water-soluble and can therefore be easily eliminated in the urine. Layne et al (1964) described a new metabolic conjugation reaction of phenolic steroids involving N-acetylglucosamine. Specifically, a double conjugate with glucuronic acid at C-3 and the N-acetylglucosamine at C-17 of  $17\alpha$ -estradiol

was identified in the rabbit urine after the animal had received  $16\text{-}^{14}\text{C}$ -estrone. Subsequent work confirmed the structure of the conjugate as estradiol-3- $\beta$ -glucuronide-17 $\alpha$ - $\beta$ -N-acetylglucosaminide (Layne, 1965; Collins et al, 1967). This double conjugate is by far the major metabolite in rabbit urine after the administration of either estrone, 17 $\beta$ -estradiol or 17 $\alpha$ -estradiol (Layne, 1965; Williams et al, 1968). In general, the 17 $\alpha$ -hydroxyl group of phenolic steroids including 17-epiestriol and 16,17-epiestriol is the preferred site for conjugation with N-acetylglucosamine (Collins et al, 1968). However, these compounds formed N-acetylglucosaminides only after prior formation of their glucuronides at C-3. This restriction is borne out by in vitro studies with homogenates of rabbit tissues showing that in order to effect the transfer of N-acetylglucosamine to the steroid aglycone both UDP-glucuronic acid (donor of glucuronic acid moiety) as well as UDP-N-acetylglucosamine (donor of N-acetylglucosamine moiety) had to be added. Otherwise, 17 $\alpha$ -estradiol 3-glucuronide has to be the substrate (Jirka & Layne, 1965). The glucuronyl transferase and N-acetylglucosaminyl transferase responsible for the transfer of the sugars were both localized in the microsomes of the rabbit liver (Collins et al, 1968). A study of the distribution of these two enzymes in various rabbit tissues showed that the liver was the most abundant source of both the transferases. Only the N-acetylglucosaminyl transferase was present in the kidney.

The observation that only the conjugate derivative of 17 $\alpha$ -estradiol was excreted in the urine when either estrone, 17 $\beta$ -estradiol or 17 $\alpha$ -estradiol was administered to the rabbit and the restriction imposed on the sequential addition of the sugars to the steroid moiety suggested a relationship between glucuronidation and oxidoreduction of these estrogens. This possibility was

explored by the localization and purification of the 17-hydroxysteroid dehydrogenases (HSDs) in rabbit tissues.

Hasnain and Williamson (1974) reported that the 105,000 g supernatant of the rabbit liver homogenate contained both 17 $\alpha$ - and 17 $\beta$ -HSD activities catalyzing the respective conversions of 17 $\alpha$ - or 17 $\beta$ -estradiols and their glucuronides into estrone and the corresponding estrone glucuronides. The activity toward 17 $\beta$ -estradiol was 1.5 times that observed with 17 $\alpha$ -estradiol whereas with the glucuronide substrate, the 17 $\alpha$ -estradiol 3-glucuronide was oxidized at about 7 times that the rate of the 17 $\beta$ -epimer. Moreover, with estrogenic substrates, the highest dehydrogenating activity was that observed with 17 $\alpha$ -estradiol 3-glucuronide. A separation of the 17 $\alpha$ -HSD from the 17 $\beta$ -enzyme was also achieved (Hasnain & Williamson, 1974). Further purification by DEAE-cellulose chromatography and column isoelectric focusing resolved the 17 $\alpha$ -HSD into multiple forms (Hasnain & Williamson, 1975) and some of the properties of the purified molecular forms have been reported (Hasnain & Williamson, 1977). In particular, there exists one enzyme form exhibiting a high specificity toward the substrate 17 $\alpha$ -estradiol 3-glucuronide when compared to other estrogenic substrates. Moreover, the different enzyme forms also catalyzed the dehydrogenation of the androgen, epitestosterone and all but one form of the enzymes showed higher activities toward epitestosterone than the estrogenic substrates.

Since the kidney is the other major steroid-metabolizing organ and presumably the final metabolic processing site before the double conjugate of 17 $\alpha$ -estradiol is excreted in the rabbit urine, we have undertaken the purification of the 17-HSD in this tissue to further establish the possible significance of the steroid conjugate, in particular 17 $\alpha$ -estradiol 3-glucuronide,

as an active metabolic intermediate in the metabolism of estrogens in the rabbit. This work has also involved an extension of the characterization of the multiple forms of the  $17\alpha$ -HSD of rabbit liver previously purified in our laboratory. Some comparative studies on the properties of the  $17\alpha$ -HSDs isolated from both the hepatic and renal tissues are also described.

B) LITERATURE REVIEW

1. Hydroxysteroid dehydrogenases (EC 1.1.1.n)

Hydroxysteroid dehydrogenases (ketosteroid oxidoreductases) are a group of enzymes catalyzing the stereospecific and usually reversible interconversion of hydroxyl and carbonyl functions located on the steroid skeleton or the side chain. These dehydrogenases are pyridine nucleotide-dependent enzymes. Dehydrogenases acting on 3 $\alpha$ -, 3 $\beta$ -, 6 $\alpha$ -, 6 $\beta$ -, 7 $\alpha$ -, 7 $\beta$ -, 11 $\alpha$ -, 11 $\beta$ -, 12 $\alpha$ -, 15 $\alpha$ -, 15 $\beta$ -, 16 $\alpha$ -, 16 $\beta$ -, 17 $\alpha$ -, 17 $\beta$ -, 20 $\alpha$ -, 20 $\beta$ -, and 21-hydroxysteroids are among those which have been demonstrated either histochemically or by the isolation of specific enzymes responsible for the oxidoreduction (Talalay, 1963; Baillie *et al*, 1966). Estrogen oxidoreductases viz. 6 $\alpha$ -, 6 $\beta$ -, 7 $\alpha$ -, 7 $\beta$ -, 11 $\beta$ -, 15 $\alpha$ -, 15 $\beta$ -, 16 $\alpha$ -, 16 $\beta$ -, 17 $\alpha$ - and 17 $\beta$ - have been reviewed by Breuer and Knuppen (1969).

A general formulation of the oxidoreduction reaction is:



2. Measurement of 17-HSD activity

Four basic enzyme assays have been used for measuring 17-HSD activity:

- (i) the continuous spectrophotometric measurement of formation and disappearance of NAD(P)H at 340 nm;
- (ii) the continuous fluorimetric measurement of NAD(P)H at the fluorescence emission maximum at 460 nm and 340 nm excitation;
- (iii) the use of radioactive substrates in the presence of saturating amounts of an appropriate coenzyme, the conversion of substrate to product can be assessed directly by counting in the liquid scintillation counter after their

separation by simple thin layer chromatography or by recrystallization of the product to constant specific activity,

(iv) a histochemical method by coupling the production of NAD(P)H to the reduction of a tetrazolium salt to its characteristic diformazan. The reaction involves the removal of electrons from a substrate and their transfer via an intermediate carrier such as PMS to reduce the tetrazolium salt (mostly widely used is the NBT chloride). The final product is an insoluble purplish blue diformazan exhibiting a broad absorption spectrum maximizing at 605 nm. Strictly, this histochemical method is used for the visualization of dehydrogenase activity in polyacrylamide gels after electrophoresis or in tissue sections. A scheme for the reaction involving structural formulae is found in Appendix I.

The above four methods will be referred to as the spectrophotometric, fluorimetric, radiochemical and histochemical methods respectively, in this thesis without further definition.

### 3. Occurrence of 17-Hydroxysteroid dehydrogenases

The 17 $\beta$ -HSD is widely distributed among different animal tissues. The largest embodiment of information on 17 $\beta$ -HSD has been gathered from studies on the soluble fraction of the human term placenta and this has been the subject of two recent major reviews (Engel & Groman, 1974; Pons et al., 1977). A historical account of C<sub>19</sub>-steroid metabolizing enzymes in mammalian tissues has also been published (Kochakian & Arimasa, 1976). More recently, Williamson (1979) provided a general review of the biochemistry of 17-HSDs.

17 $\alpha$ -Hydroxysteroid dehydrogenase activity is found primarily in those animals, chiefly the ruminants, in which the 17 $\alpha$ -epimers of estrogens (or

androgens) constitute the major hormone or the chief excreted metabolite (Velle, 1963). Among the procaryotes, the  $17\beta$ -HSD of Pseudomonas testosteroni, a soil microorganism has been studied most extensively.

However, this enzyme activity is intrinsically associated with a  $3\beta$ -HSD activity (Talalay 1963). A recent report also pointed to a general  $17\beta$ -HSD or a specific  $17\beta$ - $C_{19}$ -HSD in this microorganism (Groman & Engel, 1977).

a) Placenta

The  $17\beta$ -HSD of human term placenta is a cytoplasmic enzyme (Langer & Engel, 1958) although mitochondrial (Pollow et al, 1974) or microsomal (von Lehmann & Brueur, 1967) localizations of this enzyme have been reported. A recent assessment of the subcellular distribution of the  $17\beta$ -HSD with different buffer media attributed the mitochondrial  $17\beta$ -HSD to microsomal contamination, the latter fraction being capable of oxidizing  $C_{19}$ -steroids better than the estrogenic substrates.  $17\beta$ -Estradiol, however, is the preferred substrate for the soluble enzyme (Thomas & Veerkamp, 1976). Because of this high affinity for  $17\beta$ -estradiol, this enzyme is called  $17\beta$ -estradiol dehydrogenase. The abbreviation, EDH, will be adopted throughout this text.

(i) Isolation Procedures

No attempt will be made in this section to detail the different isolation schemes employed in the purification of EDH since many investigators have used a variety of techniques. However, some important purification steps and inherent characteristics of the enzyme will be mentioned.

EDH is a labile enzyme (Langer & Engel, 1958) and prone to cold inactivation although the process is reversible (Jarabak, et al, 1966). The enzyme can be stabilized by the addition of  $17\beta$ -estradiol,  $NAD(P)^+$  coenzymes or by high concentrations (up to 50% v/v) of glycerol. Jarabak (1969) also

noted that the frozen placentas were not a good source of the enzyme since freezing caused a loss of >80% of the enzyme activity. However, by storing the placentas in a buffer medium containing 20% glycerol and then homogenizing in the same solution, the yield of enzyme ( $\approx$ 80%) per placenta was as good as the term placenta homogenized immediately after delivery. Heavy metal ions such as  $\text{Hg}^{2+}$ ,  $\text{Cu}^{2+}$  and  $\text{Fe}^{3+}$  inactivate the EDH enzyme activity. PCMB is also inhibitory but the inhibition can be partially reversed by cysteine (Langer & Engel, 1958). It is therefore a common practice to include a chelating agent and sulfhydryl protecting reagent in the buffer used in the purification procedure. EDH also promotes the transfer of hydrogen between two nucleotide coenzymes in the presence of low amounts of  $17\beta$ -estradiol or estrone (Talalay, et al, 1958). This "transhydrogenase" activity has been shown to be due to two separate systems. The first system effects transhydrogenation in the presence of  $17\beta$ -estradiol and the  $\text{NAD(P)}^+$ -linked EDH (known as the transhydrogenase function of EDH). The other system catalyzes transhydrogenation in the presence of  $17\beta$ -estradiol but unassociated with the EDH activity. This latter system has been called the estradiol-activated transhydrogenase otherwise known as the estradiol-dependent transhydrogenase. Chromatography over hydroxylapatite separated the EDH with its transhydrogenating function from the  $17\beta$ -estradiol-activated transhydrogenase (Karavolas & Engel, 1966). The latter enzyme could also be destroyed by heat treatment (Jarabak, 1969). Using a combination of conventional techniques, chiefly chromatographic methods, homogeneous preparations of EDH have been achieved in various laboratories (Descomps et al, 1968; Jarabak, 1969; Karavolas et al, 1970; Burns et al, 1972).

An important advance was made in 1972 with the development of an affinity gel for EDH that allowed a purification of 100-fold in a single step (Nicolas et al, 1972). The gels consisted of estrone attached to Sepharose-4B via either hemisuccinyl ethylenediamine or 2-amino-caproate. Two concentrations of estrone were employed, one designated low ( $\approx 10^{-6}M$ ) and the other high ( $\approx 10^{-4}M$ ). With the low estrone concentration gel the EDH was retained in the presence of ammonium sulfate and elution was achieved by a negative ammonium sulfate gradient. With the high estrone concentration gel, the EDH was retained on the column and elution of the enzyme was effected by using estrone hemisuccinate. With a preliminary DEAE-cellulose chromatography, an ammonium sulfate precipitation, affinity chromatography and a second ion-exchange column, the EDH has been obtained in large quantities. For example, 400 mg of enzyme has been obtained from 30 kg of placental tissue (Nicolas, 1974).

Another affinity column procedure using estriol-16-hemisuccinate covalently attached to Sepharose-4B via 1,5-diaminopentane has also been proven successful in the purification of EDH (Chin & Warren, 1973). Enough pure enzyme has been obtained to achieve the first crystallization of this labile enzyme by a new method called "electrophoretic diffusion" (Chin et al, 1976). However, a modified version of the synthesis of the affinity gel has appeared, replacing the spacer arm group 1,5-diaminopentane by 1,8-diaminooctane (Chin & Warren, 1975).

(ii) Steroid substrate specificity

Studies on substrate specificity with pure EDH have shown that the enzyme is active toward those steroids possessing a  $17\beta$ -hydroxyl or a 17-ketone function as well as an aromatic A-ring (Langer et al, 1959; Karavolas & Engel, 1971). The affinity of the enzyme for  $17\beta$ -estradiol is very high, the  $K_m$

value ranging from 0.6-14.7  $\mu\text{M}$  (Warren & Crist, 1967; Jarabak & Sack, 1969; Karavolas et al, 1970; Blomquist et al, 1978 ). Among the long list of 17 $\beta$ -estradiol or estrone derivatives, equilin, equilinenin, 17 $\beta$ -dihydroequilenin (Karavolas & Engel, 1971) and 17 $\beta$ -dihydroequilin (Adams et al, 1962) are also substrates. The compound, estriol, has been of particular interest because it is the principal C<sub>18</sub>-steroid produced by the placenta. However, it is not a substrate for EDH (Langer et al, 1959; Adams et al, 1962). But the 16 $\alpha$ -hydroxyestrone (K<sub>m</sub> 16  $\mu\text{M}$ ) is readily reduced to estriol (Engel & Groman, 1974). The inability of estriol to be oxidized by EDH has been ascribed to the formation of a nonreactive complex (K<sub>i</sub>, 10.5  $\mu\text{M}$ ) probably an aftermath of the steric effect exerted by the oxygen function at C-16 (Blomquist et al, 1978 ). A similar irreversible reaction catalyzed by EDH is its interaction with 18-hydroxyestrone. The placental enzyme is unable to catalyze the oxidation of 18-hydroxy-17 $\beta$ -estradiol whereas the 18-hydroxyestrone is readily reduced (Findlay & Breuer, 1974).

The non-aromatic compounds such as testosterone, 19-nortestosterone, 5 $\alpha$ -dihydrotestosterone and 20 $\alpha$ -hydroxy-pregn-4-en-3-one previously reported to be substrates (Langer et al, 1959; Adams et al, 1962; Jarabak & Sack, 1969) were later shown to be essentially nonreactive with pure EDH (Karavolas & Engel, 1971). At best the activity of the impure enzyme preparation toward the C<sub>19</sub>- or C<sub>21</sub>-steroids relative to 17 $\beta$ -estradiol were 0.5-6% for testosterone, 0.3% for dihydrotestosterone and 20 $\alpha$ -hydroxy-pregn-4-en-3-one, and 1.5% for 19-nortestosterone. However, the possibility that EDH functions as a 20 $\alpha$ -HSD is still open to question (Little in Warren et al, 1977) in view of the kinetic evidence provided by Purdy et al (1964).

9

(iii) Coenzyme specificity

The enzyme EDH can catalyze the reduction of  $\text{NAD}^+$ ,  $\text{NADP}^+$  and a wide range of analogues. The  $K_m$  values reported for  $\text{NAD}^+$  ranged from 10-17  $\mu\text{M}$  (Warren & Crist, 1967; Karavolas et al, 1970; Biellmann & Hirth, 1975; Pons et al, 1977) whereas that of  $\text{NADP}^+$  was about 1  $\mu\text{M}$  (Jarabak & Sack, 1969; Pons et al, 1977) or 10-20  $\mu\text{M}$  (Karavolas et al, 1970; Biellmann & Hirth, 1975). The reduced nucleotides, NADH and NADPH had  $K_m$  values of 1.5-30  $\mu\text{M}$  (Talalay et al, 1958; Warren & Crist, 1967) and less than 1  $\mu\text{M}$  respectively (Talalay et al, 1958; Jarabak, 1969).

The coenzyme analogues which are active as hydrogen acceptors include those where the amide group of the nicotinamide is replaced by a methyl ketone (3-acetylpyridine adenine dinucleotide), nitrile (3-cyano-pyridine adenine dinucleotide), or thioamide (thio-nicotinamide adenine dinucleotide). Apparently the presence of a substituent at C-3 of the pyridinium ring is necessary for binding of the coenzyme to the enzyme. Variation on the adenine moiety such as nicotinamide 8-bromo-adenine dinucleotide or nicotinamide 8-thioadenine-dinucleotide are also efficient hydrogen acceptors (Biellmann & Hirth, 1975). Two coenzyme analogues in particular have been used to specifically alkylate the EDH. These are 3-chloro-acetylpyridine adenine dinucleotide (Biellmann et al, 1976) and nicotinamide-[5-bromo-acetyl-4-methyl-imidazole]-dinucleotide (Johnscher et al, 1976). The structural formulae of the above nucleotide analogues are given in Appendix II.

(iv) Inhibitors

The EDH is subject to inhibition by various nonsubstrate steroids or non-steroidal compounds. Structural analogs of  $17\beta$ -estradiol such as  $17$ -desoxyestradiol and  $17\alpha$ -estradiol, as well as certain other steroid hormones

such as androstenedione and progesterone are competitive inhibitors of  $17\beta$ -estradiol oxidation. Diethylstilbestrol [3,4-Bis(4-hydroxyphenyl)-3-hexane], an estrogenic but non-steroidal compound is also a competitive inhibitor ( $K_i$ ,  $3.3 \mu\text{M}$ ). This compound apparently binds to the enzyme with affinity as great as that of estrone ( $K_i$ ,  $2.6-3.7 \mu\text{M}$ ) (Jarabak & Sack, 1969). Blomquist et al (1978) have studied the inhibition of EDH by nonsteroidal alcohols and steroids. The nonsteroidal alcohols were shown to be weak inhibitors; benzyl alcohol, cyclohexanol, cyclopentanol and 1,9-nonandiol were competitive inhibitors at low concentrations, while phenol was a noncompetitive inhibitor. The  $K_i$  values for the nonsteroidal alcohols exceeded the  $K_m$  for  $17\beta$ -estradiol ( $0.9 \mu\text{M}$ ) by a factor of  $10^3$  to  $10^4$ . The weak inhibition seen with benzyl alcohol, cyclohexanol and cyclopentanol indicates that in these cases structural similarity to just the A or D ring of the steroid is not sufficient for tight binding. The non-aromatic steroids such as androstenedione, testosterone and  $3\beta$ -hydroxy-5-androsten-17-one have  $K_i$  values greater than  $160 \mu\text{M}$  reflecting a low affinity of these compounds for the steroid binding site. Estriol, also a nonsubstrate, has a  $K_i$  of  $10 \mu\text{M}$  indicating a greater affinity for the EDH than the androstenedione or testosterone. The most potent  $C_{18}$ -steroidal inhibitor is 1,3,5(10)-estratrien-3-ol ( $K_i$ ,  $4 \times 10^{-8} \text{ M}$ ). This compound lacks an oxygen function at C-17. The general conclusion drawn from Blomquist's studies on a series of  $C_{18}$ - and  $C_{19}$ -steroid inhibitors was that both of these classes of steroids can bind at the steroid site but the binding of non-aromatic steroids was weakened by oxygen functions at C-17 or both C-3 and C-17.

Some  $\text{NAD}^+$  coenzyme analogues have also been shown to be inhibitors with respect to  $\text{NAD}^+$  in a competitive manner. These include adenine dinucleotide, the analogue which lacks the pyridinium ring, 3-cyano-5-methyl-pyridine

adenine dinucleotide, 1,4,5,6-tetrahydro-nicotinamide adenine dinucleotide, 3-iodo-pyridine adenine dinucleotide and 5-methyl-thio-nicotinamide adenine dinucleotide (Biellmann & Hirth, 1975).

(v) Primary structure

The amino acid composition of pure EDH has been analyzed by three different groups of investigators (Jarabak & Street, 1971; Burns et al, 1972; Nicolas et al, 1972). As may be expected, they showed considerable variability (Engel & Groman, 1974). In an attempt to resolve the discrepancies, the enzymes prepared in the USA and in France were exchanged and analyzed. The updated analysis was provided by Pons et al (1977). There was complete agreement concerning cysteine content (measured as six half-cystines) while the number of other residues overlapped to a certain extent. The major differences were in the lysine, arginine and isoleucine contents. The US sample had a higher arginine to lysine ratio than the French sample. The observed differences did not appear to stem from the methodology of analysis but from the apparent genetic makeup between the two populations. Disregarding the discrepancy, the amino acid composition in all cases, revealed a higher content of (Asx+ Glx) than (Lys+Arg+His) consistent with an acidic isoelectric point(s) of the enzyme (Pollow & Pollow, 1972; Engel & Groman, 1974).

The amino-terminus of EDH has been identified as alanine (Burns et al, 1971) and a N-terminal pentapeptide (Ala-Glu-Thr-Val-Val-) has also been isolated (Burns et al, 1972). A heptadecapeptide obtained from a tryptic digest of the enzyme has also been sequenced and shown to contain a carboxymethylated cysteine essential for enzyme activity (Nicolas & Harris, 1973).

No evidence has yet been provided to show that EDH is a glycoprotein. Although 1% glucose by weight of the total protein was detected by gas liquid chromatography in one instance, the source of the sugar was attributed to the Sephadex gel which was employed in the last purification step of the enzyme (Burns et al, 1972).

(vi) Subunit structure

Evidence for a dimeric structure of EDH comes from various molecular weight determination experiments. Ultracentrifugation of the enzyme in the presence of 20% glycerol (Burns et al, 1971) or 8  $\mu$ M 17 $\beta$ -estradiol (Burns et al, 1972) both gave a value near 68,000. However, using the polyacrylamide gel electrophoresis technique in the presence of SDS, a molecular weight of about 34,000 was obtained. By the same technique, Jarabak and Street (1971) obtained a subunit molecular weight of 35,000 and after cross-linking with dimethylsuberimidate two protein bands, one corresponding to 37,000 and a second, 73,000 were observed. The molecular weight calculated using Stoke's radius and the sedimentation coefficient of the enzyme was 72,000. Recently, from a crystallized EDH sample, a subunit molecular weight of 35,000 was also noted (Chin et al, 1976). At present, there seems to be a general concensus among the major workers that the molecular weight of native EDH is near 68,000 despite earlier reports of much higher (Descomps et al, 1968) or lower (Karavolas et al, 1970) values. Molecular weight determinations by gel filtration in the presence of a substrate analogue had given higher estimates presumably due to aggregate formation. Although 17 $\beta$ -estradiol and some substrate analogues can protect the enzyme against cold inactivation, it was speculated that they might modify the physical nature of the enzyme in some way. Acidic pH may also produce aggregation (Jarabak & Street, 1971).

The existence of a concentration dependent monomer-dimer-trimer transition has also been proposed for the enzyme by Hagerman (1969) who used an impure enzyme fraction prepared in buffers containing  $17\beta$ -estradiol. The molecular weight estimates corresponding to the monomer-dimer-trimer were 41-47,000, 110,000 and 135,000 respectively. This trimerisation phenomenon may be due to the fact that  $17\beta$ -estradiol does not completely protect the enzyme from cold inactivation.

Knowing the subunit dimeric structure of EDH the next problem imposed is whether the subunits are identical. The isolation of a single N-terminal pentapeptide previously mentioned is supportive. In addition, peptide mapping studies on the chymotryptic digest of the enzyme gave only six different cysteinyl peptides from a total of twelve cysteine-containing polypeptide (Pons *et al*, 1977). However, in view of the isozymic nature of the enzyme (Engel & Groman, 1974) the question of identical subunit structure for EDH remains open.

(vii) Chemical modifications - affinity labeling

Since 1973 extensive studies on the chemical modification of EDH have been carried out using both general reagents and affinity labels in attempts to map the active site of the enzyme. A general labeling simply involves the use of a reagent specific for a certain type of amino acid residues and under appropriate conditions one can modify the available side chains bearing the same functional group. Affinity labeling, however, requires the synthesis of an appropriate substrate derivative bearing a reagent group capable of forming a covalent bond with amino acid residues present at the active site of the enzyme.

Using radioactive iodoacetic acid or N-ethyl-maleimide, Nicolas and Harris (1973) labeled two of a total of twelve cysteine residues present in the dimeric EDH enzyme. After tryptic hydrolysis of the modified enzyme, a

heptadecapeptide containing the chemically modified cysteines essential for enzyme activity was isolated and its amino acid sequence was determined. It was found that the two reactive cysteines occurred in a unique sequence in the primary structure of the enzyme. The sequence in the vicinity of the reactive cysteine was relatively hydrophobic. The alkylation was inhibited by the presence of NADP<sup>+</sup> but not by the substrate 17 $\beta$ -estradiol. The latter observation argued against the involvement of cysteine in the substrate binding site whereas the protection by NADP<sup>+</sup> suggested that the essential cysteine might be part of the coenzyme binding site. Pons et al (1973a) later reported the synthesis of an affinity label presumably directed toward the substrate binding site. The reagent was iodo-16 $\alpha$ -acetoxy-3-estrone (IAE). This reagent was a competitive inhibitor of the dehydrogenase reaction and it bound irreversibly to the enzyme. The substrate, 17 $\beta$ -estradiol exhibited a protective effect against IAE and the enzyme inactivated by IAE also lost the ability to bind the substrate. Since then many other affinity labels for EDH have been synthesized in different laboratories. The more selective reagents are as follows: 3-bromoacetoxy-estrone used by Engel and co-workers (Groman et al, 1975); 16 $\alpha$ -bromoacetoxyestradiol 3-methyl ether (Chin & Warren, 1975); 12 $\beta$ -bromoacetoxy-4-estrene-3,17-dione (Warren et al, 1977) and 4-bromoacetamidoestrone methyl ether (Bhatnager et al, 1978) used in Warren's laboratory and 3-iodoacetoxy estrone derivatives as well as 16 $\alpha$  and 16 $\beta$  estrone iodoacetates or bromoacetates used by Pons and co-workers (Pons et al, 1976). Some of the representative structural formulae of these modified steroids are provided in Appendix III.

In studies by Groman et al (1975) EDH was inactivated in minutes by 3-bromoacetoxyestrone and the inactivation was accompanied by incorporation of 1 mole of 3-bromoacetoxyestrone per mole of subunit (34,000 molecular

weight). The modified enzyme was still able to catalyze its normal reactions i.e. both dehydrogenase and transhydrogenase reactions. In addition, the normal stereospecificity of hydride transfer (B 4-pro-S) was preserved. This property of the modified enzyme was said to have demonstrated its "catalytic competence" and was considered as a new criterion for affinity labeling (Groman et al, 1975).

Warren and co-workers using 16 $\alpha$ -bromoacetoxyestradiol 3-methyl ether (Chin & Warren, 1975) and 12 $\beta$ -bromoacetoxy-4-estrene-3,17-dione (Warren et al, 1977) have identified a histidyl residue in the catalytic region of the active site of EDH. The criteria they used included the fact that the modified steroid was a substrate for the enzyme. The inactivation was a time-dependent irreversible process and the rate of inactivation was slowed by the presence of 17 $\beta$ -estradiol which competed for the steroid binding site. In the case of the 16 $\alpha$ -bromoacetoxyestradiol 3-methyl ether, both NAD(P)H slowed the inactivation of the EDH. This protective effect of the nucleotide was interpreted as resulting from cofactor induced changes in the enzyme steroid binding site, the two sites being very close to each other. Upon amino acid analysis of the hydrolysate from the inactivated 16 $\alpha$ -bromoacetoxyestradiol 3-methyl ether, both 3-carboxymethylhistidine and 1,3-dicarboxymethylhistidine were obtained; the former was found to have undergone a second alkylation resulting in the dicarboxymethylated histidine. When the amino acid hydrolysate of the enzyme modified with 12 $\beta$ -bromoacetoxy-4-estrene-3,17-dione was analyzed, 1-, 3- and 1,3-dicarboxymethylhistidine as well as  $\epsilon$ -, and  $\epsilon,\epsilon$ -dicarboxymethyllysines were found. Presumably the histidyl and lysyl residues proximated the 12-position of the steroid as the latter was bound at the active site. However it was not established whether both 16 $\alpha$ -bromoacetoxyestradiol 3-methyl ether and 12 $\beta$ -

bromoacetoxy-4-estrone-3,17-dione alkylated the same histidyl residue.

Further study in Warren's laboratory using 4-bromoacetamido-estrone methyl ether (Bhatnager et al, 1978) showed that this compound alkylated a lysyl residue (70%) and a cysteine residue (30%). The bromoacetamido derivative was chosen because of the resistance of its peptide bond to hydrolysis thus precluding the possibility of formation of the dicarboxymethylated derivative. The modified steroid was a substrate for EDH. Further, the rate of inactivation of EDH was slowed by the presence of  $17\beta$ -estradiol and  $\text{NADP}^+$ . When the enzyme was inactivated in the presence of excess nucleotide, only the lysyl residue was carboxymethylated. Presumably the accessibility of the bromoacetamido group on the steroid to the cysteinyl residue was eliminated either by direct interposition or by a conformational change when the cofactor binding site was occupied by  $\text{NADP}^+$ . Apparently, the lysyl and the cysteinyl residues were located near position 4 of the steroid i.e. at the lower portion of the A-ring.

However reports with other affinity labels including coenzyme analogues have been contrary to the findings of Warren's group. Pons et al (1973b, 1976, 1977) have identified four amino acid residues - three (one histidine, cysteine  $\text{C}_3$  and cysteine  $\text{C}_5$ ) in the active site and one (cysteine  $\text{C}_1$ ) in the neighbourhood of the active site. The histidyl residue was located very close to the A-ring probably on the " $\beta$ -face" of the steroid substrate. This conclusion was derived from the fact that labeling was reduced when the alkylating side chain was shifted toward the B or C ring. Moreover, when the alkylating side chain was in the " $\beta$ -face" of the steroid, the labeling was more specific. This histidyl residue however was not essential for enzyme activity in contrast to Warren's finding (if they were looking at the same histidine). But the two cysteinyl residues ( $\text{C}_3$  and  $\text{C}_5$ ) were catalytically important. The cysteine  $\text{C}_3$

was localized at the junction of the ring D of the steroid and the coenzyme binding site. The conclusion was drawn from the observations that there was increasing alkylation of the cysteine when the alkylating substituent of the affinity label was shifted from carbon 3 to carbon 16 of the steroid. The presence of the cysteine C<sub>3</sub> in the neighbourhood of ring D of the substrate was further supported by the results of affinity labeling using 3-chloroacetylpyridine-adenine dinucleotide, a coenzyme analogue. This analogue was active as a hydride acceptor as well as inactivating the enzyme by alkylating it (Biellmann et al, 1976). Cysteine C<sub>5</sub> was presumably closer to the catalytic site than cysteine C<sub>3</sub>. This was derived from the experiment using 3-chloroacetyl pyridine adenine dinucleotide phosphate which labeled only cysteine C<sub>5</sub> (Pons et al, 1977).

(viii) Mechanism of reaction

The reaction mechanism of EDH was thought to proceed in a random manner i.e. a binary complex formation involving either coenzyme or steroid. This conclusion was derived from kinetic analysis by measuring the isotopic exchange between substrates at equilibrium (Betz, 1971). No abortive ternary complex formation composed of enzyme and two oxidized or two reduced substrates was noted. This observation was supported by an affinity labeling study whereby NADP<sup>+</sup> exerted a protective effect against inactivation of enzyme by the oxidized inhibitor (iodoacetoxy-estrone) and NADPH by the reduced inhibitor (iodoacetoxy-estradiol). Apparently, neither iodoacetoxyestrone-enzyme-NADP nor iodoacetoxyestradiol-enzyme-NADPH complex was formed (Pons et al, 1973b). In contrast to the equilibrium rate exchange studies, kinetic analysis of measurements of the initial velocities of the EDH reaction have implied an ordered bi bi mechanism. In this case, initial steroid binding to the enzyme was deemed a prerequisite for the subsequent interaction of the coenzyme (Warren & Crist, 1967).

Three independent studies have shown that EDH is a "B" stereospecific enzyme meaning that the enzyme transfers hydride between the substrate and the B(pro-S) side of the dihydropyridine ring. In one study, Jarabak and Talalay (1960) carried out the reaction with  $17\beta$ -estradiol and NAD(P)-nicotinamide-4- $^3\text{H}$ . The resulting labeled NAD(P)H was then oxidized with glutamate dehydrogenase, an enzyme of known B-stereospecificity. On examination of the isotopic contents of the products, the tritium was found to be retained in the nicotinamide; no isotope was found in the glutamic acid. A second study by Warren et al (1967) used a variation of the above method whereby  $17\alpha$ - $^3\text{H}$ -estradiol- $17\beta$  was prepared and reacted with  $\text{NAD}^+$ . The generated, labeled NADH was then assayed with  $\alpha$ -keto-glutamate in the presence of glutamate dehydrogenase. Reduction of  $\alpha$ -ketoglutarate resulted in essentially quantitative transfer of tritium from  $\text{NAD-}^3\text{H}$  to glutamic acid. Reduction of pyruvate however yielded  $^3\text{H-NAD}$  and 'cold' lactate, lactate dehydrogenase being an A-stereospecific enzyme. A more recent analysis of the stereospecificity of hydride transfer made use of an affinity labeled enzyme (EDH covalently modified with acetoxysterone) which was incubated with (4R)- or (4S)- $[4\text{-}^2\text{H}]$  NADH. At the end of the reaction, the steroid ester was first hydrolyzed off in the presence of a strong base and then acidified to release the free radio-labeled steroid. Trimethylsilyl ether derivatives of the steroid were then made for deuterium analysis using gas-liquid-chromatography-mass spectrometry. It was found that with (4S)- $[4\text{-}^2\text{H}]$  NADH, the label was transferred resulting in  $[17\alpha\text{-}^2\text{H}]$  estradiol- $17\beta$  whereas in the case of (4R)- $[4\text{-}^2\text{H}]$  NADH the deuterium was not transferred. This experiment also illustrated the point that normal stereochemistry of hydride transfer was preserved even when the enzyme was covalently bound to ligand, (in this case also a substrate) (Groman et al, 1975).

b) Liver and kidney

Both the 17 $\alpha$ - and 17 $\beta$ -HSDs were demonstrated in the hepatic and renal homogenates of the male rabbit in Kochakian's laboratory as early as 1947 (Clark et al, 1947; Clark & Kochakian, 1947) thus establishing the equilibrium, testosterone  $\rightleftharpoons$  androstenedione  $\rightleftharpoons$  epitestosterone (Kochakian et al, 1952; Kochakian & Nall, 1953). The 17 $\beta$ -hydroxyl group of testosterone was more readily oxidized than the 17 $\alpha$ -function of epitestosterone by the crude enzyme homogenate. Ball and Breuer (1970) reported that a single 17-HSD present in the soluble fraction of the rabbit liver was responsible for the oxidation of 17 $\beta$ -estradiol or 17 $\alpha$ -estradiol to estrone, although a different fold purification of the 17 $\beta$ -enzyme and the 17 $\alpha$ -enzyme activities was reported. In contrast, the two enzyme activities were shown to be separate in the soluble fraction of the chicken liver (Ozon & Breuer, 1965). Renwick and Engel (1967) achieved a partial purification and separation of the 17 $\alpha$ - and 17 $\beta$ -enzymes from chicken liver by ammonium sulfate fractionation and Sephadex gel filtration. The two enzymes showed a marked preference for NADP<sup>+</sup> and both C<sub>18</sub>- and C<sub>19</sub>-steroids were substrates. The same authors also reported the presence of both 17 $\alpha$ - and 17 $\beta$ -enzyme activities in the crude homogenates of sheep and turkey liver but only 17 $\beta$ -enzyme activity was observed with the calf liver homogenates.

Further purification of the 17-HSD from the female rabbit liver cytosol revealed heterogeneous nature of the 17 $\beta$ -enzyme (Thaler-Dao et al, 1972) and separation of the 17 $\beta$ - from the 17 $\alpha$ -enzyme (Hasnain & Williamson, 1974). The properties of the multiple forms of these enzymes will be elaborated in Section 4 of this Introduction.

The intracellular localization of the 17 $\beta$ -HSD of liver and kidney of the guinea pig, mouse, rat, hamster, dog as well as the rabbit have also been

compared (Aoshima & Kochakian, 1963). Both the  $\text{NAD}^+$ - and  $\text{NADP}^+$ -dependent enzyme activities were present in the soluble and the microsomal fractions. The enzymes were never associated with the nuclear or mitochondrial portion of the cell. The  $\text{NAD}^+$ -enzyme activity of the liver of the guinea pig and rat primarily resided in the microsomal fraction whereas the  $\text{NADP}^+$ -enzyme was in the soluble fraction of the liver of all species. On the other hand, both enzyme activities of the kidney were localized in the soluble fraction. The specific activity of the  $\text{NAD}^+$ -dependent enzyme was highest in the liver of the guinea pig followed by the mouse and the rabbit. The  $\text{NADP}^+$ -enzyme, however, was highest in the rabbit followed by the guinea pig and the mouse. The kidneys of the guinea pig showed the highest  $\text{NAD}^+$ - and  $\text{NADP}^+$ -dependent enzyme activities although lower than those of the liver.

Studies in Kochakian's laboratory have focused primarily in the anabolic action of androgens and their role in the regulation of kidney growth particularly in the guinea pig (Kochakian, 1977). Substrate specificity studies with a partially purified soluble  $17\beta$ -HSD from the male guinea pig kidney showed that the enzyme was highly specific for  $\text{C}_{19}$ -steroids; the relative activity toward testosterone:  $5\alpha$ -dihydrotestosterone:  $5\beta$ -dihydrotestosterone:  $17\beta$ -estradiol, in the presence of  $\text{NADP}^+$  being 35:19:5:1. The  $\text{NADP}^+$ -linked enzyme activity was always higher than that of the  $\text{NAD}^+$ -linked activity. Disc polyacrylamide electrophoresis revealed the multiple nature of the enzyme (Liu & Kochakian, 1972ab). Similarly, the soluble  $\text{NADP}^+$ - $17\beta$ -HSD of male adult guinea pig liver has also been shown to exist in multiple forms (Liu & Kochakian, 1972b; Kobayashi & Kochakian, 1978). The properties of these isozymes will be treated more fully in section 4 of this Introduction.

The microsomal  $\text{NAD}^+$ -dependent  $17\beta$ -HSD of guinea pig liver has been solubilized with Triton X-100 and the enzyme obtained in phospholipid-free form (Blomquist et al, 1977). The molecular weight of the solubilized enzyme was found to be 176,000. The apparent  $K_m$  for testosterone was found to increase from  $2.2 \mu\text{M}$  to  $7.3 \mu\text{M}$  upon solubilization and the apparent  $K_m$  for  $\text{NAD}^+$  decreased from 164 to  $100 \mu\text{M}$ . The enzymes was susceptible to trypsin and phospholipase A inactivation. The extent of inactivation was not affected by the presence or absence of 2-mercaptoethanol but this sulfhydryl reagent did alter the kinetic behaviour of the enzyme toward its substrates testosterone and  $\text{NAD}^+$ . Furthermore, the  $17\beta$ -enzyme exposed to 2-mercaptoethanol exhibited substrate inhibition by testosterone, a phenomenon which may act as a control mechanism in vivo (Blomquist & Kotts, 1978),

In the above studies with the guinea pig liver or kidney, the dehydrogenation of the  $17\alpha$ -hydrosteroid catalyzed by the  $17\alpha$ -enzyme was not studied. However, the in vitro interconversion of testosterone  $\rightleftharpoons$  androstenedione  $\rightleftharpoons$  epitestosterone in liver as well as the ovary and testis slices of the guinea pig of both sexes has been demonstrated (Szamatowicz et al, 1971). In contrast, no epitestosterone was detected in the urine after the in vivo administration of androstenedione or testosterone to either male or female guinea pigs.

A brief study of the metabolism of androstenedione by mouse kidney homogenate and its subcellular fractions revealed the presence of a predominant  $17\alpha$ -HSD activity (Arimasa & Kochakian, 1973). The major metabolite formed was epitestosterone with  $5\alpha$ -androstan- $3\alpha,17\beta$ -diol and testosterone being produced in lower quantities. Incubation of testosterone with kidney homogenate also produced epitestosterone and  $5\alpha$ -androstan- $3\alpha,17\beta$ -

diol but the amounts of these metabolites were less than that obtained from androstenedione. Upon castration, testosterone metabolism by the mouse kidney was further reduced perhaps due to a decrease in 17 $\beta$ -HSD activity. The dehydrogenase activities were localized in the cytosol except that the conversion of androstenedione to testosterone in the NADH (but not the NADPH) system was localized in the microsomal fraction. Separate NADH- and NADPH-linked 17 $\beta$ -HSD systems were therefore suggested. In an earlier report, Endahl et al (1960) separated the NAD<sup>+</sup> from the NADP<sup>+</sup>-linked 17 $\beta$ -HSD activity of the guinea pig liver by centrifugation. The former was located in the microsomes and the latter in the cytosol.

A soluble NAD(P)<sup>+</sup>-dependent 17 $\beta$ -HSD from rat liver has been partially purified (Findlay & Breuer, 1974). The enzyme shared a common characteristic with the human placental EDH in that it could reduce 18-hydroxyestrone to 18-hydroxyestradiol-17 $\beta$  but could not oxidize the latter. In contrast, a microbial enzyme isolated from Pseudomonas testosteroni catalyzed the interconversion of 18-hydroxyestrone and 18-hydroxyestradiol-17 $\beta$ . These results indicated differences in the geometry of the active sites of the microbial and mammalian enzymes.

The microsomal fraction of the rat liver also contains a NAD(P)<sup>+</sup>-dependent 17 $\beta$ -HSD, NAD<sup>+</sup> being the preferred nucleotide (Laurent et al, 1978). A kinetic study of the enzyme revealed an ordered sequential mechanism of reaction with the nucleotide being bound first and the steroid product liberated first. Substrate inhibition by 17 $\beta$ -estradiol was also observed. Under the optimum conditions, the  $K_m$  of 17 $\beta$ -estradiol was found to be  $0.8 \pm 0.3 \times 10^{-5}M$  and  $K_m$  for NAD<sup>+</sup>,  $35 \pm 5 \times 10^{-5}M$ . In the rat it appeared that the 17 $\beta$ -estradiol  $\rightleftharpoons$  estrone equilibrium was an important mechanism for the modulation of estrogenic activity, estrone being more abundant than 17 $\beta$ -estradiol in the plasma of the pregnant and fetal rat.

The soluble 17 $\beta$ -HSD of monkey liver has also been partially characterized (Fan et al, 1978). The enzyme catalyzed the reduction of dehydroepiandrosterone to 5-androstene-3 $\beta$ ,17 $\beta$ -diol (Km, 50  $\mu$ M), estrone to 17 $\beta$ -estradiol (Km, 20  $\mu$ M) and androstenedione to testosterone (Km, 13  $\mu$ M). The enzyme required either NADH or NADPH preferring the latter. Both dehydroepiandrosterone and estrone served as competitive substrates for the enzyme. Androstenedione was a competitive inhibitor of the enzyme for dehydroepiandrosterone but a noncompetitive inhibitor of the enzyme for estrone. The heat-treated 50-80% ammonium sulfate fraction also contained 20 $\alpha$ -HSD activity, the stability of this enzyme was found to be similar to that of the 17 $\beta$ -HSD. Besides dehydroepiandrosterone, estrone and androstenedione were competitive inhibitors of the 20 $\alpha$ -HSD activity with progesterone as substrate. Whether the 20 $\alpha$ - and 17 $\beta$ -HSD activities of monkey liver reside in the same protein remains to be seen.

The 17 $\beta$ -HSD of human liver has been characterized to a certain extent (Littmann et al, 1971). The NADP<sup>+</sup>-linked enzyme was present in the soluble fraction whereas the NAD<sup>+</sup>-linked enzyme activity was present in both the soluble and the microsomal fractions. Both enzymes were able to convert 17 $\beta$ -estradiol to estrone and testosterone to androstenedione and were therefore quite different from the human placental soluble enzyme which was shown to be specifically a 17 $\beta$ -estradiol dehydrogenase. Littmann et al (1971) reported that the Km value for 17 $\beta$ -estradiol in the presence of NAD<sup>+</sup> was 3.5 x 10<sup>-5</sup>M in the microsomal fraction and 9.8  $\mu$ M in the soluble fraction, whereas the soluble NADP<sup>+</sup>-enzyme had a Km of 5.1  $\mu$ M for 17 $\beta$ -estradiol.

c) Gonads

Both the ovary and the testis fulfil a dual role, an endocrine function in the formation of steroid hormones and a germinal function in the production of ova or spermatozoa. Essentially every steroid in the pathway from pregnenolone to  $17\beta$ -estradiol is produced by the ovary. An ovarian  $17\beta$ -estradiol dehydrogenase has been studied in the human (Pittaway et al, 1977) and in sheep (Kautsky & Hagerman, 1970; Michel et al, 1975). The common characteristics of the ovarian enzymes include their cytoplasmic location, dual nucleotide specificity and a weak capacity to oxidize  $C_{19}$ -steroids. Different properties have been ascribed to the sheep enzyme in terms of pH optima, molecular weight and mechanism of reaction. Kautsky and Hagerman (1970) described a sequential mechanism whereas a compulsory scheme with  $NAD^+$  binding first was reported by Michel and co-workers (1975). Human ovarian and placental  $17\beta$ -enzymes share some common properties including sensitivity to sulfhydryl reagents, dual nucleotide specificity and reversibility of the reaction. However, the ovarian enzyme does not exhibit the same degree of cold inactivation as the placental enzyme. Further comparisons must await the purification of the former enzyme.

The ovary of the rabbit also contains significant  $17\beta$ -HSD activity toward both  $C_{18}$ - and  $C_{19}$ -steroids (Dennis et al, 1968). This enzyme has been shown to be associated with the  $20\alpha$ -HSD (Rodway & Rahman, 1978). The significance of the gonadal  $17\beta$ - and  $20\alpha$ -HSDs has been interpreted as a means of adjusting the biological potencies of the endocrine secretions to the requirements of the animal.

Testicular 17 $\beta$ -HSD has been investigated in a variety of animals mainly in the pig. Perhaps the most characteristic feature of animal testicular 17 $\beta$ -HSD is its microsomal localization associated mainly with the smooth or agranular endoplasmic reticulum (Inano & Tamaoki, 1974; 1975; Tamaoki & Inano, 1975; Inano et al, 1977; Cooke & Gower, 1977). Some of the properties of the purified enzyme have been summarized by Tamaoki and Inano (1975). NADP(H) was the preferred cofactor and androstenedione, estrone and dehydroepiandrosterone were equally good substrates. Like the human placental EDH, the testicular 17 $\beta$ -HSD is a "B" stereospecific enzyme (Inano & Tamaoki, 1975). Using gel filtration the molecular weight of the enzyme was found to be 35,000. By active enzyme centrifugation method, the monomeric form of the enzyme (35,000 MW) was found to be the active species (Inano et al, 1977). An unusual property reported for the testicular 17 $\beta$ -HSD from rat and human was that the enzyme activity was enhanced by either testosterone or androstenedione (Oshima et al, 1975, 1977). These studies were performed with the enzyme still attached to the microsomal membranes. The possibility of the presence of isozymes of human testicular 17 $\beta$ -HSD was also raised in view of the two apparent Km values for the substrate dehydroepiandrosterone, one at low concentrations and the other at high concentrations. This phenomenon was also observed with canine testicular 17 $\beta$ -HSD (Pittaway, 1978). Indeed, two distinct testicular 17 $\beta$ -enzymes were described in the rat testis, one in the interstitial and the other in the seminiferous tubules (Murono & Payne, 1976). The two enzymes when assayed for the reduction of androstenedione to testosterone, had different pH optima, Km values and responded differently to testosterone and its metabolites.

The interstitial tissue enzyme was inhibited by testosterone and several  $5\alpha$ -reduced metabolites of testosterone ( $5\alpha$ -androstan- $3\alpha,17\beta$ -diol being most potent) and by estrogens ( $17\beta$ -estradiol being most potent). In the seminiferous tubules only a high concentration of  $5\alpha$ -dihydrotestosterone inhibited the  $17\beta$ -HSD activity. Furthermore,  $5\alpha$ -androstan- $3\alpha,17\beta$ -diol and testosterone which acted as potent inhibitors of the interstitial enzyme activity, stimulated this enzyme activity in the seminiferous tubules.

These differential effects of steroid metabolites led the authors to speculate that testosterone biosynthesis in the rat testis may be regulated locally in the two testicular compartments without an apparent direct involvement of the pituitary gonadotrophins.

The  $17\beta$ -HSD of dog testis and human testicular  $17\beta$ -HSD share several common characteristics. Androstenedione, estrone and dehydroepiandrosterone, were all substrates (Pittaway *et al*, 1976; Pittaway, 1978), androstenedione having the lowest  $K_m$  value. The apparent  $K_m$  values of the  $17\beta$ -HSD for androstenedione and dehydroepiandrosterone were similar in both species as was the observation of two  $K_m$  values for dehydroepiandrosterone (Oshima & Ochiai, 1973; Oshima *et al*, 1975). The importance of the study of testicular  $17\beta$ -HSD will be elaborated in section 7.

d) Blood

Portius and Repke (1960) studied the occurrence of  $17\beta$ -HSD in the erythrocytes of 15 animal species. The  $17\beta$ -HSD activity was found to be highest in rats and lowest in man. On the other hand,  $17\alpha$ -HSD was found in the erythrocytes of ruminants such as cows, sheep and goats. Working with human erythrocytes and labeled substrates, Migeon *et al* (1962) showed that the  $17\beta$ -HSD was NADP(H)-dependent and that the interconversion of


estrone and  $17\beta$ -estradiol was related to the activity of the pentose phosphate pathway. Thus subjects with a glucose 6-phosphate dehydrogenase deficiency had a marked, but not complete, decrease in ability to interconvert estrone and  $17\beta$ -estradiol. The  $17\beta$ -enzyme has been partially purified from the membrane-free hemolysate (Mulder et al, 1972). It exhibited a broad substrate specificity including  $17\beta$ -estradiol, testosterone, dehydroepiandrosterone and the sulfate conjugate of dehydroepiandrosterone and estrone.  $20\alpha$ -Hydroxy-pregn-4-en-3-one was also oxidized but the activity was lost with purification. The enzyme showed an almost absolute requirement for NADP(H). The molecular weight of the enzyme calculated from Sephadex G-150 filtration was found to be about 64,000.

There are indications that the  $17\beta$ -HSD in the blood of pregnant women is of placental origin (Lubbert, 1977). The enzyme, stabilized by 40% glycerol and 40  $\mu$ M  $17\beta$ -estradiol in potassium phosphate buffer at pH 7.4, was found to generally increase with the progression of gestation and the enzyme activity was highest at term. After the expulsion of the placenta (post-partum) the enzyme activity rapidly decreased. Moreover, the serum  $17\beta$ -enzyme shared some common properties with the human placental enzyme such as its stabilization by glycerol and substrate. The  $K_m$  values for  $17\beta$ -estradiol with  $NAD^+$  and  $NADP^+$  as coenzymes were almost identical to those reported by Langer et al (1959) and Jarabak and Sack (1969) for the purified placental EDH.

Using affinity chromatography on Cibacron Blue F3GA coupled to Sepharose, Heyns and de Moor (1974) purified a  $17\beta$ -HSD from rat erythrocytes to 7200-fold with respect to the crude hemolysate. However, a  $3\beta$ -HSD

enzyme activity was found to coincide with the  $17\beta$ -enzyme activity when the enzyme(s) was monitored on polyacrylamide gel electrophoresis. Both activities were NADP(H)-linked and their  $K_m$  values for  $\text{NADP}^+$  as well as for NADPH were very similar. The effect of pH on their activity and protection by the presence of coenzyme were similar in both cases. The enzyme had a molecular weight of about 70,000 estimated by Sephadex G-100 chromatography (Heyns & de Moor, 1974).

e) Uterus



Because the uterus is a target tissue for the female sex hormones, the study of the  $17\beta$ -HSD activity in this tissue has been of considerable interest. Both the human endometrium and myometrium have been shown to metabolize  $17\beta$ -estradiol or estrone, the former tissue having 40 times the capacity of the latter. Moreover, the endometrium favored the oxidative reaction (Sweat et al, 1967). It has also been pointed out that the  $17\beta$ -estradiol was oxidized about twelve times more rapidly by crude homogenates of secretory than of proliferative endometrium. Using histochemical staining techniques, Scublinsky et al (1976) found that the  $17\beta$ -enzyme of human endometrium was localized in the glandular epithelium of the secretory endometrium; negative staining was observed with the proliferative endometrium. The increase in the enzyme activity following ovulation is of interest since the change in this enzyme activity to a great extent is directly related to the change in concentrations of progesterone in this tissue (Tseng & Gurpide, 1974).

The  $17\beta$ -HSD is ubiquitous in the cells of the human secretory endometrium. Pollow et al (1975b, 1976a) reported that the highest specific activity of the enzyme was mainly located in the mitochondrial and microsomal fractions

while in the cytosol and nuclear fractions the activity was about fifty times less. The soluble enzyme from the secretory endometrium has been partially characterized (Pollow, et al, 1975c). Testosterone and androstenedione also served as substrates though they were interconverted more slowly than  $17\beta$ -estradiol and estrone. The optimum pH for oxidation and reduction were 9.5 and 6 respectively. The  $K_m$  value for  $17\beta$ -estradiol was  $3.3 \mu M$ . Like the soluble  $17\beta$ -enzyme of human placenta the endometrial enzyme displayed cold inactivation and protection by coenzymes,  $17\beta$ -estradiol or glycerol. Sulfhydryl reagents inhibited enzyme activity. The enzyme also preferred  $NAD^+$  to  $NADP^+$  as a hydrogen acceptor. However, the  $17\beta$ -HSD of human endometrium and human placenta appeared to be immunologically different (Tseng et al, 1977). The EDH activity of the placenta both in early pregnancy and at term was some 27 times higher than that of the secretory endometrium.

A partial purification of the soluble  $17\beta$ -HSD of the secretory endometrium by ammonium sulfate precipitation, gel filtration, ion exchange chromatography and isoelectric focusing indicated heterogeneity of the enzyme (Pollow et al, 1976b). This will be elaborated under section 4 in which the isozymic pattern of the normal and neoplastic endometrium are compared. The molecular weight of the soluble enzyme from the secretory endometrium was estimated to be 50-54,000, significantly lower than the value for the human placental enzyme.

The mitochondrial  $17\beta$ -HSD of the human secretory endometrium displayed similar characteristics to those of the soluble enzyme with respect to the steroid substrate and coenzyme specificities, kinetic parameters, pH optima and sensitivity to sulfhydryl reagents (Pollow et al, 1976a). Within the mitochondria, the enzyme activity was localized in the outer membrane.

Most of the  $17\beta$ -enzyme activity of neoplastic endometrium like normal secretory endometrium was located in the mitochondrial and microsomal fraction (Pollow et al, 1975ab). However, depending on the stage of the tumour, the enzyme activity pattern seemed to change. The enzyme activity appeared to decrease with decreasing differentiation of the tumour (from well differentiated to undifferentiated carcinomata). Moreover, after treatment of patients with progestational (gestagen) agents the  $17\beta$ -enzyme of the well-differentiated carcinomata was seen to increase significantly, rising to or above the level in normal secretory endometrium. This observation as well as other hormonal treatments of the human neoplastic endometrium will be discussed further under the section on Regulation of  $17\beta$ -HSD.

f) Other tissues

The human skin is capable of interconverting  $17\beta$ -estradiol and estrone and the  $17\beta$ -enzyme activity varies with the body site of the skin. For example, in neonatal foreskin, abdominal skin (Weinstein et al, 1968) axillary and pubic skin the oxidative direction was favoured, whereas in facial and scalp skin the reductive direction was preferred (Hodgins & Hay, 1976). Apparently there are two forms of  $17\beta$ -HSD in human skin, a microsomal NAD(H)-dependent enzyme of the forehead skin and a soluble NADP(H)-dependent enzyme of the axillary skin. The former enzyme had a lower apparent  $K_m$  value for testosterone than for androstenedione and the latter appeared to have a higher affinity for androstenedione. The  $17\beta$ -HSD has also been demonstrated in the microsomal fraction of rat skin. The enzyme preferred  $NAD^+$  to  $NADP^+$  and both  $17\beta$ -estradiol and testosterone were substrates (Davies et al, 1972).

In the female rabbit the interconversion of  $17\beta$ -estradiol and estrone has also been shown in the eye tissues, the cornea being most active. The  $17\alpha$ -enzyme activity was negligible (Starka & Obenberger, 1975). The anterior pituitary of male or female rabbits is also capable of metabolizing  $17\beta$ -estradiol or testosterone (Reddy et al, 1974). The preimplantation embryos of virgin female rabbits also exhibited  $17\beta$ -dehydrogenase activity thus providing an evidence for steroidogenesis in this organism (Dickmann et al, 1975).

The  $17\beta$ -HSD activities have been localized histochemically in a variety of tissues including the human placenta (Mellgren & Eide, 1972) and human endometrium (Scublinsky et al, 1976). Pearse (1972) provided a review of the histochemical demonstration of various  $17\beta$ -HSDs and other steroid dehydrogenases.

#### 4. Isozymic nature of mammalian $17$ -hydroxysteroid dehydrogenases

The occurrence of isozymes or multiple molecular forms of enzymes catalyzing the same reaction in the same cell or organism is a widely accepted phenomenon. The first indication of the isozymic nature of  $17\beta$ -HSD of mammalian origin, demonstrated by electrophoresis coupled to a histochemical staining procedure, was that reported by Kochakian and co-workers (Liu & Kochakian, 1972ab). Disc gel electrophoresis of kidney cytosol from adult male guinea pigs gave 3 major and 2 minor bands which possessed both  $\text{NAD}^+$ - and  $\text{NADP}^+$ -linked  $17\beta$ -HSD activities. These enzyme forms were charge isomers with a mean molecular weight of 33,000. One of the major enzyme forms was obtained in an electrophoretically pure state with a molecular weight of 31,000 (Stevenson & Kochakian, 1974). This particular major enzyme form (later designated as  $I_1$ ) and five of the

seven other forms have been further characterized in terms of their isoelectric points, coenzyme and substrate specificities and their kinetic constants (Shen & Kochakian, 1978). These enzymes were isolated in a highly pure state by an extensive combination of anion and cation exchange chromatography. Among these multiple forms, one enzyme, designated I<sub>0</sub>, appeared to be a 3 $\alpha$ ,17 $\beta$ -C<sub>19</sub>-HSD with additional preference toward an A/B trans configuration of the steroid substrate and an apparent absolute requirement for NADP<sup>+</sup>. The K<sub>m</sub> value for testosterone and 5 $\alpha$ -dihydrotestosterone reflected a lower affinity of this enzyme for these substrates as compared to the other enzyme forms, for example, I<sub>1</sub> had K<sub>m</sub> values 2-4 times lower than that exhibited by I<sub>0</sub> toward the two substrates. Enzyme form I<sub>1</sub> but not others (except I<sub>0</sub>) also had appreciable activity with androsterone in the presence of NADP<sup>+</sup>. Three of the multiple forms also had very similar amino acid compositions, alanine being the N-terminal amino acid in two cases. All of the purified enzymes contained a similar number of total (4-6) and exposed (2-3) sulfhydryl groups. Although it was earlier reported (Kochakian et al, 1973) that one of the enzymes disappeared after castration and reappeared after testosterone treatment, a later report (Shen & Kochakian, 1978) did not confirm this apparent androgen dependence phenomenon.

The 17 $\beta$ -HSD of the female guinea pig kidney has also been purified after the enzyme was induced by testosterone treatment up to 50 days at which time the enzyme activity paralleled with that of the normal male (Shen & Kochakian, 1979). The induced enzyme had a molecular weight of 32,000 and a single protein and enzyme staining band on gel electrophoresis. The pure enzyme dissociated into three electrophoretic bands on removal of 2-

mercaptoethanol from the solution and was restored by replacement of the mercaptoethanol. Storage of the cytosol at 4°C for 48 hours with or without 2-mercaptoethanol also yielded multiple bands. The multiple forms of the male guinea pig kidney exhibited similar electrophoretic behaviour (Shen & Kochakian, 1978). The steroid specificity, Km value, apparent isoelectric point, pH optimum and NAD(P)<sup>+</sup> requirement of the induced enzyme from female kidney were practically identical with those of the purified major enzyme form (I<sub>1</sub>) of the male guinea pig kidney. It was speculated that the male and female kidneys contained an identical gene for this particular enzyme form.

The male guinea pig liver 17β-HSD of the cytosol has also been resolved into multiple forms, one major and six minors (Kobayashi & Kochakian, 1978). Two separate groups of enzymes appeared to exist according to the amino acid composition and substrate specificities. One group could utilize either NAD<sup>+</sup> or NADP<sup>+</sup> and appeared to be specific for 5β-androstanes. The second group had a high preference for NAD<sup>+</sup> and 5α-androstane substrates. The molecular weight estimated for four of the enzymes was about 32,000. The other three showed the presence of 2 subcomponents of 24,000 and 11,000. Some comparisons between the 17β-HSD of this study and similar enzymes reported by others could be made. During investigation of a soluble 3-hydroxyhexobarbital dehydrogenase from guinea pig liver which catalyzed the reversible oxidation of 3-hydroxyhexobarbital to 3-oxohexobarbital, it was found that the pure enzyme could also catalyze the dehydrogenation of a variety of androgens having a 17β-hydroxyl group (Kageura & Toki, 1975). (3-Hydroxyhexobarbital, [5-(3<sup>1</sup>-hydroxyl-1<sup>1</sup>-cyclohexen-1<sup>1</sup>-yl)-1,5-dimethylbarbituric acid], is a microsomal oxygenation product of hexobarbital).

Yet another 17 $\beta$ -HSD was discovered from the same source and purified (Kageura & Toki, 1977). This enzyme termed a "new testosterone 17 $\beta$ -dehydrogenase" could not metabolize 3-hydroxyhexobarbital. Moreover, the new enzyme oxidized testosterone less rapidly than did the hexobarbital dehydrogenase. The two enzymes also exhibited different activity for the metabolism of 5 $\alpha$ - and 5 $\beta$ -congeners of the 17 $\beta$ -hydroxysteroids but were similar with respect to coenzyme requirement (preferring NADP<sup>+</sup>), pH optima and molecular weight. The "new testosterone 17 $\beta$ -dehydrogenase" mimicked two of the liver enzyme forms described by Shen & Kochakian (1978) with respect to dual nucleotide specificity and preference for 5 $\beta$ -androstane steroids such as 5 $\beta$ -dihydrotestosterone and 5 $\beta$ -androstane-3 $\alpha$ ,17 $\beta$ -diol. Perhaps it is noteworthy that the highest dehydrogenase activity in the female guinea pig liver was obtained with 5 $\beta$ -dihydrotestosterone. The importance of these enzymes which exhibited high activity toward 5 $\beta$ -androstanes is not clear since these steroids are biologically inactive.

Thaler-Dao et al (1972) initially reported that there were at least two different NAD(P)<sup>+</sup>-linked 17 $\beta$ -HSDs in the soluble fraction of the female rabbit liver. Chromatography on DEAE-cellulose and gel electrophoresis showed one enzyme form had activity toward C<sub>18</sub>- and C<sub>19</sub>-steroids. The other obtained in a homogenous state had a high affinity for testosterone and in addition exhibited a high 3 $\alpha$ -HSD activity. No 17 $\alpha$ -HSD activity was detected. However, Hasnain & Williamson (1974) working with the same animal system reported the separation of the 17 $\beta$ - and 17 $\alpha$ -HSD activities. Separate 17 $\beta$ -HSD activities toward 17 $\beta$ -estradiol and 17 $\beta$ -estradiol 3-glucuronide were also obtained. In contrast, Takenoshita and Toki (1978)

found that the rabbit liver cytosol contained several enzyme activities for the dehydrogenation of  $5\beta$ -androstane- $3\alpha,17\beta$ -diol to  $5\beta$ -androstan- $3\alpha$ -ol- $17$ -one. One of the activities was not separable from the 3-hydroxyhexobarbital dehydrogenase in the course of purification and on gel electrophoresis. However, the 3-hydroxyhexobarbital dehydrogenase was separated distinctly from the testosterone  $17\beta$ -dehydrogenase by triethylaminoethyl-cellulose column chromatography. In contrast with the guinea pig liver, 3-hydroxyhexobarbital and testosterone were metabolized by different enzymes and testosterone functioned only as a poor substrate for rabbit liver 3-hydroxyhexobarbital dehydrogenase. It may also be noted that 3-hydroxyhexobarbital dehydrogenase obtained from rabbit liver cytosol was separable from alcohol dehydrogenase and that horse liver alcohol dehydrogenase was unable to oxidize 3-hydroxyhexobarbital (Toki & Tsukamoto, 1964).

The soluble  $17\alpha$ -HSD of female rabbit liver was resolved by DEAE-cellulose chromatography and column isoelectric focusing into 8 molecular forms (Hasnain & Williamson, 1975). Five of the enzymes were obtained in a homogenous state. One enzyme form specific for  $17\alpha$ -estradiol 3-glucuronide was also demonstrated. These enzymes were charge isomers with an average molecular weight of 39,600. The amino acid composition of the different enzymes were very similar. All but one form of the  $17\alpha$ -HSDs also showed greater activities toward epitestosterone than toward the estrogenic substrates (Hasnain & Williamson, 1977).

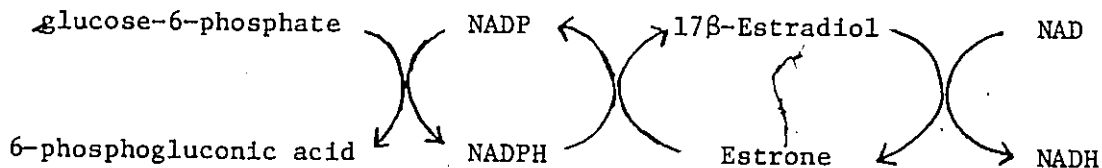
The normal and neoplastic endometrium from human were shown to have different molecular forms of the  $17\beta$ -HSD (Pollow et al, 1976b). Isoelectric focusing in a sucrose density gradient of the  $17\beta$ -HSD from the secretory

endometrium gave three enzymatically active bands with both testosterone and 17 $\beta$ -estradiol as substrates in the presence of NAD(P)<sup>+</sup>. On the other hand, the activity patterns from the endometrial carcinoma gave 2-7 bands depending on the substrate and coenzyme. These findings suggested the possibility of different molecular forms of the 17 $\beta$ -enzyme in normal and neoplastic endometrium.

Engel and Groman (1974) reported that isoelectric focusing of the soluble 17 $\beta$ -estradiol dehydrogenase from human placenta in polyacrylamide gels produced multiple bands. Under dissociating conditions in 8M urea three protein bands were seen. However, in the native state, three major and two faint bands were detected by substrate staining. The pattern was interpreted as being due to three different monomers present in unequal amounts that interacted to form six dimers. This is disconcerting in view of the identical dimeric structure of the enzyme (Pons et al, 1977). Nevertheless it was argued that whatever the isozymic structure of the enzyme was, the primary structure of the subunits might differ slightly so as not to affect the active site. All of the properties of the EDH described have characterized the active dimeric structure of the enzyme. It would be interesting to see the characterization of each isozymic form of the enzyme.

##### 5. Steroid-mediated transhydrogenase reaction

An unusual property of the human placental EDH is its intrinsic ability to carry out a transhydrogenation according to the following scheme:



The NADPH formed by a generating system such as the oxidation of glucose 6-phosphate to 6-phosphogluconic acid catalyzed by glucose 6-phosphate dehydrogenase is continuously oxidized by catalytic amounts of estrone which are converted into 17 $\beta$ -estradiol by EDH. Estrone is regenerated by the same enzyme in the presence of NAD<sup>+</sup>.

There was an earlier apparent conflict between two groups of investigators concerning the mechanism of the stimulation by 17 $\beta$ -estradiol of the enzymatically catalyzed transfer of hydrogen from NADPH to NAD<sup>+</sup> in human placenta extracts. Briefly, Talalay et al (1958) maintained that the 17 $\beta$ -estradiol functioned as a coenzyme during the transfer of hydrogen from NADPH to NAD<sup>+</sup>; this transfer was enzymatically catalyzed by the EDH which possessed dual pyridine nucleotide specificity and which also effected the interconversion of estrone and 17 $\beta$ -estradiol. Later, Jarabak et al (1962) achieved a 2500-fold purification of the EDH and reported that there was no separation of the dehydrogenase from the transhydrogenase activity over the entire eight-step purification procedure. These workers concluded that the EDH was responsible for most, if not all, of the 17 $\beta$ -estradiol-mediated transfer of hydrogen between the pyridine nucleotides in the human placental extracts. In contrast, Hagerman and Vिलlee (1959) maintained that there was an estrogen-dependent transhydrogenase distinct from the EDH, the latter presumably consisted of two discrete proteins one specifically required NAD<sup>+</sup> and the other NADP<sup>+</sup> as hydrogen acceptors. A later report (Hagerman & Vилlee, 1965) however inferred the presence of two transhydrogenase systems in the soluble extracts of the human placenta, one due to the substrate-activated transhydrogenase catalyzed by EDH and the other due to a specific estrogen-dependent transhydrogenase. Using hydroxylapatite column

chromatography Karavolas and Engel (1966) separated the "transhydrogenase function of EDH" from the "17 $\beta$ -estradiol activated (or dependent) transhydrogenase". The former enzyme activity could be eluted off at a lower phosphate concentration than the latter enzyme. In the 17 $\beta$ -estradiol activated transhydrogenase, there was no simultaneous 17 $\beta$ -estradiol and estrone interconversion. In contrast, Hagerman (1969) claimed the preparation of EDH free of the unique transhydrogenase activity. The enzyme preparation was shown to exhibit a concentration-dependent monomer-dimer-trimer transition in which the monomer catalyzed both steroid dehydrogenation and substrate-activated transhydrogenation. On the other hand, the trimer catalyzed only substrate-activated transhydrogenation.

Some fundamental differences between the transhydrogenase function of EDH and the substrate activated transhydrogenase activities have been reported (Karavolas et al, 1969). With the latter enzyme there was no isotopic effect observed with 17 $\alpha$ -<sup>2</sup>H-estradiol-17 $\beta$  nor was there a loss of deuterium from the 17 $\alpha$ -position of the 17 $\beta$ -estradiol during transhydrogenation. The 17 $\beta$ -estradiol activated transhydrogenase was also unstable to heating to 55<sup>o</sup>C, the differential destruction being made use of by Jarabak et al (1966) in their purification procedure for the EDH. The conditions under which the two transhydrogenase systems were stimulated were quite different. Both were activated by 17 $\beta$ -estradiol and neither by 17 $\alpha$ -methylestradiol. Diethylstibestrol stimulated only the 17 $\beta$ -estradiol activated transhydrogenase. Finally, estrone stimulated the transhydrogenase of the dehydrogenase system but had no effect upon the 17 $\beta$ -estradiol activated transhydrogenase.

The stereospecificity of hydrogen transfer to or from the pyridine ring of NAD(H) was "B" stereospecific in both the dehydrogenase and the transhydrogenase reactions (Jarabak & Talalay, 1960). Recently, the intrinsic transhydrogenase activity of the EDH and the transfer of the 4-(pro-S) proton of NADH was further demonstrated by affinity labeling whereby it was also shown that reversible enzymatic interconversion of covalently bound estrone was still possible (Groman et al, 1975).

EDH cannot form an abortive ternary complex with NADPH in the presence of 17 $\beta$ -estradiol nor with NAD<sup>+</sup> in the presence of estrone (Betz, 1971). Pons et al (1977) have suggested that this property of the EDH along with its dual pyridine nucleotide specificity are involved in the transhydrogenase function of this enzyme.

In addition to the human placental EDH, certain other mammalian or microbial HSDs are capable of catalyzing transhydrogenation in the presence of low levels of the appropriate steroid substrates. The 3 $\alpha$ -HSD of rat liver and the 3 $\alpha$ - and 3(17) $\beta$ -HSDs of Pseudomonas testosteroni are examples (Talalay, 1963). Although the two latter microbial enzymes are highly specific for NAD<sup>+</sup> and do not react at all with NADP<sup>+</sup>, transhydrogenation could still be demonstrated. Thus, the microbial 3 $\alpha$ -HSD catalyzed an androsterone-dependent transhydrogenation between NADH and the 3-acetylpyridine, 3-pyridine aldehyde, and thionicotinamide analogs of NAD<sup>+</sup>. Similarly, the 3(17) $\beta$ -HSD promoted the reduction of 3-pyridine aldehyde adenine dinucleotide by NADH in the presence of low levels of androstenedione.

Several criteria have to be met in order to demonstrate successfully the transhydrogenase reaction by hydroxysteroid dehydrogenases (Talalay, 1963). These include a comparable rate of reaction with NAD<sup>+</sup> and NADP<sup>+</sup> in the dehydrogenase reaction, ability of the steroid to undergo oxidoreduction by

the specific enzyme, proper concentration of the steroid and proper relative concentrations of the donor and acceptor nucleotides (as low as  $10^{-9}$ M.). For example, the very high affinities of an enzyme for  $\text{NADP}^+$  and  $\text{NADPH}$  required that only very low concentrations of these nucleotides be used for transhydrogenase experiments involving other nucleotides of lesser affinity. In this case, hydrogen transfer from the enzymatically generated  $\text{NADPH}$  to  $\text{NAD}^+$  would occur efficiently provided the acceptor nucleotide was present in large concentration relative to the donor. On the other hand, transhydrogenation of  $\text{NADH}$  to  $\text{NADP}^+$  would readily be demonstrated when  $\text{NADH} \gg \text{NADP}^+$  (Talalay, 1963). In most studies on  $17\beta$ -HSDs, the transhydrogenase reaction was not considered. However in others it was either not rigorously examined e.g. porcine testicular microsomal  $17\beta$ -enzyme (Inano & Tamaoki, 1975) or the activity was not demonstrable at physiological concentrations of the reactants e.g. guinea pig liver  $\text{NADP}^+$ -specific  $17\beta$ -HSD (Viljee & Spencer, 1960).

The discovery of the  $17\beta$ -estradiol activated transhydrogenase and the transhydrogenase function of EDH in the placenta had led to an earlier proposal that the transhydrogenations could be a mechanism of hormonal action, regulating the levels of oxidized and reduced pyridine nucleotides and thus the amount of  $\text{NADPH}$  available for biosynthetic purposes (Talalay & Williams-Ashman, 1960). But this concept has been overshadowed by the development of the receptor theory and the finding of hormonal activity in the immature rat uterus where the  $17\beta$ -estradiol-estrone interconversion does not occur. (For further discussion see Williams-Ashman & Liao, 1964; Engel, 1970). Recent investigations point to a major mechanism of action of the steroid hormones by the regulation of specific gene expression (Chan et al, 1978). However, Pons et al (1977) asserted that while the transhydrogenation might not be a general

mechanism of estrogen action, it might function in this manner in the placenta where the estrogen content is high and the affinity of the transhydrogenase for  $17\beta$ -estradiol is high ( $K_m$  0.2  $\mu M$ ).

6) Regulation of  $17\beta$ -HSDs

Studies on normal human endometrium have shown that the  $17\beta$ -HSD activity was maximal during the luteal phase of the menstrual cycle (Tseng & Gurpide, 1974). The correspondence between the  $17\beta$ -enzyme levels and the plasma concentrations of progesterone following ovulation suggested that this hormone could have an inductive effect upon the endometrial enzyme. Indeed, this was shown to be the case by both in vivo and in vitro studies. Administration of medroxyprogesterone acetate (6 $\alpha$ -methyl-17 $\alpha$ -acetoxyprogesterone or Provera) to women during the proliferative phase resulted in a significant increase in  $17\beta$ -enzyme activity, while in vitro induction of the enzyme was seen when the proliferative endometrium was incubated for two to three days in medium containing progesterone at physiological levels. Norgestrel (17 $\alpha$ -ethynyl-18-methyl-19-nortestosterone), a synthetic progestin had a similar effect showing that the stimulation of the  $17\beta$ -enzyme was not due to substrate induction. This possibility was considered since progesterone might act as a substrate for the enzyme. Furthermore, addition of  $17\beta$ -estradiol to the culture medium did not influence the activity of the enzyme. The induction of  $17\beta$ -enzyme by progesterone was inhibited by puromycin or actinomycin D suggesting that RNA synthesis was required for induction (Tseng & Gurpide, 1975a). Other progestational agents which were effective inducers include 17 $\alpha$ -hydroxyprogesterone, medroxyprogesterone, R5020 and norethynodrel. In

contrast, testosterone and cortisol, like  $17\beta$ -estradiol, were not inducers (Tseng et al, 1977).

Concomitant with an increase of endometrial  $17\beta$ -HSD during the luteal phase was a reduction of  $17\beta$ -estradiol receptors. The decline in the receptor level was due to the influence of progesterone (Tseng & Gurpide, 1975b). While the increase in  $17\beta$ -enzyme activity led to an acceleration of the conversion of  $17\beta$ -estradiol to estrone, the decrease in the intracellular levels of  $17\beta$ -estradiol and its receptors led to reduced binding. Both effects thus tended to reduce the response of endometrium to circulating  $17\beta$ -estradiol and explained the antagonism of progesterone to estrogen action.

The effects of progestins on the  $17\beta$ -HSD and  $17\beta$ -estradiol receptor levels were also observed in endometrial carcinoma (Pollow et al, 1975a; Tseng et al, 1977) i.e. the  $17\beta$ -enzyme activity of post-menopausal, hyperplastic or adenocarcinomatous endometrium was found to be as low as that in proliferative endometrium while the concentration of  $17\beta$ -estradiol receptor was not very different from the normal proliferative endometrium. In these cases, progesterone which was the necessary agent for the induction of  $17\beta$ -enzyme activity was absent. It is currently accepted that chronic estrogenic stimulation of the endometrium, in the absence of progesterone, favors the development of endometrial hyperplasia (Tseng et al, 1977). Malathi and Gurpide (1977) observed that the rat prostatic and human hyperplastic prostatic conversions of testosterone to androstenedione were also increased with the addition of progesterone to the incubation mixture. However, the increase was not due to stimulation of the  $17\beta$ -enzyme activity but was a consequence of the inhibitory effect of progesterone on the conversion of testosterone to  $5\alpha$ -dihydrotestosterone, the latter being the active androgen in the prostate.

Moreover, upon administration of Provera the induction of the prostatic 17 $\beta$ -HSD activity was not as significant as the increase of 17 $\beta$ -estradiol dehydrogenase activity observed in the case of the human endometrium. Thus, it was suggested that the progestin-induced changes in the prostatic 17 $\beta$ -enzyme activity might not be physiologically as important in the regulation of androgen action in prostatic tissue as the induction of 17 $\beta$ -dehydrogenase enzyme in the regulation of estrogen action in human endometrium.

The "induction" of the 17 $\beta$ -HSD of the female guinea pig kidney has also been described (Shen & Kochakian, 1979). Upon administration of testosterone, a progressive increase of the 17 $\beta$ -enzyme activity from trace levels to the normal male kidney levels was achieved after 50 days. The stimulating effect of testosterone was specific for testosterone and 5 $\alpha$ -dihydrotestosterone. Apparently, the enzyme could regulate the level and metabolic action of testosterone in the kidney of the female guinea pig. In contrast, the liver did not respond to the testosterone treatment indicating separate control mechanisms for the two organs.

Kochakian et al (1973) reported that castration produced a partial decrease in the 17 $\beta$ -enzyme activity of the male kidney with a restoration to normal on administration of testosterone propionate. The difference was attributed to the disappearance and appearance of an enzyme form detectable by substrate staining on polyacrylamide gels. However, Shen and Kochakian (1978) in a recent report did not confirm this gel pattern although they did observe a decrease in the concentration of the 17 $\beta$ -enzyme activity after castration. The partial loss of enzyme activity after castration suggested the involvement of other steroid production sites other than the testis such as the adrenal cortex.

In contrast to the stimulatory effect of androgens described above, this class of steroids has been reported to have a repressive influence on the renal cytoplasmic  $17\beta$ -enzyme activity of the male rat (Ghraf et al, 1977). Castration or treatment of intact male animals with  $17\beta$ -estradiol elevated the enzyme activity of the male animals to that of the female. Hypophysectomy of normal or castrated rats of either sex led to a drastic fall in enzyme activity demonstrating the role of the hypophysis in the regulation of this androgen-dependent enzyme. The hypophyseal factors involved are growth hormone and prolactin both of which when administered caused a significant increase in the enzyme activity. No effect on the enzyme activity occurred in response to treatments with other hormone preparations including testosterone or gonadotropins. Removal of the adrenals or the thyroid gland, also resulted in a significant decrease in activity in both sexes. A complex endocrine management, therefore seems to be involved in the regulation of the soluble  $17\beta$ -enzyme of rat kidney.

The soluble  $17\beta$ -enzyme of the rat liver is an estrogen-dependent enzyme (Ghraf et al, 1975). This is characterized by a masculinization of the enzyme activity of female animals upon gonadectomy or testosterone administration. Gonadectomy of the male animal had no effect on the liver  $17\beta$ -enzyme. However, intact male animals reacted to the administration of  $17\beta$ -estradiol by a feminization of this enzyme activity. It has not been established whether the hypophysis plays a role in the regulation of this enzyme.

The possibility that the dehydrogenase activities of  $17\beta$ -hydroxysteroids may be regulated by changes in the concentration of certain intracellular substances has also been explored. Shaw and Jeffrey (1976a) have studied the influence of some 20 nucleotides on the human placental EDH and found that ATP

inhibited the oxidation of both  $17\beta$ -estradiol and  $20\alpha$ -hydroxy-4-pregnen-3-one with  $\text{NAD}^+$  as coenzyme. They suggested that this inhibition by ATP could also occur in vivo. However, the effects of ATP differed depending on the coenzyme employed (Shaw & Jeffrey, 1976b). It was shown that ATP inhibited the reduction of estrone,  $16\alpha$ -hydroxyestrone or dehydroepiandrosterone more strongly with NADH as coenzyme than with NADPH. Earlier, the same authors had excluded the direct effect of prostaglandins on the placental enzyme (Shaw & Jeffrey, 1975).

Yet another point of control might be exerted by the testosterone-estradiol-binding globulin or the steroid-binding  $\beta$ -globulin present in the plasma (Heyns, 1977). This binding-globulin binds selectively  $\text{C}_{18}$ - or  $\text{C}_{19}$ - $17\beta$ -hydroxysteroids and therefore may influence directly the enzymatic reactions of steroids. In one isolated case, the effect of the steroid-binding globulin and albumin (which also binds steroids) on the oxidoreduction of four  $\text{C}_{19}$ - $17\beta$ -hydroxysteroids by the  $3(17)\beta$ -HSD of *Pseudomonas testosteroni* was investigated (Hampl & Starka, 1975). It was found that there was a decreased yield of products in the presence of the binding globulin in both directions of the reversible reaction. This finding was unexpected in the case of the reductive reaction because of the high affinity of the binding protein for the product (e.g. testosterone) but not the substrate (e.g. androstenedione). In this case, an increase of the reaction rate would be expected. Undoubtedly, the role of the steroid binding  $\beta$ -globulin warrants further studies.

#### 7. Clinical implications and applications

A congenital deficiency of testicular  $17\beta$ -HSD has been shown to contribute to a condition called male pseudohermaphroditism (MP) (Saez et al, 1971, 1972; Givens et al, 1974; Pittaway et al, 1976; Akesoda et al, 1977; Virdis et al, 1978).

Some common features of MP patients include the following: 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 with gynecomastia (breast development) at puberty (Saez et al, 1971, 1972; Akesoda et al, 1977) or without (Givens et al, 1974).

With testicular 17 $\beta$ -HSD deficiency in MP, there is marked elevation of plasma androstenedione but low plasma testosterone. In vitro studies with testis slices incubated with androstenedione or testosterone support the enzymatic defect (Givens et al, 1974; Akesoda et al, 1977; Pittaway et al, 1976). Plasma estrogen concentrations are sometimes elevated in these patients as a consequence of the conversion of excessive circulating androstenedione to estrone and then to estradiol. The clinical manifestation is gynecomastia. However, in those cases where virilization occurs but gynecomastia does not develop, estrogen levels are probably lower than in other reported cases.

Defects in the synthesis of testosterone may result from other key enzymes involved in the biosynthesis of testosterone. The efficient enzymes may involve cholesterol 20,22-desmolase or 20 $\alpha$ ,22-hydroxylase, 3 $\beta$ -HSD, 17 $\alpha$ -hydroxylase or 17,20-desmolase. A review of the testosterone enzyme deficiency is provided by Imperato-McGinley and Peterson (1976). In addition, the authors described a 5 $\alpha$ -reductase deficiency in which the basic defect resides in the hormone responsive tissue. This form of MP has normal testosterone level but decreased dihydrotestosterone plasma level. The decreased dihydrotestosterone formation may therefore affect the male differentiation of the external genitalia. In association with the disorders of function at the androgen dependent-target tissues, defective androgen receptor proteins may also play a role (refer to Imperato-McGinley & Peterson, 1976; Pittaway & Stage, 1978 for further discussion).

Another pathological condition associated with feminization of the male is the occurrence of liver disease in chronic alcoholics. Other characteristics include breast development and testicular atrophy. An increase in plasma concentration and production rate of  $17\beta$ -estradiol and a decrease in plasma concentration, metabolic clearance and production of testosterone, among many other factors, have been noted (Gorden et al, 1976). Increased circulating  $17\beta$ -estradiol may be due to an increased peripheral conversion of androgenic precursor hormones testosterone and androstenedione, via the action of aromatase enzyme. Indeed an elevated aromatase activity has been demonstrated in the chronic alcoholic rat liver together with an increased plasma  $17\beta$ -estradiol but a decreased testosterone level (Gordon et al, 1979). Another enzyme activity, though not examined, that is expected to increase under the same conditions is the  $17\beta$ -HSD. This is in keeping with the fact that in ethanol-treated animal there is an increase in hepatic reducing equivalents of NADH which would favor the formation of estradiol as compared to estrone i.e. there will be an increase in the formation of  $17\beta$ -hydroxy metabolites and a corresponding fall in the  $17\beta$ -ketosteroids. Indeed this has been shown in a superfused guinea pig liver where the increased concentration of NAD during ethanol metabolism has caused an increase in the reduction of  $17\beta$ -ketosteroid sulfates to the corresponding  $17\beta$ -hydroxysteroids (Admirand et al, 1970).

Epitestosterone has been demonstrated in normal human urine by several investigators but the large increase over normal values found in the urine of patients with hirsutism and virilism (de Nicola et al, 1966) may be a reflection of the enzyme system responsible for its metabolism. Epitestosterone is virtually devoid of androgenic properties, therefore its biological role may be of considerable interest.

One recent direct clinical application of the study of the human placental EDH has been its use in the determination of estrogens in plasma and urine (Nicolas et al, 1979). The enzymatic assay makes use of the transhydrogenase function of the EDH whereby the transhydrogenase activity is directly related to the estrogen concentration. It was claimed that the method was highly specific for C<sub>18</sub>-steroids in view of the specificity of the EDH for these substrates; moreover, the specificity for transhydrogenase activity was higher than the specificity for dehydrogenase activity since the 3-sulfate and 3-glucuronide of 17 $\beta$ -estradiol which were good substrates for the dehydrogenase activity did not work for the transhydrogenase activity. In addition, estrone and 17 $\beta$ -estradiol could be determined together or separately if the former was previously reacted with hydrazine. The sensitivity of the method was reported to be near 10 picograms in plasma and 1 mg in urine with a precision of 10% in both cases. It is noteworthy that the determination can be done directly on hydrolyzed urine without extraction.

The serum 17 $\beta$ -HSD level has also been examined for possible diagnostic value during pregnancy in women since the enzyme probably originates from the placenta and the fetus (Lubbert, 1977). There has been good correlation between the enzyme level and the status of pregnancy. For example, there is an increase of enzyme activity during pregnancy and a rapid disappearance of activity after expulsion of the placenta. Also in acute damage of the placenta, the serum enzyme activity increases while in chronic damage the activity drops. Further, in imminent inevitable abortions, the enzyme levels were significantly higher than normal. However, the diagnostic value of the serum 17 $\beta$ -HSD warrants further studies, the biggest problem of stabilization of the enzyme activity being just overcome.

Studies on estrogen metabolism in normal and neoplastic endometrium and the influence of progestins on the 17 $\beta$ -HSD and 17 $\beta$ -estradiol receptor levels

described in section 6 have contributed to the understanding of the therapeutic use of progestational agents in various stages of endometrial carcinoma. It was suggested that a short term treatment with progestins and evaluation of the histologic changes by the histochemical procedure described by Scublinsky and co-workers (1976) and of induction of the  $17\beta$ -enzyme either in vivo or in vitro might be useful in predicting the responsiveness of endometrial cancer to hormonal therapy (Gurpide et al, 1977).

The microbial  $17\beta$ -HSD, including  $20\beta$ -HSD and  $3\alpha$ -HSD, have been immobilized on cyanogen bromide-activated Sepharose and used for the large scale transformation of steroids in water-organic solvent systems (Carrea et al, 1979). Ordinarily, the use of enzyme-catalyzed reactions for the transformation of steroids for preparative purposes is hindered by the low solubility of the steroids in aqueous buffers and by the availability and instability of the enzymes. The findings that immobilized enzymes retained a high activity for long periods of time (for example 60% of the original  $17\beta$ -HSD remained after two months of continuous use in the water-ethyl acetate system) has made the use of such immobilized enzymatic systems practical.

## CHAPTER II

### MATERIALS AND GENERAL METHODS

#### A) MATERIALS

##### 1. Isotopically labeled compounds:

[1-<sup>14</sup>C] Acetic anhydride (SA, 27 mCi/mmol), 5α-[1α,2α(n)-<sup>3</sup>H] Androstan-3α, 17β-diol (SA 41 Ci/mmol), 5α-[1α,2α(n)-<sup>3</sup>H] Androstan-3β,17β-diol (SA 40 Ci/mmol), 17β[6,7-<sup>3</sup>H] Estradiol (SA 40-60 Ci/mmol), [6,7-<sup>3</sup>H] Estrone (SA 44 Ci/mmol) and [6,7-<sup>3</sup>H] Estrone sulfate (potassium salt) (SA 1.1 Ci/mmol) were purchased from Amersham Corporation, Oakville, Ontario.

[1,2-<sup>3</sup>H(N)] Androstenedione (SA 40-60 Ci/mmol), [1,2-<sup>3</sup>H(N)]-Androsterone (SA 44.5 Ci/mmol), [1,2-<sup>3</sup>H(N)] Dihydrotestosterone (SA 50.6 Ci/mmol), [1,2-<sup>3</sup>H(N)] Epitestosterone (SA 50 Ci/mmol), [1,2-<sup>3</sup>H(N)] Etiocholanolone (SA 45 Ci/mmol), [7-<sup>3</sup>H(N)]-Pregnenolone (SA 17.2 Ci/mmol) and [1,2-<sup>3</sup>H(N)] Testosterone (SA 50-60 Ci/mmol) were purchased from New England Nuclear, Lachine, Quebec.

##### 2. Unlabeled steroids:

Androsterone, estrone sulfate (potassium salt) and 5α-androstan-3β,17β-diol were from Research Plus Steroids Lab. Inc. Benville, New Jersey.

5α-androstan-17α-ol-3-one, 5α-androstan-3α,17β-diol, 4-androsten-3β,17α-diol, 17α-estradiol 3-acetate, 17α-estradiol 3-trimethylacetate, 17-epiestriol, 16, 17-epiestriol, 6-keto-17α-estradiol, 5α-pregnan-3α-ol-20-one, 5α-pregnan-3α,20α-diol, 5α-pregnan-20α-ol-3-one, 5α-pregnan-20β-ol-3-one, 5α-pregnan-3α,20β-diol, 5β-pregnan-20β-ol-3-one, 5β-pregnan-3,20-dione and 17α-hydroxyprogesterone were purchased from Steraloids Inc. Wilton, NH.

17α-estradiol, 17β-estradiol, estrone, estrone-β-D-glucuronide (sodium salt), testosterone, epitestosterone, androstenedione, 5α-dihydrotestosterone, androstane-dione, etiocholanolone, etiocholan-3, 17-dione, pregnenediol, 5β-pregnan-3α,20β-diol, 5β-pregnan-3α-ol-20-one, 5β-pregnan-20α-ol-3-one, 5-pregnen-3β,20β-diol, 5-pregnen-3β,20α-diol & pregnenolone were obtained from Sigma Chemical Co. St. Louis, Missouri.

3. Coenzymes:

NAD, (Grade III from yeast), NADP (monosodium salt), NADPH (tetrasodium salt) and UDP-glucuronic acid were also from Sigma Chemical Co., St. Louis, Missouri.

4. Chromatographic and electrophoretic chemicals:

Sephadex G-75, G-200, both of superfine grade were from Pharmacia, Montreal, Quebec.

Ultradex, ampholine pH range 2-10; 4-6 and 5-7 were from LKB, Sweden distributed by Fisher Scientific Co., Ltd., Ottawa, Ontario.

Acrylamide, bisacrylamide, TEMED were from Eastman Kodak Co., Rochester, New York.

DEAE-cellulose (DE-52) was from Mandel Scientific Co., Montreal, Quebec.

Silica gel (Kieselgel N) without binder was from Macherey Nagel & Co., Germany.

5. Protein standards, enzymes and related compounds:

Ribonuclease, chymotrysinogen A, aldolase and ovalbumin were from Pharmacia Molecular Weight Calibration Kit.

Bovine serum albumin, dithiothreitol, Coomassie Brilliant Blue R250, NBT, PMS were from Sigma Chemical Co., St. Louis, Missouri.

Ketodase ( $\beta$ -glucuronidase) was from General Diagnostic, Warner-Lambert Co., Morris-Plains, New Jersey.

Papain was from Worthington Biochemicals Corp., Freehold, New Jersey.

PCMB was from General Biochemicals, Chagrin Falls, Ohio.

Biuret reagent was from Fisher Scientific Co., Ottawa, Ontario.

SDS (sequanal grade) was from Pierce Chemical Co., Rockford, Illinois.

Urea (ultra pure) was from Schwarz/Mann, Orangeburg, New York.

$\epsilon$ -DNP-L-Lysine HCl was from United States Biochemical Corp., Cleveland, Ohio.

6. Scintillation fluor and fluid:

PPO was purchased from Kent Laboratories Ltd., Vancouver, British Columbia.

Aquasol was from New England Nuclear, Lachine, Quebec.

7. Buffer salts and solvents:

All salts and solvents were of high purity preparations or of reagent grades. 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

$17\alpha$ -[6,7- $^3\text{H}$ ] Estradiol and  $17\alpha$ -[6,7- $^3\text{H}$ ] estradiol 3-glucuronide were prepared from the double conjugate  $17\alpha$ -[6,7- $^3\text{H}$ ] estradiol 3-glucuronide 17-N-acetylglucosaminide excreted in the urine of a New Zealand white female rabbit injected intravenously with 5 mCi of  $17\beta$ -estradiol or estrone (SA 40-60 Ci/mmol). The details of the methods for the isolation of the double conjugate have been described by a number of workers (Layne et al, 1965; Collins et al, 1967; Gwilliam et al, 1974). The double conjugate was incubated with hyaluronidase to remove the N-acetylglucosamine and produce the 3-glucuronide derivative of  $17\alpha$ -estradiol. Further treatment of the latter steroid monoconjugate with ketodase or  $\beta$ -glucuronidase removed the sugar moiety at C-3 to produce free

17 $\alpha$ -estradiol. The purity of the desired radioactive substrates was monitored by t.l.c. employing solvent systems such as benzene-ethyl acetate (7:3, v/v) or benzene-ethanol-methyl ethyl ketone-water (3:3:3:1, by volume). The specific activities of the purified tritiated substrates were adjusted to 4.5  $\mu\text{Ci}/\mu\text{mol}$  by the addition of the respective radioinert steroids.

17 $\beta$ -[6,7- $^3\text{H}$ ] Estradiol 3-glucuronide and [6,7- $^3\text{H}$ ] estrone 3-glucuronide were prepared by incubation of 17 $\beta$ -[6,7- $^3\text{H}$ ] estradiol and [6,7- $^3\text{H}$ ] estrone respectively 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. The aqueous layer was then adjusted to pH 2 and ethyl acetate was added to extract radioactive 17 $\beta$ -estradiol 3-glucuronide or estrone 3-glucuronide. Purification of the substrates was done by t.l.c. in benzene-ethanol-methyl ethyl ketone-water (3:3:3:1, by volume). The specific activities of the purified substrates were adjusted to 4.5  $\mu\text{Ci}/\mu\text{mol}$  by the addition of radioinert 17 $\beta$ -estradiol 3-glucuronide or estrone 3-glucuronide.

All other radioactive steroids from commercial source were monitored for their purity before they were used as substrates. All but two steroids were used without further purification. The two exceptions were radioactive 5 $\alpha$ -androstan-3 $\alpha$ ,17 $\beta$ -diol and 5 $\alpha$ -androstan-3 $\beta$ ,17 $\beta$ -diol which were purified to 92% and 95% respectively by t.l.c. in benzene-acetone (4:1, v/v).

For routine enzyme assays all the stock solutions of the radioactive steroids were made up in methanol to a specific activity of 4.5  $\mu\text{Ci}/\mu\text{mol}$  by the addition of radioinert steroids. The final steroid concentration was 0.1  $\mu\text{mol}/\text{ml}$ . These stock solutions were stored in the freezer at  $-20^{\circ}\text{C}$  but warmed to room temperature before use.

## 2. Radioactivity measurement

A Mark III Liquid Scintillation System Model 6880 (Searle Analytic Inc.) was used for all radioactivity counting. The scintillation mixtures used were 10 ml. toluene containing 40 mg PPO for non-polar samples and 10 ml of aquasol (New England Nuclear) or aquasol-xylene (1:1, v/v) were used for aqueous samples. The counting efficiency was determined by the channels ratio method and the quenching calibration curves were obtained with tritium or carbon-14 quench standards.

## 3. Enzyme assays

### a) With hydroxysteroids and NAD(P)<sup>+</sup>

The incubation mixture consisted of steroid substrate (SA 4.5  $\mu\text{Ci}/\mu\text{mol}$ ), 3 picomole in 30  $\mu\text{l}$  methanol; NAD(P)<sup>+</sup>, 0.5  $\mu\text{mol}$  (final concentration 167  $\mu\text{M}$ ) in 2.0 ml of 0.1 M glycine-NaOH buffer pH 9.5 and 1-500  $\mu\text{l}$  enzyme. The final volume was made up to 3.0 ml by the addition of 0.15 M KCl. Samples were incubated for 30 minutes in a water-bath maintained at 37°C. The amount of enzyme added to each incubation mixture was adjusted so that the extent of substrate oxidation did not exceed 25% ensuring that initial rates were measured. However, for assaying column fractions, especially at peak tubes, the extent of substrate oxidation sometimes exceeded 25%. In the control experiments, the enzyme was omitted.

### b) With ketosteroids and NADPH

The conditions and concentrations were as above except that the ketosteroid, NADPH and 0.1 M Tris-maleate buffer of pH 5.85 were used. The control experiment was also carried out in the absence of enzyme.

c) Treatment of incubated reaction mixtures

Incubations with C<sub>18</sub>-, C<sub>19</sub>- and C<sub>21</sub>-steroids were terminated by the extraction of the steroid substrate and product with 5 ml of ethyl acetate. The organic layer was removed and evaporated to dryness under nitrogen. A couple drops of methanol containing the relevant radioinert steroid standards was added to the ethyl acetate extract residue. The mixture was then spotted on thin layer plates coated with silica gel N and the mixture subsequently separated by chromatography in the solvent systems described in a later section.

In incubations with estrogen glucuronides, the reactions were stopped by the addition of 0.25 ml 1N HCl and extraction with 5 ml ethyl acetate. The organic layer was removed and evaporated to dryness under nitrogen. The residue was then incubated 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 extracted with ethyl acetate (5 ml) and analyzed by t.l.c.

Estrone sulfate was solvolysed by the method of Segal et al (1960). Solid sodium chloride (0.53 gm) was added to the reaction mixture (3 ml) to give a final salt concentration of 3 M. The aqueous mixture was then extracted with ethyl acetate (6 ml). To hydrolyze ~~the~~ sulfate, 0.1 ml of 0.012 M sulfuric acid in ethyl acetate (final concentration 0.2 mM) was added to the organic layer and the acidified mixture left for 24 hours at 37°C. A volume of water was then added to remove excess salt and the ethyl acetate extract containing the free steroids evaporated down to dryness and analyzed accordingly.

In all cases the recovery of extracted radioactivity after incubation or hydrolysis was better than 80%.

d) Thin layer chromatography and solvent systems

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 120°C for at least one hour before use. Samples were spotted 3 cm from the lower edge of the plate and 2 cm apart. Chromatography was done at room temperature. 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 for 5-10 minutes at 120°C. The spots corresponding to the added steroid standards were scraped into scintillation vials. Methanol (0.2 ml) was added to elute the radioactivity from the silica gel. Scintillation fluid (10 ml) was added and the radioactivity was measured in the scintillation counter.

The following solvent systems were employed to separate the various steroids or pairs of steroids representing the substrate and the corresponding product(s). The R<sub>f</sub> values are as noted.

- 1) Benzene-acetone (4:1, v/v)

17α-Estradiol (0.55)		
17β-Estradiol (0.50)	.....	Estrone (0.74)
Epitestosterone (0.48)		
Testosterone (0.49)	.....	Androstenedione (0.72)
Androsterone (0.66)	.....	Androstanedione (0.86)
5α-Androstan-3α,17β-diol (0.47)		
5α-Androstan-3β,17β-diol (0.41)	.....	5α-Dihydrotestosterone (0.67)
Etiocholanolone (0.56)	.....	Etiocholan-3,17-dione (0.86)
Pregnenolone (0.69)		
	.....	5-Pregnen-3β,20α-diol (0.45)
	.....	5-Pregnen-3β,20β-diol (0.50)
- ii) Benzene-ethanol-methyl ethyl ketone-water (3:3:3:1, by volume)

Estrone 3-glucuronide (0.38)	.....	Estrone (0.84)
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- iii) Isopropanol-chloroform-methanol-water (5:5:2.5:1, by volume)

Estrone 3-sulfate (0.63)	.....	Estrone (0.90)
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e) Expression of enzyme activity

The % conversion of substrate to product ( $A \rightarrow B$ ) was calculated by the formula:  $\frac{B}{A+B} \times 100$ . Enzyme activity is expressed as units per ml or units per mg protein. Owing to the low amounts of enzyme, milliunits or  $\mu$ units are often used. One  $\mu$ unit of activity is defined as the amount of enzyme catalyzing the oxidation or reduction of 1 picomole of steroid substrate per minute under the specified conditions of the assay.

4. Protein determination

The Biuret method (Gornall et al, 1949) was used for crude protein fractions. For the more purified fractions, the protein concentrations were estimated by UV absorption at 280 and 260 nm related by the equation:  $\text{mg protein/ml} = 1.55 A_{280} - 0.76 A_{260}$  (Layne, 1957). When monitoring the protein content in column fractions the absorbance at 280 nm was read. A Cary 15 spectrophotometer was used for all the UV readings.

5. Purification of kidney hydroxysteroid dehydrogenases

All procedures for the purification of the 17-HSDs were carried out at about 4°C. Essentially the steps employed in the purification of the liver 17-HSD (Hasnain & Williamson, 1974, 1975) were adopted with a few modifications.

In a typical experiment, three female virgin New Zealand white rabbits 2-3 months old were sacrificed by cervical dislocation. The kidneys were removed, freed of adherent tissues by decapsulating the outer membrane and rinsed in cold 0.25 M sucrose. The tissue (about 1 gm/kidney) was minced with scissors and a 20% (w/v) homogenate was prepared in 0.25 M sucrose containing 0.5 mM DTT using a Sorvall omnimixer for 1 minute at top speed. The homogenate

was centrifuged at 10,000g for 30 minutes in a Sorvall RC2-B centrifuge to remove nuclei and mitochondria. The resulting supernatant was centrifuged at 105,000 g for one hour in a Beckman ultracentrifuge to separate the microsomal fraction and the soluble fraction (105,000 g supernatant). The soluble fraction was then subjected to calcium phosphate gel fractionation.

When the microsomes were needed, the microsomal pellet was washed by resuspending in 0.25 M sucrose - 0.5 mM DTT and centrifuging at 105,000 g for an additional hour. The resulting pellet was suspended in buffer to give a final volume equivalent to the weight of the original tissue.

#### 6. Calcium phosphate gel fractionation

The 17-HSD activity of the 105,000 g supernatant was partially purified by adsorption and desorption from calcium phosphate gel prepared according to the method of Swingle and Tiselius (1951). A solution of 0.5 M potassium phosphate buffer, pH 7.0, was added to the 105,000 g supernatant to a final concentration of 0.5 mM. In a typical fractionation procedure, this supernatant (12-15 mg protein/ml) was mixed with a 3% calcium phosphate gel suspension, 1 ml of gel being used for each 6-8 mg of kidney supernatant protein. The mixture was stirred in an ice-bath for 45 minutes and then centrifuged at 2500 g for 10 minutes. The resulting supernatant was discarded and the pellet resuspended in a volume of 70 mM potassium phosphate buffer, pH 7.0 containing 0.5 mM DTT equal to that of the original calcium phosphate gel suspension. The mixture was again stirred for about 45 minutes and centrifuged. The supernatant was filtered through glass wool to remove fatty materials. The filtrate was then concentrated to about 10 ml by ultrafiltration in an Amicon Diaflo cell equipped

with a PM-10 membrane. The concentrate was allowed to stand at 4°C overnight to remove dissolved gases. Any insoluble material was removed from the enzyme fraction by centrifugation just prior to Sephadex gel column chromatography.

7. Sephadex G-75 (superfine) column chromatography

Gel filtration in Sephadex G-75 superfine was carried out in a column 5 x 90 cm. The equilibrating and elution buffer was 10 mM Tris-HCl-0.5 mM DTT, pH 8.0. The enzyme sample (up to one gm of protein) obtained after calcium phosphate gel filtration was applied onto the column through a flow adapter. Elution was carried out at a flow rate of 20-25 ml/hr. Fractions of 160 drops (about 10 ml) were collected. After chromatography, every fourth fraction after the void volume was assayed for HSD activity. The active fractions were then pooled and concentrated by ultrafiltration.

8. DEAE-cellulose column chromatography

The pre-swollen microgranular form of DE-52 (100 g) was used without precycling but fully equilibrated first with concentrated buffer (0.1 M Tris-HCl, pH 8.0) followed by 10 mM Tris-HCl, pH 8.0. After packing, the DEAE-cellulose column (2.5 x 40 cm) was equilibrated with the starting buffer, 10 mM Tris-HCl-0.5 mM DTT-30 mM NaCl, pH 8.0. The concentrated hydroxysteroid dehydrogenase fraction (200-250 mg protein in about 10 ml buffer) obtained after Sephadex gel filtration was then applied onto the anion exchange column. Protein elution was begun with 100 ml of starting buffer followed first by a 1.2 litre linear gradient of sodium chloride from 30 mM-60 mM in 10 mM Tris-HCl-0.5 mM DTT, pH 8.0 and then by a 1.2 litre linear gradient of 60 mM-120 mM NaCl in the same Tris buffer. During this chromatography, the initial flow rate was adjusted to

about 50 ml/hr and approximately 10 ml fractions were collected. After chromatography the various HSD activities were monitored in every third tube.

### 9. Isoelectric focusing

A Desaga flat-bed isoelectric focusing system was used. Two types of granulated gels were employed as the anti-convective media: Sephadex G-200 superfine or Ultrodex.

#### a) Preparation of gel slurry

Method 1: Isoelectric focusing in Sephadex G-200 superfine was carried out essentially as described by Radola (1973, 1974). A glass trough consisting of a 40 x 20 glass plate and a 1 x 1 cm frame was used to contain the gel suspension. This gel suspension usually prepared in a 500 ml conical flask with a side-arm consisted of the following:

Sephadex G-200 (superfine)	8.8 g	(4%, w/v)
distilled water	220 ml	
Ampholine (40% w/v) pH 5-7	11 ml	(2%, w/v)
pH 2-10	1.1 ml	(0.2%, w/v)
DTT	17 mg	(0.5 mM)

Although in some experiments up to 300 ml distilled water was used, the proportions of the different ingredients were adjusted accordingly. The gel suspension was deaerated for a few minutes and then poured into the plate, spread evenly and dried to the correct consistency. The criterion adopted was a flat and firm surface when the plate was inclined at an angle. The final gel layer was 2-3 mm in thickness. With the aid of a 1 cm wide spatula, a slot of 15 cm long was made in the gel layer in the middle of the plate. This

could accommodate up to 4 ml of sample suspension. In experiments where two samples were run concurrently, two 5 cm slots clearly spaced from each other as well as from the edges of the plate were made.

Method 2: This is an improved method in which Ultrodex (LKB), a specially prepared and purified dextran gel was used. The composition and preparation of the gel slurry was as described in Method 1 except that the gel slurry was dried to a predetermined limit (LKB Instruction note 2117-510) allowing a standardized water content in the final gel composition. The following calculations are made:

Weight of gel slurry + flask = a gm  
Weight of flask after pouring off slurry = b gm  
Weight of gel slurry added to plate = a-b = c gm  
% allowed for water evaporation = 30% for 35% evaporation limit  
(or 35% for 39% limit)  
Final weight of slurry allowed = 70% x c = d or 65% x c = d  
Final weight of plate + slurry allowed = d + e gm where e = weight of the empty plate

This procedure was used in most of the electrofocusing experiments presented in this thesis.

b) Preparation of enzyme sample

Prior to isoelectric focusing, the enzyme samples were dialyzed against 1 mM Tris-HCl-0.5 mM DTT, pH 8.0 and concentrated to 1-4 ml by ultrafiltration. Sephadex gel G-200 (superfine) or Ultrodex (30 mg/ml enzyme) was slowly added and the slurry carefully mixed. A couple drops of ampholyte of the desired pH range was also added. The resulting slurry was then applied into the slot using a pasteur pipette. Care was taken to avoid introducing air bubbles.

c) Focusing conditions

Focusing was carried out at 4°C. Platinum ribbon electrodes (Desaga) were used and contact with the gel surface was effected by a 1.5 x 20 cm strip of Whatman 3 MM chromatography paper soaked with the electrolytes, 0.2 M sulfuric acid at the anode and 0.4 M ethylenediamine at the cathode. A field strength of 15-17 volt/cm was applied across the plate overnight (20-24 hours). The voltage gradient was then stepped up to 25 volt/cm for an additional 7-8 hour. The criterion of complete focusing was the attainment of a steady current.

d) Preparation of samples after focusing

After focusing was completed, the gel layer was cut widthwise into 1 cm segments. The gel was removed from the plate with a spatula into culture tubes (16 x 100 mm) and 2 ml of cold distilled water was added into each tube to suspend the ampholyte and protein. This suspension was later used for the measurement of pH and enzyme activity. Alternatively, the protein-containing focused gel fractions were suspended with an aliquot (1-2 ml) of 10 mM Tris-HCl-0.5 mM DTT-20% glycerol. This was used for enzyme activity assays. In addition, the non-protein containing gel fractions were scraped accordingly and eluted with 1 ml of 10 mM KCl (Righetti & Drysdale, 1976). This is to ensure adequate conductivity for pH measurements.

e) pH gradient measurements

A digital pH ion meter (Fisher Accumet model 520) fitted with a combination microelectrode (Brinkman Instruments) was used. The test tube containing the gel slurry was immersed in an ice-bath and the pH electrode was placed in the slurry. The sample was equilibrated for 5 minutes before each

pH measurement. The pH corresponding to the focused protein peak was taken as its apparent isoelectric point.

f) Enzyme assay

Aliquots of the gel suspension fractions were assayed for various HSD activities. The presence of the gels or ampholytes did not affect the radiochemical assay.

g) Separation of protein from ampholine and gel

The fractions comprising each peak of HSD activity were pooled into small glass columns closed at the bottom with glass-wool. The gels were eluted with two volumes of buffer, 0.1 M Tris-HCl-0.5 mM DTT; pH 8.0. A high ionic strength was used to disrupt any protein-ampholyte complexes formed by electrostatic interaction (Righetti & Drysdale, 1976). The eluates were concentrated by ultrafiltration. Extensive ultrafiltration was then carried out to remove carrier ampholyte using 10 mM Tris-HCl-0.5 mM DTT, pH 8.0 as the dialyzing buffer.

10. Polyacrylamide disc gel electrophoresis

Electrophoresis was carried out essentially as described by Davies (1964).

10.5% acrylamide separating gels were prepared from the following stock solutions

and proportions: 1 part of a stock solution containing 48 ml 1N HCl,  
36.6 gm Tris and 0.23 ml TEMED made up to  
100 ml with distilled water (pH 8.9).

3 parts of a stock solution containing 28 gm  
acrylamide and 0.74 gm Bis made up to 100 ml  
with distilled water.

4 parts of a 0.14% ammonium persulfate solution  
(made fresh).

The gels were polymerised in 9.0 cm lengths in 12.5 x 0.6-cm-internal-diameter glass columns. The gels were prerun at a current of 3 milliamperes per tube

for 2½ hours without tracking dye. The buffer in the upper and lower reservoirs of the electrophoretic apparatus (E.C. Chemical Corporation) for both the prerun and the actual run contained 6.0 gm of Tris, 28.8 gm of glycine/litre and was diluted 1 : 1 with distilled water before use. Following the prerun and removal of buffer from the upper reservoir and gel surface, 1 µl of tracking dye made up of 0.01% bromophenol blue in distilled water was dispensed onto the surface of the gel. This was overlaid with 25-100 µl of sample solution (10-800 µg protein). When dilution of the protein sample was necessary, this was achieved by mixing in appropriate aliquots of the reservoir buffer (before dilution) containing about 20% glycerol. Viscous samples such as the crude cytosolic fractions were applied without the addition of glycerol. Electrophoresis was carried out at 4°C at an initial current of 2 mA/tube and then at 3 mA/tube until the tracking dye had travelled to within 5 mm of the gel bottom (about 2 hours).

a) Substrate staining

Immediately following electrophoresis each gel was removed and transferred to individual tubes (16 x 250 mm) containing ice-cold 0.1 M glycine-NaOH buffer, pH 9.5. This is to allow the buffer to diffuse into the gels assuring the appropriate pH along the entire gel (Gabriel, 1971). During this equilibrating period (about 30 minutes) the following staining reagent mixture, a modification of the method described by Pollow et al (1976b), was prepared. For each gel the staining reagent contained 5 µmol NADP<sup>+</sup>, 0.27 µmol PMS and 5 µmol NBT and 925 µmol glycine-NaOH buffer pH 9.5 in a final volume of 15 ml. To this, 6 µmol steroid substrate (androsterone for 3α-HSD activity and epitestosterone for 17α-HSD activity) dissolved in 0.65 ml methanol was added. For the control gels,

steroid was omitted but the methanol included. For the detection of dehydrogenase activities in crude preparations, 30  $\mu$ mol sodium cyanide (final concentration 2 mM) was included in the staining mixture. The above solutions were prepared under subdued light and the solution kept at room temperature. The gels were incubated in the appropriate staining mixture in a closed dark chamber (Fisher Isotemp Incubator) maintained at 37°C. Enzyme activity was detected by the formation of purplish-blue diformazan band(s). A typical staining period was 1 hour. However, when necessary, the staining period was lengthened or shortened to produce the suitable band intensity. Immediately after staining, the gels were removed and washed thoroughly with distilled water to remove the staining fluid and in particular the fine particles produced by light-induced reduction of the tetrazolium salt. Densitometric scanning of the gels was carried out within two hours with a Gilford 240 spectrophotometer equipped with a linear transport attachment at a wavelength of 600 nm and a slit width of 0.05 x 2.36 mm. The scanning speed was 0.5 cm/minute and the recording chart speed was 0.5 min/inch. Gels were stored in 7.5% acetic acid.

b) Protein staining

Gels were stained for protein with Coomassie brilliant blue R-250 (Bulletin AN 32, Ortec Inc., 1970). The gels were first fixed in 12.5% trichloroacetic acid for 30 minutes followed by staining with 0.2% aqueous Coomassie brilliant blue R-250 in 45% ethanol-10% acetic acid for an additional half hour. Both these steps were performed at 65°C. Initial destaining was carried out in 25% ethanol-10% acetic acid also at 65°C for half an hour but further destaining was performed at room temperature. These gels were stored in 10% acetic acid.

11. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis

Acrylamide gels (10%) were prepared according to Weber et al (1972) in 8 x 0.6 cm-internal-diameter glass columns. The following protein standards were used: ribonuclease (MW 13,700), chymotrypsinogen A (25,700), aldolase (40,000), ovalbumin (45,000) and bovine serum albumin (68,000). These standards as well as the protein samples were denatured in 0.01 M sodium phosphate, pH 7.0, containing 1% SDS and 1 mM DTT and heated in a 100°C bath for 3 minutes. Protein (2-10 µg) was mixed with glycerol 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 ½ hour and then at 8 mA/tube until the dye front had reached the bottom of the gel (about 5½ hours). After electrophoresis the length of each gel and the distance to the center of the dye band on each gel were measured. Staining was achieved in 0.25% Coomassie brilliant blue R-250-45% methanol-9% acetic acid for 2 hours at room temperature. The gels were destained in 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. These gels were stored in 7.5% acetic acid.

12. Peptide mapping

a) Labeling procedure

Protein samples (250 µg - 1 mg) and carrier-ampholyte (0.5 ml of pH range 5-7) were lyophilized prior to acetylation. Each sample was then dissolved in 2.5 ml of 8 M urea and the mixture acidified to destroy any cyanate formed. The mixture was transferred to a reaction vessel. Sodium borate was added as

buffer and the pH was adjusted to pH 9.5 with 1N KOH. Acetonitrile (100  $\mu$ l) containing 0.2 mCi  $^{14}$ C-acetic anhydride (SA 27 mCi/mmol) was added to the reaction mixture. A second 100  $\mu$ l aliquot of this mixture was added five minutes later. Unlabeled acetic anhydride in 4 portions of 10  $\mu$ l volume were then added. About five minute reaction time was allowed between each addition. During the acetylation, all the solutions were maintained at pH 9.0 using a pH stat with 5 N KOH as titrant. The reaction mixture was also stirred throughout. In the case of the ampholyte sample, a considerable amount of base had to be added to bring the pH to 9.5. Also in this case, a total of 80  $\mu$ l of unlabeled acetic anhydride was added in separate portions.

After acetylation, the samples were acidified with concentrated HCl. About 1 mg bovine serum albumin was added to each fraction and each solution was placed in a pre-soaked dialysis tubing and dialyzed against 4 litres of distilled water with a few changes.

b) Digestion of acetylated samples

After thorough dialysis, the acetylated samples were freeze-dried and the contents dissolved in 1-5 ml of 10% ammonium bicarbonate solution. Aliquots of these samples were digested for 2 hours at 37 $^{\circ}$ C with papain in the presence of excess 2-mercaptoethanol. The amount of papain used for digestion was equivalent to a quarter of the protein content of the sample. The digestion was stopped by freezing and the frozen samples were lyophilized.

c) High-voltage electrophoresis

Each proteolytic digest of acetylated samples was dissolved in a few drops of pH 6.5 buffer (acetic acid-pyridine-water, 0.3:10:90, by volume) and then spotted in the middle of a sheet of Whatman 3 MM paper 56 x 15 cm. Enough

radioactivity was spotted to enable subsequent detection by autoradiography. A drop of a mixture of the dye markers xylene cyanol FF (blue) and  $\epsilon$ -DNP-L-lysine HCl (yellow) was also spotted along the origin on each side of the paper. Electrophoresis was performed at pH 6.5 (acetic acid-pyridine-water, 0.3:10:90, by volume) at 3000V for about 1 hour or until the dyes were about 10 cm apart.

Second-dimensional electrophoresis was achieved by cutting out the radioactive strip from the first electrophoresis and stitching it onto a sheet of Whatman 1 MM paper (45 x 56 cm) 15 cm from the anode end. The dye markers were applied as before and electrophoresis was carried out at pH 2.1 (acetic acid-formic acid-water, 4:1:45, by volume).

Electrophoresis at pH 3.5 (water-acetic acid-pyridine, 19:1:0.1, by volume) was also carried out on Whatman 1 MM paper. However, a longer run, about 2 hours was necessary for better resolution of the peptides.

d) Autoradiography

After electrophoresis, the chromatogram was air-dried. The midpoints of the dye markers plus other identification marks were 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 original electrophoretogram.

CHAPTER III

PRELIMINARY INVESTIGATIONS ON 17-HYDROXYSTEROID DEHYDROGENASES FROM RABBIT  
KIDNEY

1. The soluble and microsomal 17-hydroxysteroid dehydrogenases of female rabbit kidney - substrate specificity and nucleotide requirement

The 105,000 g supernatant and the microsomal pellet prepared from rabbit kidney homogenates were assayed for both 17 $\alpha$ - and 17 $\beta$ -hydroxysteroid dehydrogenase activities. Table I shows the relative enzyme activities toward various estrogenic substrates and the androgens, epitestosterone and testosterone. In the soluble fraction with NADP<sup>+</sup> as coenzyme, the activity toward these substrates decreases in the order: epitestosterone > 17 $\alpha$ -estradiol 3-glucuronide > 17 $\alpha$ -estradiol > testosterone > 17 $\beta$ -estradiol > 17 $\beta$ -estradiol 3-glucuronide, demonstrating a higher 17 $\alpha$ -enzyme activity than 17 $\beta$ -enzyme activity. The activity obtained with epitestosterone is about 1.6 times greater than that obtained with 17 $\alpha$ -estradiol 3-glucuronide and 19 times greater than that obtained with 17 $\alpha$ -estradiol. Although testosterone is oxidized at a rate of about 24 times less than its androgen counterpart, epitestosterone, its rate still exceeds that of the free 17 $\beta$ -estradiol or its glucuronide.

In the soluble fraction with NAD<sup>+</sup> as coenzyme, the activity toward these substrates decreases in the order: 17 $\alpha$ -estradiol 3-glucuronide > epitestosterone > testosterone > 17 $\beta$ -estradiol > 17 $\alpha$ -estradiol > 17 $\beta$ -estradiol 3-glucuronide. The soluble 17 $\alpha$ -enzyme invariably shows a higher activity with

TABLE 1

Substrate Specificity and Nucleotide Requirement of the Soluble and Microsomal 17-Hydroxysteroid Dehydrogenases of Female Rabbit Kidney

Substrate	Specific Activity ( $\mu$ units/mg protein)			
	NAD <sup>+</sup>		NADP <sup>+</sup>	
	Soluble	Microsomal	Soluble	Microsomal
17 $\alpha$ -Estradiol	12.7	0.7	47.3	1.8
17 $\beta$ -Estradiol	20.1	84.6	26.1	60.4
17 $\alpha$ -Estradiol 3-glucuronide	140.6	6.9	549.0	33.4
17 $\beta$ -Estradiol 3-glucuronide	11.8	7.7	17.7	16.0
Epitestosterone	57.9	5.5	886.0	59.1
Testosterone	52.4	47.7	36.5	9.7

A unit of enzyme activity is defined as the amount of enzyme catalyzing the oxidation of 1 picomole of substrate per minute under the specified conditions of the assay.

NADP<sup>+</sup> than NAD<sup>+</sup>. For example, the oxidation of epitestosterone in the presence of NADP<sup>+</sup> is 15 times greater than with NAD<sup>+</sup>.

While the 17 $\alpha$ -enzyme activity toward all substrates is found predominantly in the soluble fraction, the distribution of the 17 $\beta$ -enzyme activity is dependent on the substrate employed. The activity toward 17 $\beta$ -estradiol is higher in the microsomal fraction whereas the activity toward testosterone is higher in the soluble fraction. Both the soluble and the microsomal testosterone dehydrogenase and the microsomal 17 $\beta$ -estradiol dehydrogenase activities appear to prefer NAD<sup>+</sup> whereas the soluble 17 $\beta$ -estradiol dehydrogenase shows no obvious preference for either nucleotide.

2. Comparison of the NADP<sup>+</sup>-dependent 17-hydroxysteroid dehydrogenase activities in the soluble fraction of male and female rabbit kidney

The male and female rabbits used in these studies were approximately two months old. The 105,000 g supernatants prepared from kidney homogenates were assayed for the NADP<sup>+</sup>-dependent 17-HSD activities with both estrogen and androgen substrates. Table 2 lists the similarities in the dehydrogenase activities of the kidneys of both sexes. No significant differences are observed. The specific activity of the male kidney 17 $\alpha$ -estradiol dehydrogenase is 47.9  $\mu$ units per mg protein, agreeing very well with the cumulative specific activity of 40.7  $\mu$ units per mg protein of the female rabbit kidney toward 17 $\alpha$ -estradiol.

3. Effect of glycerol and freezing on the soluble 17-hydroxysteroid dehydrogenase activities of female rabbit kidney

Two homogenates one with 20% glycerol (v/v) and one without glycerol were prepared, in the usual homogenizing buffer (0.25 M sucrose + 0.5 mM DTT) from

TABLE 2

The Relative Activities of the Soluble NADP<sup>+</sup>-dependent 17-Hydroxysteroid Dehydrogenases of Male and Female Rabbit Kidney

---

Substrate	Activity Relative to 17 $\alpha$ -Estradiol In Female Kidney	
	Female *	Male
17 $\alpha$ -Estradiol	1.0	1.2
17 $\beta$ -Estradiol	0.5	0.6
17 $\alpha$ -Estradiol 3-glucuronide	12.0	12.2
17 $\beta$ -Estradiol 3-glucuronide	0.4	0.4
Epitestosterone	20.3	27.3
Testosterone	0.7	0.7

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\*The values are from means of eight experiments.

10-20  $\mu$ l aliquots of the soluble fraction were usually used for the assay with 17 $\alpha$ -estradiol.

each kidney of the same animal. The 105,000 g supernatants were then prepared as described in the General Methods (p.59). Table 3 compares the effect of inclusion of glycerol in the buffer on the soluble 17 $\alpha$ - and 17 $\beta$ -HSD enzyme activities. No significant difference is evident with the relative enzyme activities prepared in the presence or absence of 20% glycerol. However, storage of the crude cytosol at 4 $^{\circ}$ C, in the presence of 20% glycerol was observed to protect the enzyme significantly, the half life being about a month (not shown). In contrast, the fraction stored without glycerol was essentially devoid of enzyme activity after 17 days. However, the 17 $\alpha$ -HSD activity of the crude cytosol was stable to freezing. As much as 62% of the original activity remained after two months of storage in the freezer at -20 $^{\circ}$ C when assayed with epitestosterone and 17 $\alpha$ -estradiol. Freezing in the presence of 20% glycerol, however, did not further protect the enzyme stability.

#### 4. 17-Hydroxysteroid dehydrogenase activity as a function of pH

The pH dependence of the female rabbit kidney 17-HSD was determined by incubating aliquots of the 105,000 g supernatant with substrates 17 $\alpha$ -estradiol 3-glucuronide and 17 $\beta$ -estradiol at several pH values in the range pH 7.0 to 11.0. The activity curves of the oxidative reactions with the two substrates are shown in Figure 1. Both the 17 $\alpha$ - and 17 $\beta$ -enzyme activities exhibit a broad pH profile. With glycine-NaOH as buffer both enzymes have pH optima at about 9.5 whereas with Tris-HCl buffer, the pH optimum ranges from 9-10 for the 17 $\alpha$ -enzyme and from 9.5-10.0 for the 17 $\beta$ -enzyme. With glycine buffer at pH greater than 10 both enzyme activities decrease rapidly.

TABLE 3

Effect of 20% Glycerol on the Soluble 17-Hydroxysteroid Dehydrogenase  
Activities of Female Rabbit Kidney

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Substrate	Activity Relative to 17 $\alpha$ -Estradiol without Glycerol	
	Without Glycerol 1 Kidney*	With Glycerol 1 Kidney*
17 $\alpha$ -Estradiol	1.0	1.2
17 $\beta$ -Estradiol	0.4	0.4
17 $\alpha$ -Estradiol 3-glucuronide	10.4	11.6
17 $\beta$ -Estradiol 3-glucuronide	0.3	0.3
Epitestosterone	18.8	22.8
Testosterone	0.5	0.6

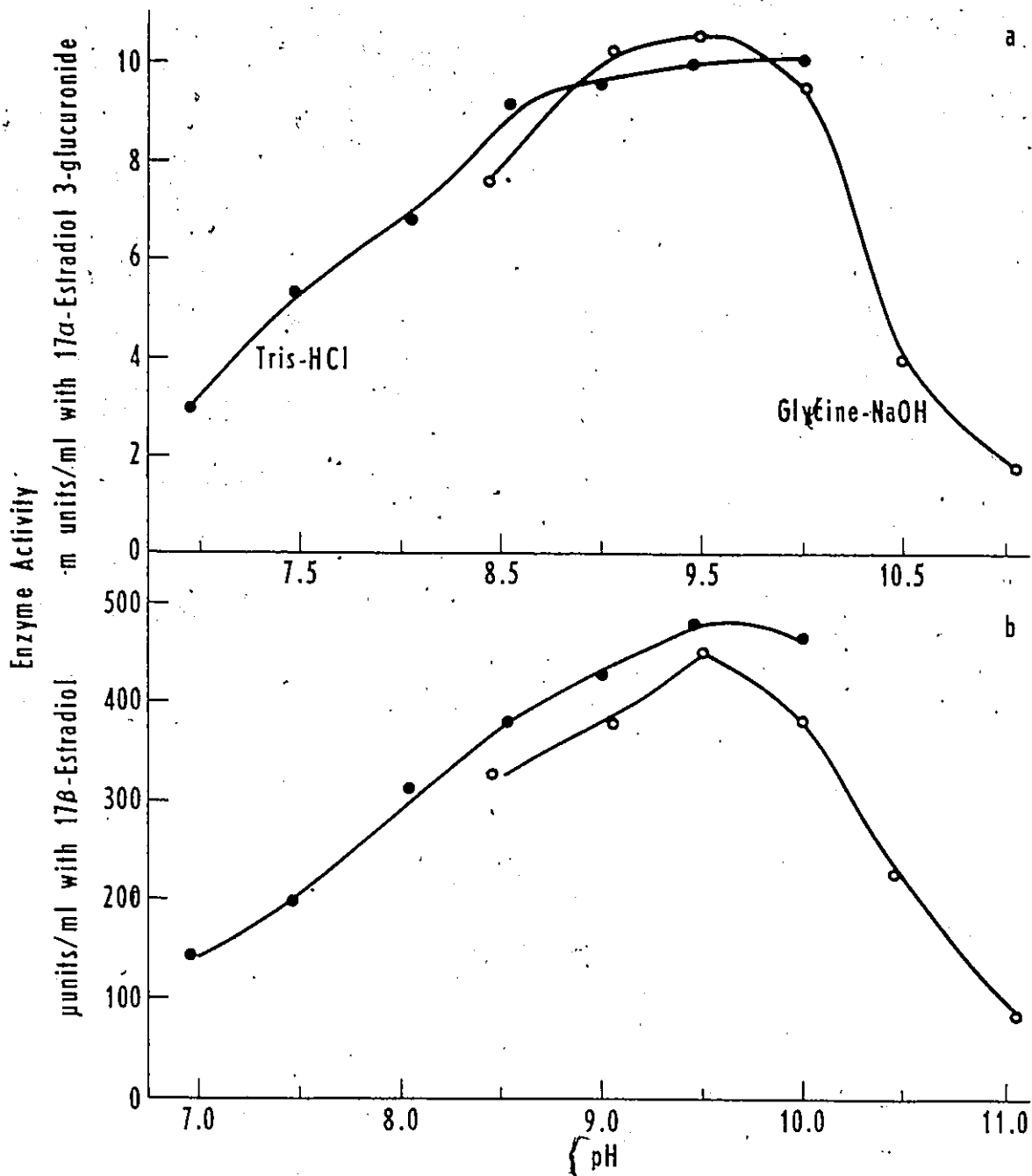
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\*The ratios to 17 $\alpha$ -estradiol were calculated from means of two separate experiments. The average specific activity toward 17 $\alpha$ -estradiol is 52.3  $\mu$ units/mg protein in the absence of glycerol and 60.7  $\mu$ units/mg protein in the presence of glycerol.

FIGURE 1

pH Activity Curves

The crude soluble fraction of female rabbit kidney was assayed for  $17\alpha$ -estradiol 3-glucuronide and  $17\beta$ -estradiol dehydrogenase activities from pH 7.0 to 11.0. 0.1 M Tris-HCl was used over the pH range 7.0 to 10.0 and 0.1 M glycine-NaOH from pH 8.5 to 11.0. The data are from a set of triplicate experiments. 5  $\mu$ l and 50  $\mu$ l enzyme aliquots were used for the substrates  $17\alpha$ -estradiol 3-glucuronide and  $17\beta$ -estradiol respectively.



5. Isoelectric focusing of the crude soluble 17-hydroxysteroid dehydrogenases of the female rabbit kidney, pH range 2-10

Figure 2 demonstrates the heterogeneity of both the 17 $\alpha$ - and 17 $\beta$ -HSDs. Three to four peaks of enzyme activities are apparent with epitestosterone and 17 $\alpha$ -estradiol 3-glucuronide focusing within the pH range 5-6. However, with testosterone three major enzyme peaks are separated and their apparent isoelectric points are quite distinct from the 17 $\alpha$ -enzyme. Thus, the major 17 $\alpha$ - and 17 $\beta$ -HSD activities are separable on the basis of their ionic properties.

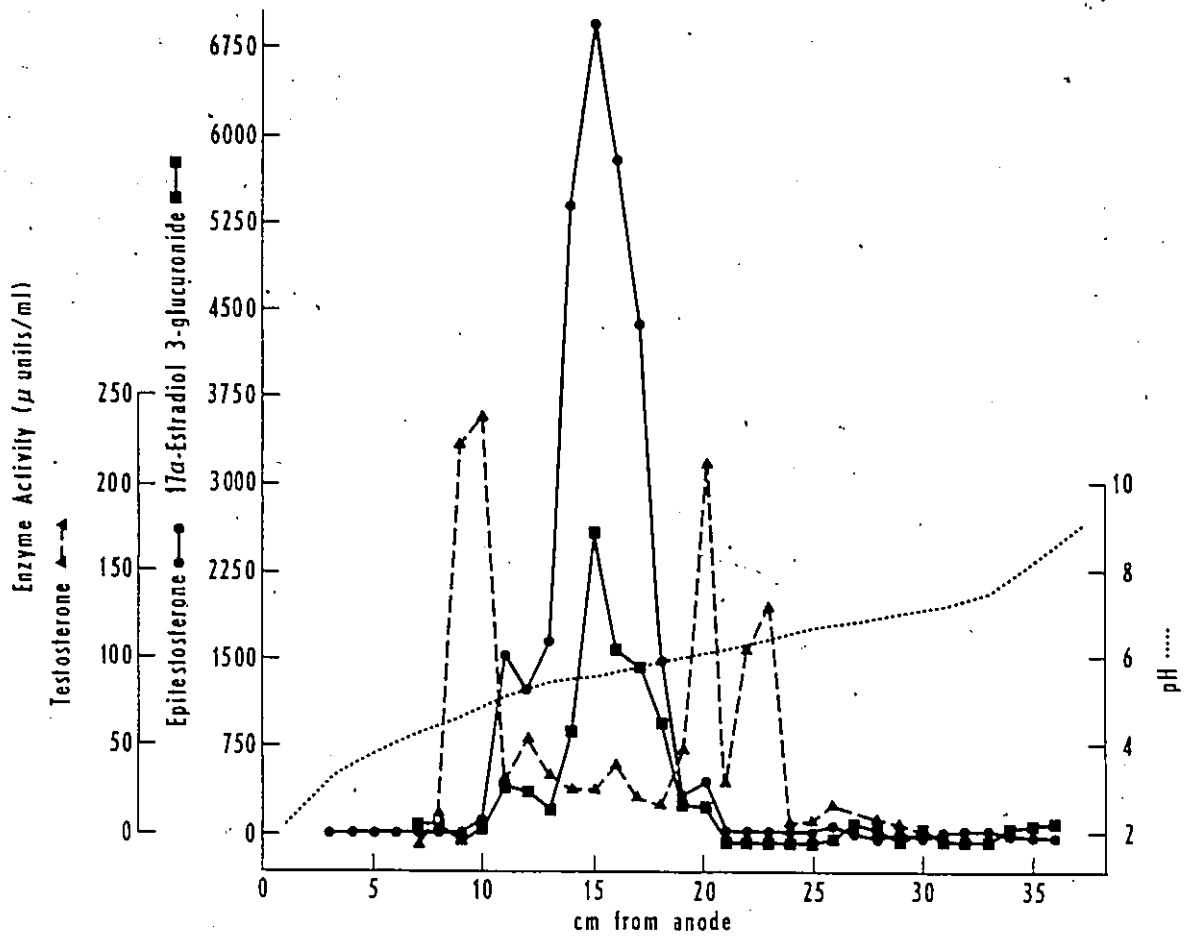
DISCUSSION

Both the NAD(P)<sup>+</sup>-dependent 17 $\alpha$ - and 17 $\beta$ -hydroxysteroid dehydrogenase activities are present in the female rabbit kidney subcellular fractions. The soluble fraction contains the majority of the NADP<sup>+</sup>-dependent 17 $\alpha$ -HSD activities toward the estrogenic and androgenic substrates. These activities are generally higher than the soluble 17 $\beta$ -enzyme. The relatively low 17 $\beta$ -HSD activities of the cytosol is not a consequence of cold-inactivation as indicated by the addition of 20% glycerol during fractionation. The property of cold-inactivation and protection by glycerol is characteristic of the human placental 17 $\beta$ -estradiol dehydrogenase (Jarabak et al, 1966). In fact, the 17 $\beta$ -estradiol dehydrogenase of the rabbit kidney is mainly localized in the microsomal fraction and it prefers NAD<sup>+</sup> to NADP<sup>+</sup>. In contrast, the 17 $\beta$ -HSD of the soluble fraction exhibits highest activity toward testosterone and can utilize either NAD<sup>+</sup> or NADP<sup>+</sup>. The preference for NADP<sup>+</sup> of the soluble 17 $\alpha$ -enzyme of the rabbit kidney is also exhibited by the corresponding enzyme present in the liver of this animal (Hasnain & Williamson, 1974). The utilization of either NAD<sup>+</sup> or NADP<sup>+</sup>

FIGURE 2

Isoelectric Focusing of the Crude Soluble 17-Hydroxysteroid Dehydrogenases  
of Female Rabbit Kidney, pH 2-10

The soluble fraction was prepared from 17.6 g of kidneys in 0.25 M sucrose containing 0.5 mM DTT. The fraction was concentrated to 5.2 ml and dialysed against 1 mM Tris-HCl - 0.5 mM DTT, pH 8.0. About 4 ml of the dialysate containing 780 mg protein was applied on to the 1 x 16 cm trough located at the middle of the 0.5 cm thick granulated gel plate. The gel medium was prepared by mixing 12 g Sephadex G-200 superfine in 300 ml distilled water, 6 ml each of ampholyte pH range 5-7 and 2-10, and 23 mg DTT. The gel slurry was deaerated, poured into the 20 x 40 cm gel plate and then air-dried to the consistency as described in the General Methods (Method 1, p.62 ). Focusing was carried out initially at 600V (39 mA) for about 16 hours during which time the voltage and current changed to about 800V and 4.8 mA respectively. The final voltage applied was 1000V at 6 mA which then remained constant. At the end of 7 hours, each 1 x 16 cm gel slurry was removed into separate test tubes to which 2 ml of distilled water was added. Aliquots of the gel suspension, 100- $\mu$ l for testosterone and 5  $\mu$ l for epitestosterone and 17 $\alpha$ -estradiol 3-glucuronide were used for enzyme assays. The gel suspensions were also used for pH determinations.



by the kidney soluble  $17\beta$ -estradiol dehydrogenase is also a property of the corresponding liver enzyme (Hasnain, 1975) and the human placental  $17\beta$ -estradiol dehydrogenase (Karavolas & Engel, 1971), among many others. This dual nucleotide specificity of the rabbit kidney  $17$ -HSD may also mean another catalytic property of the enzyme i.e. acting as a transhydrogenase, the latter function being inherent of the human placental  $17\beta$ -estradiol dehydrogenase (Jarabak *et al.*, 1962; Karavolas & Engel, 1966).

The variety of hydroxysteroid dehydrogenase activities in the rabbit kidney cytosol indicates that the kidney plays an active role in the inactivation of estrogens (and perhaps androgens) in this animal. Further, the differences in the oxidative rates of the various substrates suggest the presence of different  $17$ -HSDs in the rabbit kidney cytosol. Preliminary isoelectric focusing revealed the presence of multiple forms of both the soluble  $17\alpha$ - and  $17\beta$ -HSDs in the kidney. The presence of large amounts of nucleic acids in the crude 105,000 g supernatant may have contributed to the blurred pattern seen in Figure 2 (Righetti & Drysdale, 1976). A consistently higher ratio of absorbance at 260 nm to that at 280 nm (1.21-1.33 : 1) of the crude cytosol reflects this high nucleic acid content. Despite the poor resolution of the isoelectric focusing, the ionic property of the crude  $17\alpha$ -HSDs of the kidney cytosol displays characteristics similar to the liver  $17\alpha$ -HSDs (Hasnain, 1975). However, the kidney  $17\beta$ -enzyme differs from the liver  $17\beta$ -enzyme described by Hasnain, in that the former enzyme possesses a very acidic enzyme form not found in the liver.

The soluble  $17\beta$ -HSD activity of rabbit kidney is equivalent in male and female. This is in contrast to the guinea pig kidney NAD(P)<sup>+</sup>-linked  $17\beta$ -HSD activity, the female exhibits only a trace of activity for several  $C_{19}$ -hydroxysteroids when compared to the male (Shen & Kochakian, 1978). However, the administration of testosterone produces progressive induction of the soluble  $17\beta$ -enzyme level to that observed in the male animal (Shen & Kochakian, 1979).

Because the properties of the rabbit kidney 17-HSDs (such as substrate specificity and pH optima) are similar to the liver enzymes of this animal, the purification of the kidney enzymes was initiated so that a more rigorous comparison of these activities in the two tissues could be made. These studies were primarily concerned with the 17 $\alpha$ -HSD because of its high activity in this tissue relative to the 17 $\beta$ -enzyme. However, the latter enzyme activity was also monitored for comparative purposes.

CHAPTER IV

PURIFICATION OF THE SOLUBLE NADP<sup>+</sup>-DEPENDENT 17 $\alpha$ -HYDROXYSTEROID DEHYDROGENASES  
OF FEMALE RABBIT KIDNEY

1. Calcium phosphate gel fractionation

Absorption and desorption from calcium phosphate gel of the 105,000 g supernatant of rabbit kidney resulted in about 1.5 fold purification of the 17 $\alpha$ -enzyme (Table 4) removing most, if not all, of the hemoglobin associated with the crude fraction. Approximately 36% of the original contaminating protein was removed in this case. The recovery of the 17 $\alpha$ -enzyme activity was better than 83% depending on the substrates used. The 17 $\beta$ -enzyme was also retained in this fractionation. The recovery of the 17 $\beta$ -enzyme activity was more varied ranging from 37% when assayed with 17 $\beta$ -estradiol to 89% with testosterone.

2. Sephadex G-75 (superfine) column chromatography

Gel filtration of the concentrated enzyme fraction obtained by calcium phosphate gel fractionation on a column of Sephadex G-75 (superfine) eluted the 17 $\alpha$ -enzyme as a single peak; the activity profile with 17 $\alpha$ -estradiol is shown in Figure 3. Other 17 $\alpha$ -enzyme activities with epitestosterone and 17 $\alpha$ -estradiol 3-glucuronide co-chromatographed with this peak. Furthermore, the bulk of the 17 $\beta$ -enzyme was also retained in this peak although a small proportion of the 17 $\beta$ -enzyme migrated slightly faster than the main peak free of the 17 $\alpha$ -enzyme. This phenomenon was observed with both 17 $\beta$ -estradiol and

TABLE 4

Purification Scheme of Female Rabbit Kidney Soluble 17-Hydroxysteroid Dehydrogenases

Step	Total mg Protein	Substrate*	Total Activity (units)	Specific Activity** (units/mg protein)	Purification-fold	Y Recovery	
						Activity	Protein
105,000 g	2338	EpIT	1,950,000	835	-	100%	100%
		E <sub>2</sub> αGA	1,230,000	526	-		
		E <sub>2</sub> α	108,300	46.3	-		
		T	45,500	19.5	-		
		E <sub>2</sub> β	45,000	19.2	-		
		E <sub>2</sub> βGA	35,000	15.0	-		
Calcium phosphate gel fractionation	1491	EpIT	1,743,000	1169	1.4	89.3	63.8
		E <sub>2</sub> αGA	1,020,000	684	1.3	82.9	
		E <sub>2</sub> α	106,800	71.6	1.5	98.6	
		T	40,300	27	1.4	88.6	
		E <sub>2</sub> β	16,500	11.1	-	36.7	
		E <sub>2</sub> βGA	23,350	15.8	1.1	67.1	
Sephadex G-75 effluent	160	EpIT	1,286,000	8627	9.6	65.9	6.8
		E <sub>2</sub> αGA	898,000	5354	10.2	69.7	
		E <sub>2</sub> α	102,000	638	13.8	94.3	
		T	25,300	132	6.8	55.7	
		E <sub>2</sub> β	7,500	46.8	2.4	16.7	
		E <sub>2</sub> βGA	7,700	48.1	3.2	21.9	
DEAE-cellulose chromatography Peak I	24.2	EpIT	345,500	14,301	17.1	17.7	1.0
		E <sub>2</sub> αGA	385,200	15,944	30.3	31.3	
		E <sub>2</sub> α	18,500	765	16.5	17.1	
		T	2,760	114	5.8	6.1	
Peak II & III	24.6	EpIT	460,300	18,731	22.4	23.6	1.1
		E <sub>2</sub> αGA	147,500	6,003	11.4	12.0	
		E <sub>2</sub> α	30,600	1,245	26.9	28.3	
		T	6,320	257	13.2	13.9	
Peak IV	12.6	EpIT	150,000	11,872	14.2	7.7	0.5
		E <sub>2</sub> αGA	15,800	1,252	2.4	1.3	
		E <sub>2</sub> α	5,900	468	10.1	5.5	
		T	3,580	284	14.6	7.9	

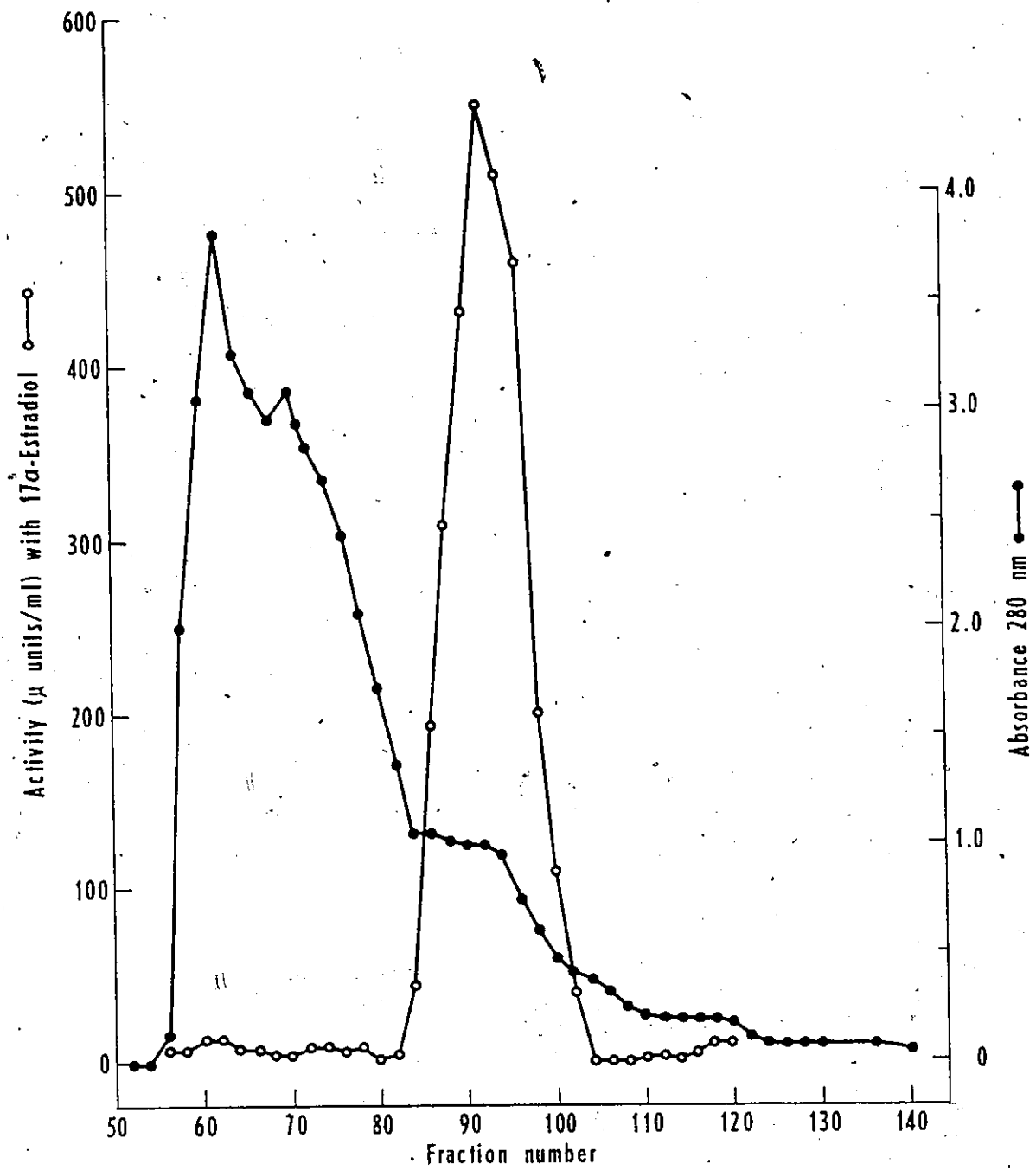
\*The abbreviated substrates are: EpIT - epitestosterone; E<sub>2</sub>αGA - 17α-estradiol 3-glucuronide; E<sub>2</sub>α - 17α-estradiol; T - testosterone; E<sub>2</sub>β - 17β-estradiol and E<sub>2</sub>βGA - 17β-estradiol 3-glucuronide.

\*\*The specific activity values were calculated before the total activities were rounded-off.

FIGURE 3

Sephadex G-75 (superfine) Column Chromatography of Rabbit Kidney  
17-Hydroxysteroid Dehydrogenase Activity

The 17-HSD activity fractionated by calcium phosphate gel was further purified by gel filtration on a Sephadex G-75 (superfine) column, 5 x 90 cm. The equilibrating and elution buffer was 10 mM Tris-HCl-0.5 mM DTT, pH 8.0. 0.1 ml aliquots of the column fractions (about 10 ml) were used in the assays with  $17\alpha$ -estradiol.



testosterone as substrates (not shown). This minor peak of  $17\beta$ -enzyme activity was discarded.

The gel filtration gave a purification of 10-14 fold for the  $17\alpha$ -enzyme activities (Table 4). About 90% of the protein present in the calcium phosphate fraction was removed by this step while retaining 74-96% of the  $17\alpha$ -enzyme activity from the preceding step. The recovery is dependent on the substrate assayed. On the other hand, the recovery of the  $17\beta$ -enzyme activity ranged from 33-63%, the overall recovery from the original crude high speed supernatant being 17% with  $17\beta$ -estradiol or 56% with testosterone.

3. DEAE-cellulose column chromatography -  $17\alpha$ - and  $17\beta$ -hydroxysteroid dehydrogenase activities

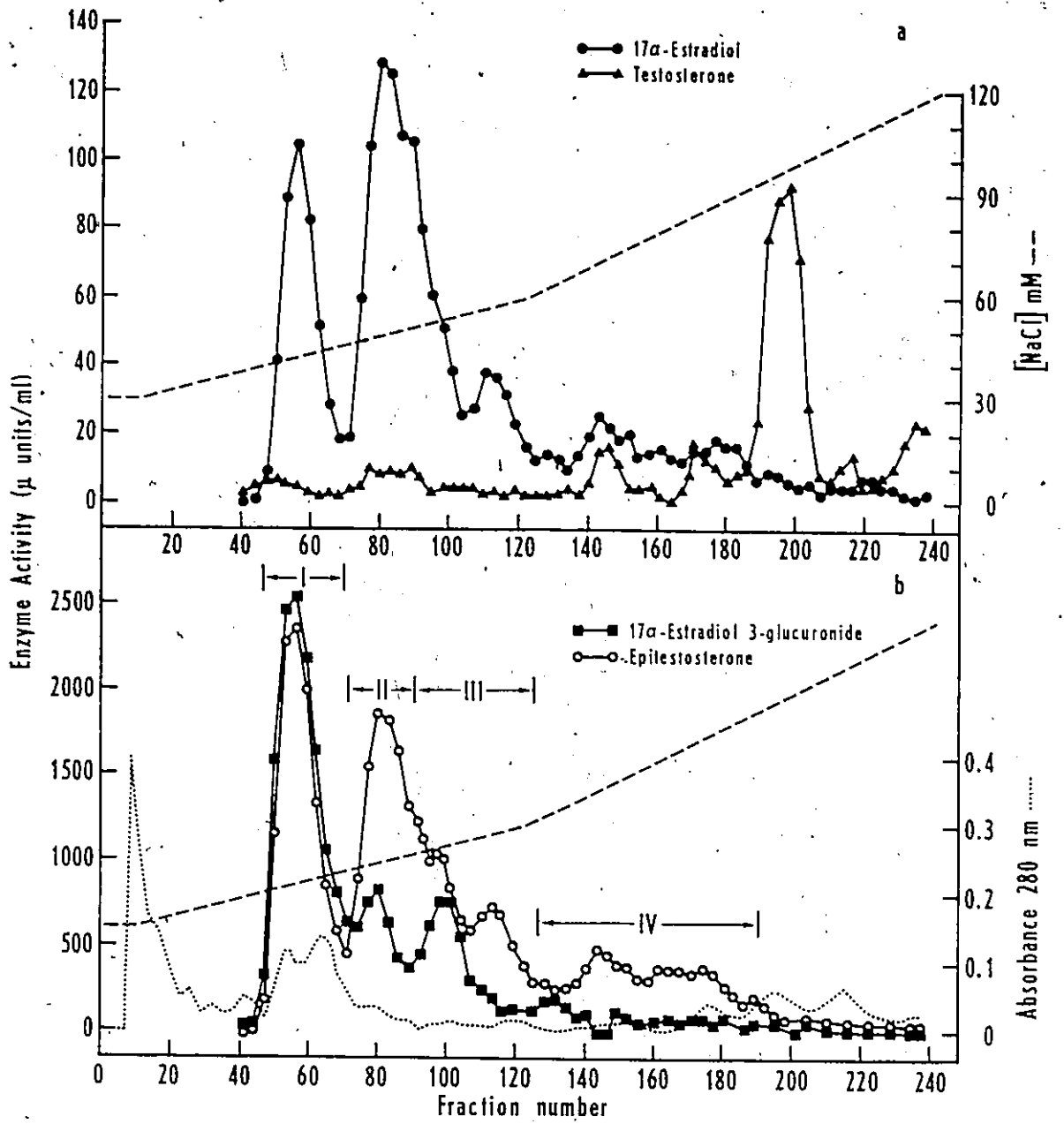
Chromatography on DEAE-cellulose of the  $17$ -HSD fraction from Sephadex gel filtration revealed the multiple nature of the  $17\alpha$ -enzyme (Figure 4). A single peak of  $17\alpha$ -HSD activity (Peak I) toward epitestosterone and  $17\alpha$ -estradiol 3-glucuronide, (Figure 4b) and  $17\alpha$ -estradiol (Figure 4a) was eluted at a salt gradient between 40 mM and 46 mM (fractions 46-70). The remainder of the first salt gradient, between 46-60 mM NaCl (fractions 71-125) eluted the contiguous peaks II and III, more evident with the  $17\alpha$ -estradiol 3-glucuronide as substrate; a second enzyme peak (fractions 107-125) within peak III was also apparent. This peak was not present when enzyme activity was assayed with  $17\alpha$ -estradiol 3-glucuronide.

The second linear salt gradient, 60-120 mM NaCl, eluted off the "residual"  $17\alpha$ -enzyme activity in a broad peak IV spanning fractions 126-190. Very little  $17\beta$ -enzyme activity as evidenced by the assay with testosterone (Figure 4a) was associated with these fractions or with those fractions eluted off in the first

FIGURE 4

DEAE-cellulose Column Chromatography of Rabbit Kidney  
17 $\alpha$ - and 17 $\beta$ -hydroxysteroid Dehydrogenases

The 17-HSD activity partially purified by Sephadex gel filtration and calcium phosphate gel fractionation was chromatographed on a DEAE-cellulose column (2.5 x 40 cm). The enzyme activities were eluted in a 1.2 litre linear gradient of 30-60 mM NaCl in the 10 mM Tris-HCl-0.5 mM DTT, pH 8.0 buffer followed by a second 1.2 litre linear gradient of 60-120 mM NaCl in the same buffer. Ten ml fractions were collected. Aliquots of enzyme used in the assays with epitestosterone and 17 $\alpha$ -estradiol 3-glucuronide were 30  $\mu$ l; 17 $\alpha$ -estradiol, 300  $\mu$ l and testosterone, 500  $\mu$ l.



salt gradient. However, a major  $17\beta$ -enzyme peak was evident between fractions 190-210. No significant  $17\alpha$ - or  $17\beta$ -enzyme activity was further eluted when 200 ml of the eluting buffer containing 200 mM NaCl was passed through the column.

This ion-exchange chromatography step resulted in a total activity recovery of about 45-51% of the  $17\alpha$ -enzyme and an overall protein recovery of about 2.6% of the original starting material. The highest purification achieved in these peaks was 22-fold for epitestosterone, 30-fold for  $17\alpha$ -estradiol 3-glucuronide and 27-fold for  $17\alpha$ -estradiol. The extent of purification of enzyme activities for each substrate varied among the different enzyme peaks (Table 4).

Addition of 20% glycerol (v/v) in the eluting buffer reduced the flow rate of the column considerably. Moreover, it modified the elution pattern of the enzyme activity peaks by "squeezing" them essentially entirely into the first salt gradient effluents while retaining the same number of enzyme activity peaks. The fold of purification of the various enzyme activities under these peaks did not improve. Therefore the addition of glycerol to the column buffer was discontinued.

#### 4. DEAE-cellulose column chromatography - $3\alpha$ - and $17\alpha$ -hydroxysteroid dehydrogenase activities

In view of the numerous reports on the bi-specificity of steroid dehydrogenases, eg. the  $3(17)\beta$ -HSDs from *Pseudomonas testosteroni* (Marcus & Talalay, 1956) and rat erythrocytes (Heyns & de Moor, 1974), the  $3\alpha,20\beta$ -HSD from *Streptomyces hydrogenans* (Gibb & Jeffrey, 1971) the debatable  $17\beta,20\alpha$ -HSD from human term placenta (Purdy et al, 1964) and especially the  $3\alpha,17\beta$ -HSD from female rabbit liver cytosol (Thaler-Dao et al, 1972), the activities of the partially purified  $17\alpha$ -HSDs toward 3-hydroxysteroids was examined.

The protein fraction for the DEAE-cellulose chromatography was obtained by calcium phosphate gel fractionation and Sephadex gel filtration as described earlier. The salt gradient employed for elution of the column was that described in Figure 4. The enzyme activity patterns of the steroid dehydrogenases assayed with two  $3\alpha$ -hydroxysteroid substrates, androsterone and  $5\alpha$ -androstan- $3\alpha,17\beta$ -diol and a  $17\alpha$ -hydroxysteroid represented by epitestosterone are shown in Figure 5. It is evident that both the  $3\alpha$ - and  $17\alpha$ -enzyme activities exhibit the same elution profile. In addition, the  $3\alpha$ -HSD activities are considerably higher than that of the  $17\alpha$ -enzyme, a feature which allows the detection of a fifth peak (peak V, fractions 181-220) not obvious with any of the  $17\alpha$ -hydroxysteroid substrates. This peak V contains the major (though negligible with respect to the  $3\alpha$ - or  $17\alpha$ -enzyme activities)  $17\beta$ -enzyme activity exhibited with  $5\alpha$ -dihydrotestosterone shown in the inset in Figure 5. With  $5\alpha$ -androstan- $3\alpha,17\beta$ -diol as substrate no conversion to androstanedione, a possible product from oxidation of both  $3\alpha$ - and  $17\beta$ -hydroxyl groups was detected under the conditions of the assay.

The distribution of the epitestosterone, androsterone and  $17\alpha$ -estradiol 3-glucuronide dehydrogenases in the various DEAE-column peaks is represented in Table 5. About half of the total  $17\alpha$ -estradiol 3-glucuronide activity is present in peak I whereas the highest activities for epitestosterone and androsterone are found in peak III. The distribution of the latter two activities is similar in peaks I to IV. Only 1-2% of the total  $17\alpha$ -enzyme is found in peak V. In contrast, about 8% of the total  $3\alpha$ -HSD is contained in this peak.

The ratio of the total activities of epitestosterone :  $17\alpha$ -estradiol 3-glucuronide in the DEAE-column effluents is 2:1 whereas that of androsterone : epitestosterone is 2.4 : 1. The latter ratio is in good agreement with those

FIGURE 5

DEAE-cellulose Column Chromatography of Rabbit Kidney  
3 $\alpha$ -, 17 $\alpha$  and  $\beta$ -hydroxysteroid Dehydrogenases

The conditions for chromatography were as described for Figure 4. The enzyme aliquots used in the assays for androsterone, 5 $\alpha$ -androstan-3 $\alpha$ ,17 $\beta$ -diol and epitestosterone dehydrogenase activities in the first salt gradient eluates (fractions 10-129) were 5, 10 and 20  $\mu$ l respectively. For the second salt gradient eluates (fractions 130-233), the amounts of enzymes used were 10, 20 and 50  $\mu$ l with respect to the above substrates in the same order. For 5 $\alpha$ -dihydrotestosterone dehydrogenase, 0.5 ml aliquot of enzyme was used.

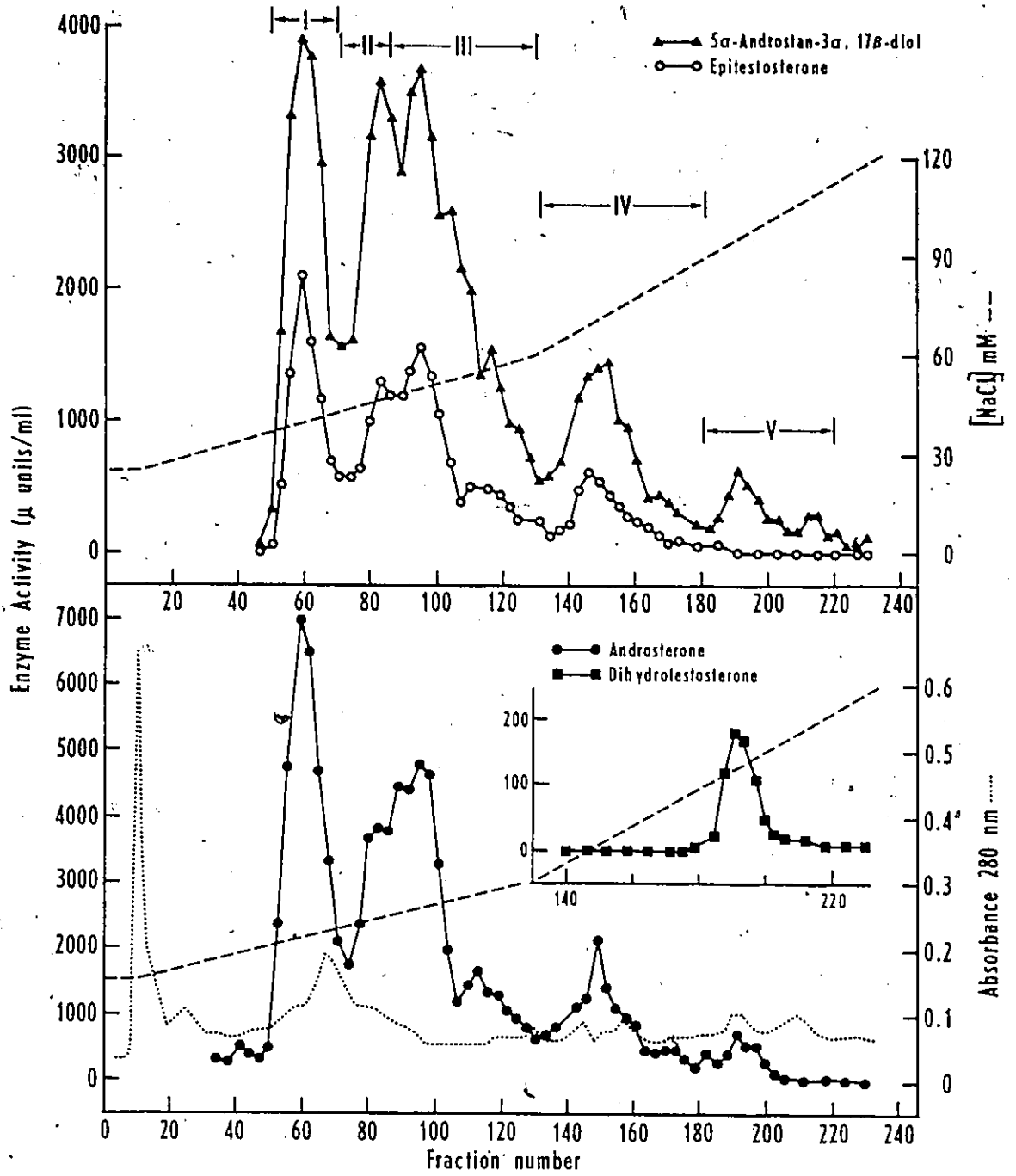


TABLE 5

Distribution of Rabbit Kidney 3(17) $\alpha$ -Hydroxysteroid Dehydrogenases  
in the Various DEAE-cellulose Column Peaks

Substrate	% of Total Activity DEAE Peak					Total munits
	I	II	III	IV	V	
17 $\alpha$ -Estradiol 3-glucuronide	48.6	18.4	26.7	5.4	0.9	500
Epitestosterone	23.8	14.7	38.5	20.8	2.2	960
Androsterone	24.4	17.6	32.0	18.4	7.6	2270

The designations of the DEAE-column peaks are as described in Figure 5. The enzyme activities under each peak were pooled separately and each fraction concentrated by ultrafiltration to about 2 ml. For enzyme assays a 20 x dilution of the concentrate was made and the following aliquots of enzymes were used: androsterone, 1-2  $\mu$ l; epitestosterone, 2-4  $\mu$ l; and 17 $\alpha$ -estradiol 3-glucuronide, 2-4  $\mu$ l for peak I, 5-10  $\mu$ l for peaks II and III and 20-40  $\mu$ l for peaks IV and V.

obtained in the preceding purification steps. The crude high speed supernatant fraction displayed an androsterone : epitestosterone ratio of 2.5 : 1; the calcium phosphate gel fraction 2.2 : 1 and the Sephadex gel filtration fraction 2.9 : 1.

The substrate specificities of the rabbit kidney 3(17) $\alpha$ -HSDs after DEAE-cellulose chromatography is summarized in Table 6. When 17 $\alpha$ -enzyme activity is measured with estrogen substrates, peak I exhibits a much higher specificity toward the glucuronide derivative of 17 $\alpha$ -estradiol than other peaks. Peak II shows the highest 17 $\alpha$ -enzyme activity toward the androgen, epitestosterone. Measurement of 3 $\alpha$ -HSD activity with steroid substrates of the 5 $\alpha$ -configuration with trans A/B ring fusion, viz. androsterone and 5 $\alpha$ -androstan-3 $\alpha$ ,17 $\beta$ -diol, show that this activity is much higher than the 17 $\alpha$ -enzyme activity. The activity ratios of androsterone : epitestosterone in peaks I to IV range from 2.5-3.7 : 1 whereas that of 5 $\alpha$ -androstan-3 $\alpha$ ,17 $\beta$ -diol : epitestosterone is 1.6-2.0 : 1. Etiocholanolone which has the same functional groups as androsterone but has a 5 $\beta$ -structure with cis A/B ring junction, is a poor substrate.

Only trace amounts of 17 $\beta$ -enzyme activity are present in peaks I to IV, the ratio of testosterone : epitestosterone being less than 0.02 : 1 (Table 4). However, in peak V the ratio of testosterone : epitestosterone was found to be 0.14 : 1 (not shown). This peak also appeared to be more "specific" for androsterone than for epitestosterone, the ratio of androsterone to epitestosterone being about 5 : 1 compared to the lower ratios obtained with the other enzyme peaks.

TABLE 6

Substrate specificities of the Rabbit Kidney 3(17) $\alpha$ -Hydroxysteroid  
Dehydrogenases After DEAE-cellulose Chromatography

Substrate	Specific Activity (nmole of product/min/mg protein)					
	Peaks:	I	II	III	IV	V
17 $\alpha$ -Estradiol		0.6	1.5	0.7	0.4	0.2
17 $\alpha$ -Estradiol 3-glucuronide		16.8	7.4	6.1	1.5	0.9
Epitestosterone		12.3	20.0	16.3	9.7	4.3
Androsterone		45.2	49.1	47.3	26.8	23.5
5 $\alpha$ -Androstan-3 $\alpha$ ,17 $\beta$ -diol		23.8	35.1	32.4	15.2	9.8
Etiocholanolone		1.4	3.1	2.3	1.0	0.5

The designations of the DEAE-column peaks are as described in the text. The enzyme activity under each peak was first pooled and then concentrated to less than 2 ml. For the assay with epitestosterone 2-4  $\mu$ l of ten-time-diluted samples of enzyme peaks I to V were used. Aliquots of enzymes used with other assay substrates were then calculated accordingly.

5. Isoelectric focusing experiments of rabbit kidney 3(17) $\alpha$ -hydroxysteroid dehydrogenases

a) Isoelectric focusing of DEAE-column peak I

The enzyme fraction from peak I obtained by DEAE-cellulose chromatography was subjected to isoelectric focusing over the pH range 5-7. The focused pattern is shown in Figure 6. Two main peaks of enzyme activities IA and IB toward 3 $\alpha$ -hydroxysteroids (Figure 6a) and 17 $\alpha$ -hydroxysteroids (Figure 6b) are separated. The majority of the enzyme activity with respect to all substrates resides in peak IB. However peak IA varied in different preparations depending on how well the DEAE-column peaks I and II were separated or on the manner the fractions under these peaks were pooled. This suggested that the focused peak IA was a contaminant from DEAE peak II. This point will be discussed in a later section.

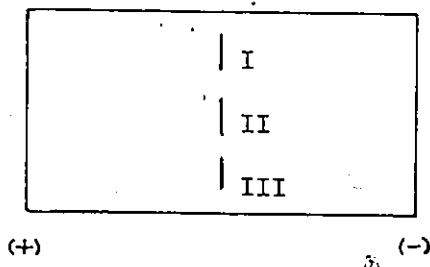
b) Isoelectric focusing of DEAE-column peak II

In the same electrofocusing experiment just described for DEAE-column peak I, a sample of the partially purified DEAE-column peak II enzyme was applied in a different track on the same gel slab. The enzyme activity pattern after focusing is shown in Figure 7. With 5 $\alpha$ -androstan-3 $\alpha$ ,17 $\beta$ -diol and epitestosterone as substrates, a single broad peak (peak II) is obtained. A similar peak is displayed by androsterone substrate but shoulders (peaks IIa and IIb) on each side of the main peak are evident. The minor peak IIb is also exhibited by 17 $\alpha$ -estradiol 3-glucuronide substrate, the major activity being found under the main peak II, although slightly shifted. Further, the relative activity toward 17 $\alpha$ -estradiol 3-glucuronide dehydrogenase is considerably less than that toward epitestosterone or the 3 $\alpha$ -hydroxysteroids. The activity profile with 17 $\alpha$ -estradiol (not shown) followed that of epitestosterone.

FIGURE 6

Isoelectric Focusing of 3(17) $\alpha$ -HSD Activity in DEAE-cellulose  
Chromatography Peak I

Electrofocusing of peak I enzyme activity was done simultaneously with those of peak II and III from the same column. After removal of salt by dialysis about 0.8 ml fraction of each column peak I, II and III containing 81, 83 and 96 milliunits of activity with epitestosterone respectively were each mixed with about 24 mg Ultrodex and 0.1% of pH 5-7 ampholyte. The samples were applied in the respective slots (1 x 4 cm) on the gel plate shown in the following diagram.



The gel slurry consisted of 12 g Ultrodex in 300 ml distilled water, 15 ml ampholyte pH 5-7 and 23 mg DTT evaporated near the cracking limit (35%). In this case, 30% evaporation was allowed. Initial focusing was carried out at 600V (10 mA). After 19 hours at 640V (3.8 mA) the voltage was increased to 1000V (6 mA) which remained relatively steady. After 6 hours, 1 x 4 cm strips of gels were scraped into separate tubes and the gel slurry mixed with 1 ml of 10 mM Tris-HCl-0.5 mM DTT-20% glycerol, pH 8.0. The fractions from the edges were scraped accordingly for pH determinations. These were suspended in 1 ml 10 mM KCl solution. The gel suspensions containing the focused proteins were carefully well mixed and aliquots of the suspensions were assayed for enzyme activities. For DEAE-column peak I focused fractions, the following enzyme aliquots were used: epitestosterone, 10-30  $\mu$ l; androsterone, 5-20  $\mu$ l; 5 $\alpha$ -androstane-3 $\alpha$ ,17 $\beta$ -diol, 10  $\mu$ l and 17 $\alpha$ -estradiol 3-glucuronide, 10-30  $\mu$ l.

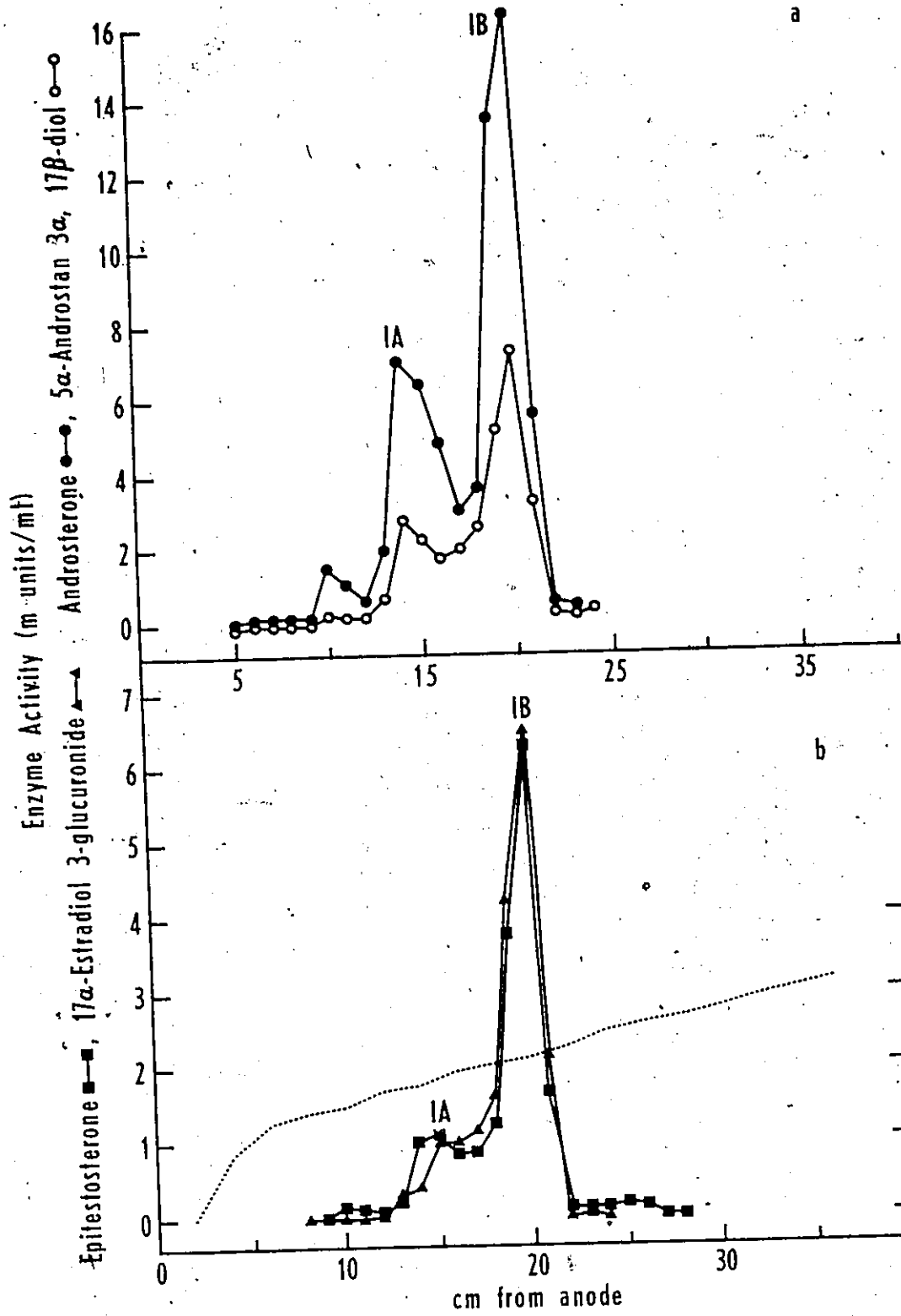
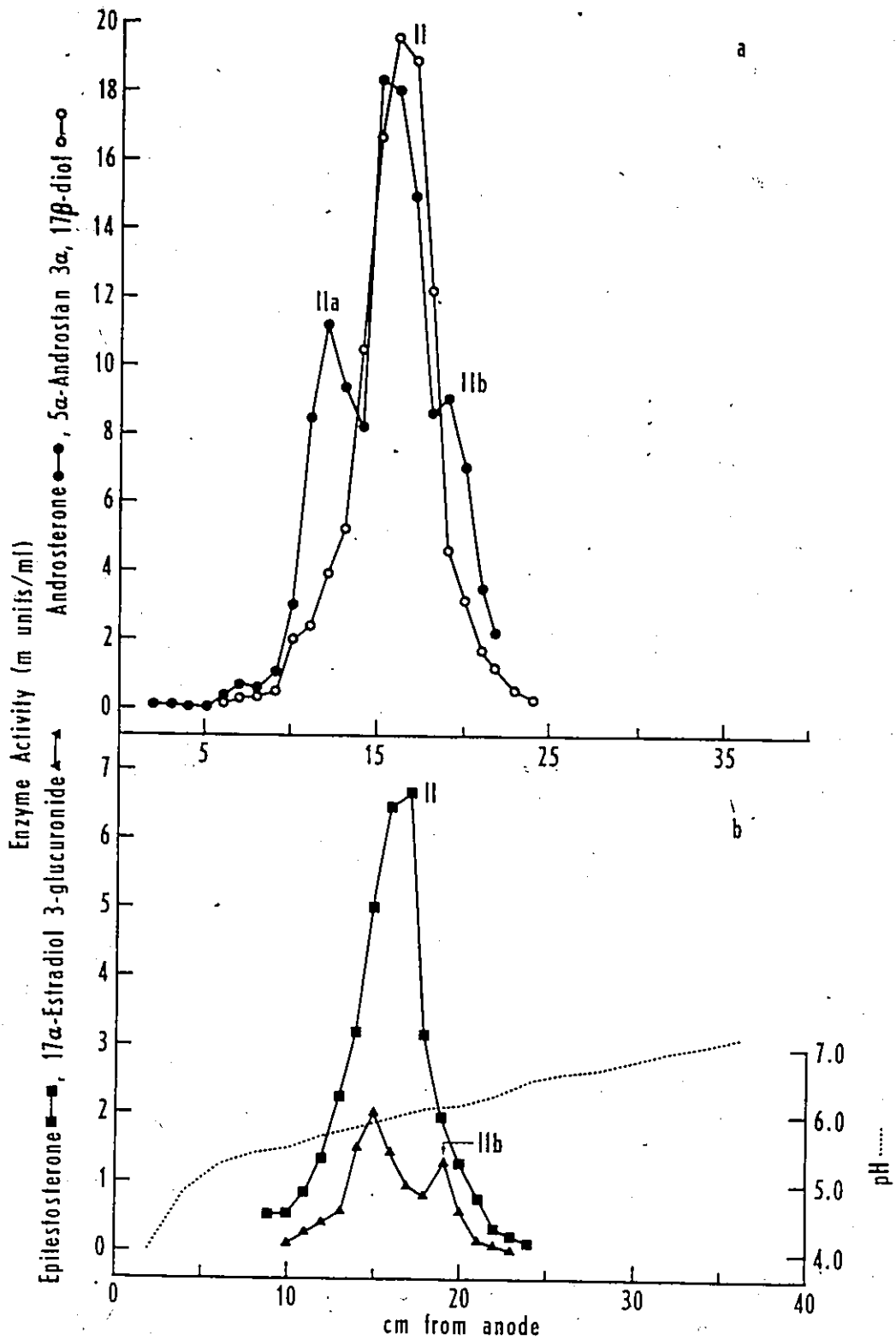


FIGURE 7

Isoelectric Focusing of 3(17) $\alpha$ -HSD Activity in DEAE-cellulose  
Chromatography Peak II

The preparation of enzyme sample and focusing conditions are as described in Figure 6. The following enzyme aliquots were used for assays with the various substrates: epitestosterone, 10  $\mu$ l; androsterone, 4  $\mu$ l; 5 $\alpha$ -androstan-3 $\alpha$ ,17 $\beta$ -diol, 2  $\mu$ l and 17 $\alpha$ -estradiol 3-glucuronide, 30  $\mu$ l.



c) Isoelectric focusing of DEAE-column peak III

Concurrent with the electrofocusing of DEAE-column peaks I and II enzymes we have also applied in a third track, a sample of the partially purified peak III enzyme fraction. This fraction is resolved into two major peaks, IIIA and IIIB (Figure 8). Both peaks including two minor ones are active toward the  $3\alpha$ - and  $17\alpha$ -hydroxysteroid substrates. The  $17\alpha$ -estradiol 3-glucuronide dehydrogenase activity in peak IIIB is much less than the other hydroxysteroid dehydrogenase activities. This peak might correspond to the area within DEAE-column peak III (Figure 4b) which exhibited a low  $17\alpha$ -estradiol 3-glucuronide dehydrogenase activity.

d) Isoelectric focusing of DEAE-column peak IV

The electrofocusing of the partially purified DEAE-column peak IV enzyme fractions was carried out in conjunction with a sample of the DEAE-column peak I enzyme the latter fraction being used as a point of reference for the apparent isoelectric point determinations. The DEAE-column peak IV enzyme, upon focusing is resolved into three main peaks IVA, IVB and IVC all of which are active toward the  $3\alpha$ - and the  $17\alpha$ -hydroxysteroid substrates (Figure 9). The glucuronide derivative of  $17\alpha$ -estradiol was also oxidized, but poorly (not shown). A very acidic peak in the vicinity of pI 4.5 is also observed with androsterone substrate.

Since the two focused peaks, IA and IB, of DEAE-column peak I in this same experiment (not shown) were found in exactly the same fractions as in the previous run shown in Figure 6, the isoelectric focusing profile of the DEAE-column peak IV can be correlated to those of the peaks I, II and III illustrated in Figures 6, 7 and 8.

FIGURE 8

Isoelectric Focusing of 3(17) $\alpha$ -HSD Activity in DEAE-cellulose  
Chromatography Peak III

The preparation of enzyme sample and focusing conditions are as described in Figure 6. The following enzyme aliquots were used for assays with the various substrates: epitestosterone, 10  $\mu$ l; androsterone and 5 $\alpha$ -androstan-3 $\alpha$ ,17 $\beta$ -diol, 5  $\mu$ l; and 17 $\alpha$ -estradiol 3-glucuronide, 30  $\mu$ l.

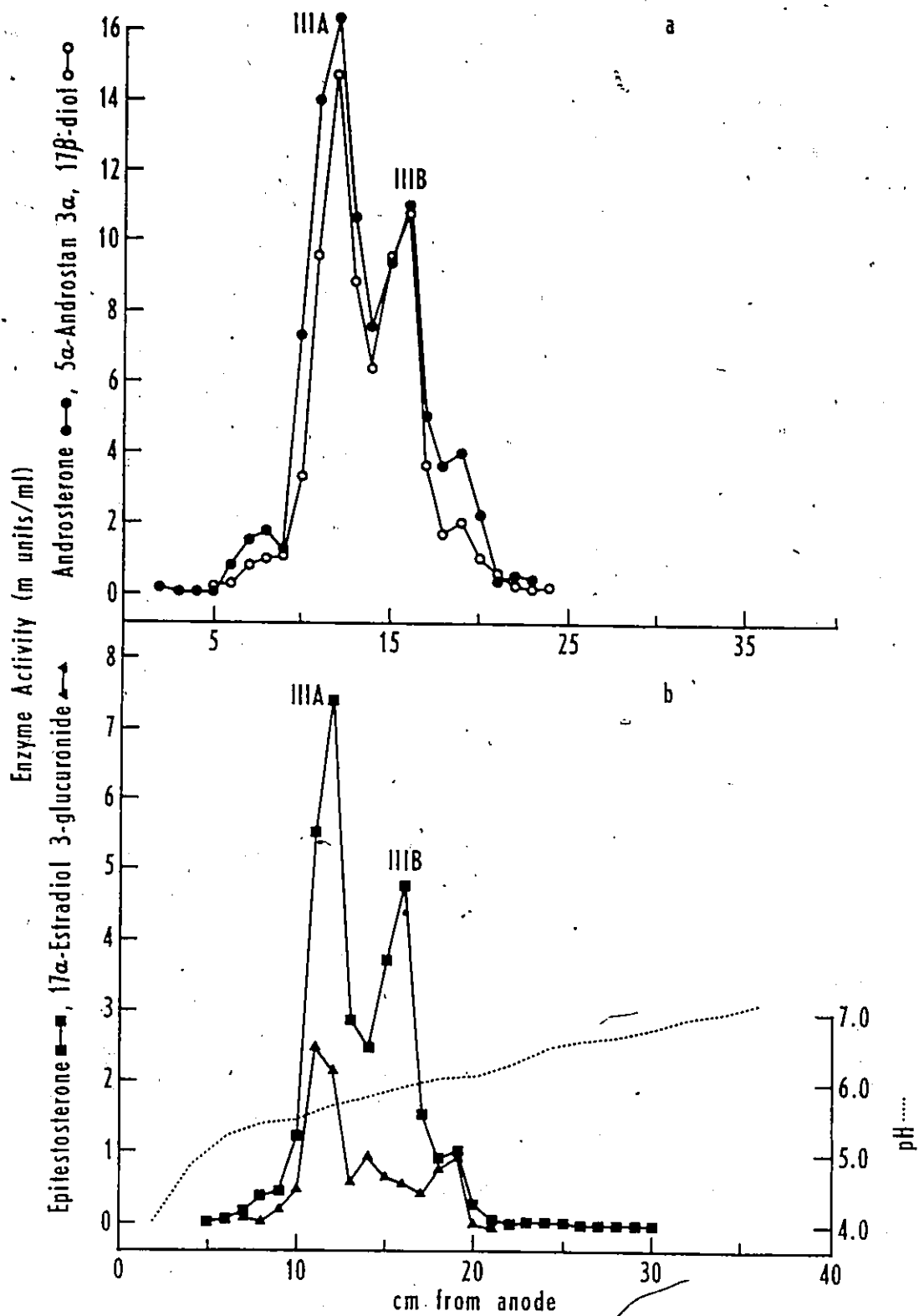


FIGURE 9

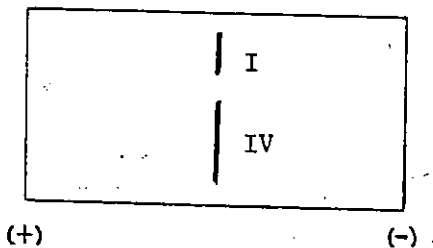
Isoelectric Focusing of 3(17) $\alpha$ -HSD Activity in DEAE-cellulose  
Chromatography Peak IV

This peak was focused in conjunction with the DEAE-column peak I. Both samples were treated according to the standard procedure. The particulars are:

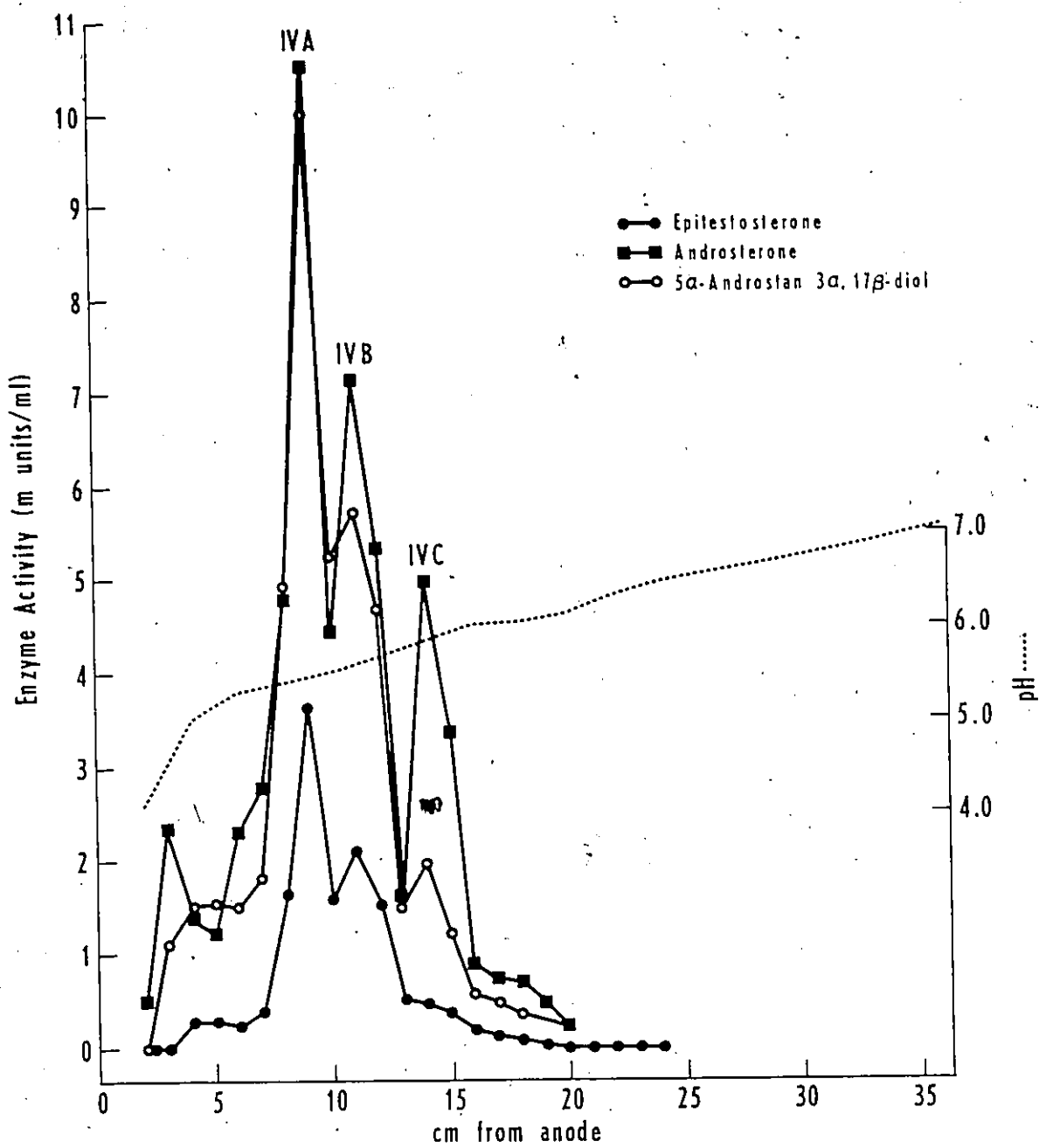
Peak I 0.8 ml containing about 81 milliunits of activity with epitestosterone.

Peak IV 1.7 ml containing about 51 milliunits of activity with epitestosterone.

The samples were applied in slots as illustrated.



Preparation of the gel slurry and conditions of focusing were as described in legend for Figure 6. Treatment of the gel slurries after focusing was also as described previously. Aliquots of enzyme suspensions used in the assay were as follows:  
epitestosterone, 20  $\mu$ l; androsterone, 4  $\mu$ l; 17 $\alpha$ -estradiol 3-glucuronide, 100  $\mu$ l and 5 $\alpha$ -androstan-3 $\alpha$ ,17 $\beta$ -diol, 5  $\mu$ l.



e) A composite isoelectric focusing profile of DEAE-column peaks I, II, III, and IV

Figure 10 shows a direct comparison of the isoelectric points of the focused fractions of the anion exchange column peaks I, II, III and IV assayed with epitestosterone substrate. Four distinct enzyme forms, IVA, IIIA, II and IB are featured, their apparent isoelectric points being 5.54, 5.67, 5.98 and 6.11 respectively. On the other hand, the pI of the focused peak IVB overlaps with enzyme form IIIA whereas enzyme forms IIIB and IA overlap with II, allowing for the experimental error of  $\pm 1$  cm fractionation. The additional peaks are probably not "true" molecular enzyme forms but stem from cross contamination taking into consideration the contiguous nature of the DEAE-cellulose chromatography enzyme elution profiles (Figures 4 & 5). In particular, this has been established for enzyme form IA which is actually part of enzyme form II. Supporting evidence from gel electrophoresis is presented in Chapter V.

An identical composite isoelectric focusing pattern was obtained for substrate  $5\alpha$ -androstan- $3\alpha,17\beta$ -diol. Similar profiles were also obtained with substrates androsterone and  $17\alpha$ -estradiol 3-glucuronide except for the additional peaks IIa and IIb (Figure 7) and other less established differences. It is also noteworthy that the focused enzyme forms increase in acidity in good correlation with the elution pattern of the DEAE-cellulose column peaks with increasing salt concentrations.

Monitoring the peak tube enzyme by electrophoresis in polyacrylamide gels stained for protein and with steroid substrate we have obtained pure samples of enzyme forms IA, IB, II and IIIA. Figure 11 shows the single protein band obtained with each of the above mentioned proteins. These proteins bands also stained for enzyme activity (see Chapter V).

When the purified enzyme forms of IB, II and IIIA were subjected to electrophoresis in the presence of SDS, no apparent subunit structure of these

FIGURE 10

A Composite Isoelectric Focusing Profile of  $17\alpha$ -HSD Activities  
in DEAE-cellulose Column Chromatography Peaks I, II, III and IV

The activity patterns obtained with epitestosterone in Figures 6, 7, 8  
and 9 were superimposed to provide the composite picture shown.

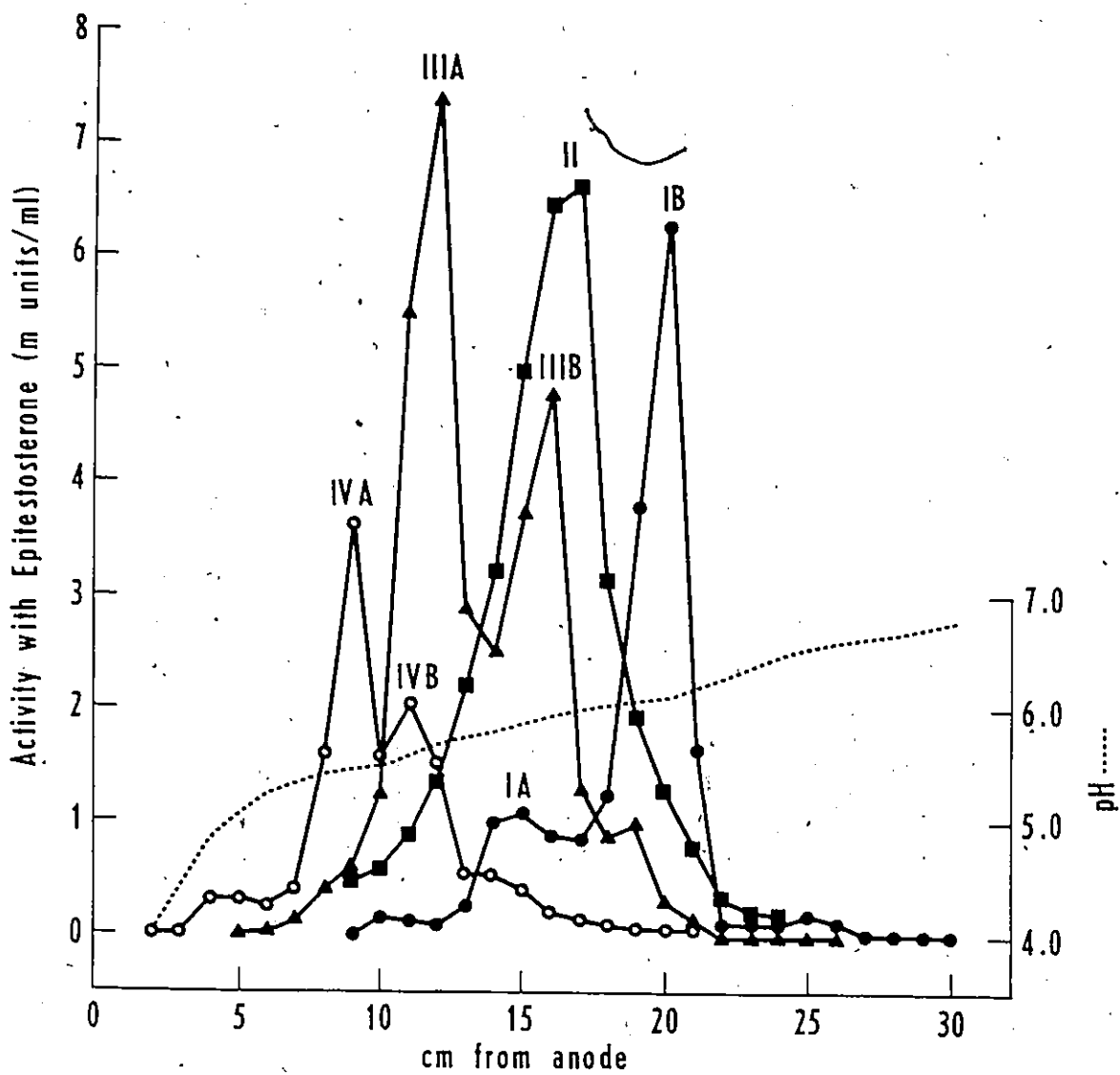
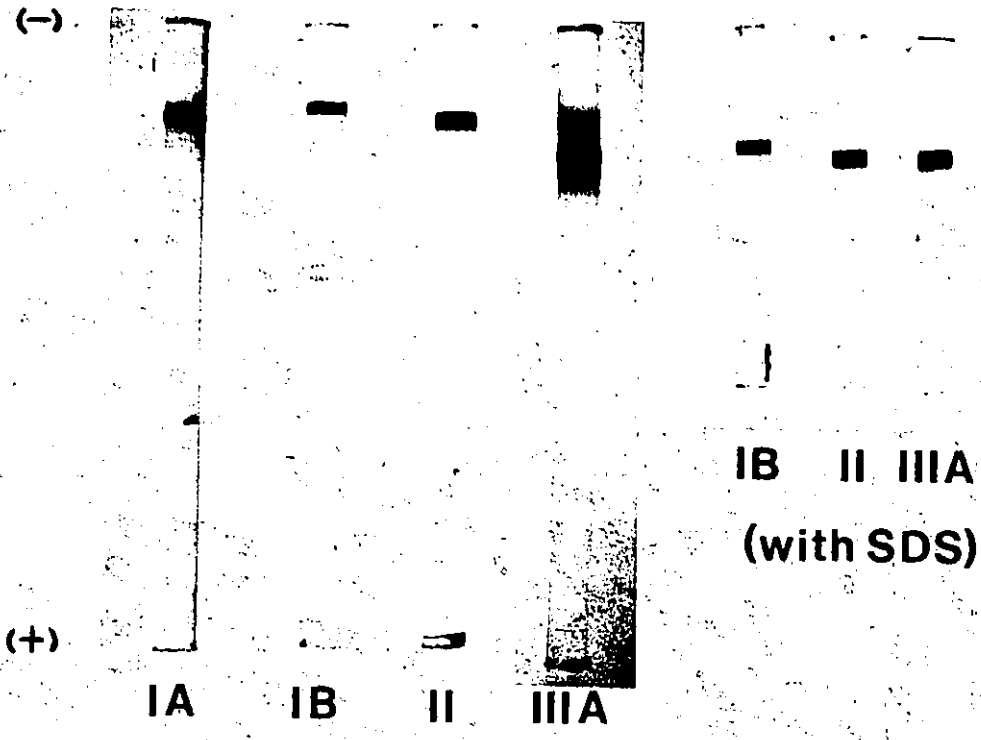


FIGURE 11

(SDS) Polyacrylamide Gel Electrophoresis of 3(17) $\alpha$ -Hydroxysteroid  
Dehydrogenases after Isoelectric Focusing

Electrophoresis in 10.5% acrylamide disc gels were carried out according to Davies (1964) as described in the General Methods (p.65 ). 10-25  $\mu$ g proteins determined by the UV absorption method (Layne, 1957) were applied to the gels. In the case of SDS-acrylamide gels, 5-10  $\mu$ g proteins were applied. The procedure of Weber et al (1972) was followed.



proteins was observed (Figure 11). These proteins migrated as single bands with mobilities close to those of chymotrysinogen A (45,000 MW) and the monomeric subunit of aldolase (40,000 MW). A molecular weight of 40,000, 41,000 and 41,000 was estimated for enzyme forms IB, II and IIIA respectively.

The apparent molecular weights of the less purified proteins of IIIB and IVA were also estimated to be 40,500 and 41,000 respectively. However, these values were only calculated from the most intensely-stained protein band on the gels (not shown).

Table 7 summarizes the substrate specificities of some of the multiple forms of the hydroxysteroid dehydrogenases after isoelectric focusing. With  $17\alpha$ -hydroxysteroids, the activities of all enzymes except IB, are much higher with epitestosterone than with the two estrogen substrates. Enzyme IB displays the characteristic high activity toward  $17\alpha$ -estradiol 3-glucuronide. When the oxidation of  $3\alpha$ -hydroxysteroids of the  $5\alpha$ -androstane series at C-3 are compared with the  $17\alpha$ -hydroxysteroids at C-17, androsterone is the best substrate for all the enzymes although enzymes IIIB and IVA appear to have an equal rate with  $5\alpha$ -androstan- $3\alpha,17\beta$ -diol. Apparently, the  $5\alpha$ -structure of the androstanes is important for the  $3\alpha$ -HSD activity because etiocholanolone which has a  $5\beta$ -structure is poorly oxidized. We have also tested  $5\alpha$ -androstan- $3\beta,17\beta$ -diol and testosterone as possible substrates, but neither the  $3\beta$ -hydroxyl group of the former steroid or the  $17\beta$ -hydroxyl groups of both steroids were oxidized to any significant extent. This shows that the  $3\beta$ - or  $17\beta$ -HSD activities are not associated with the  $3(17)\alpha$ -HSD activities.

Enzyme form IIIb which has an overlapping pI value with enzyme II exhibits similar substrate specificity characterized by a low  $17\alpha$ -estradiol 3-glucuronide dehydrogenase activity but the  $3\alpha$ -HSD toward androsterone and  $5\alpha$ -androstan- $3\alpha,17\beta$ -diol of enzyme II is higher than that of enzyme IIIB. A similar situation is

TABLE 7

Substrate Specificities of the Multiple Forms of the 3(17) $\alpha$ -Hydroxysteroid Dehydrogenases of Rabbit Kidney Cytosol after Isoelectric Focusing

Substrate	Specific Activity Relative to 17 $\alpha$ -Estradiol					
	Enzyme*					
	IB	II	IIIA	IIIB	IVA	IVB
17 $\alpha$ -Estradiol	1.0	1.0	1.0	1.0	1.0	1.0
17 $\alpha$ -Estradiol 3-glucuronide	33	4.0	13	1.5	1.1	3.8
Epitestosterone	28	16	30	12	27	25
Androsterone	120	71	95	42	63	86
5 $\alpha$ -Androstan-3 $\alpha$ ,17 $\beta$ -diol	72	65	63	46	63	58
Etiocolanolone	2.1	1.6	2.1	1.5	1.6	1.8

\*Enzymes forms IB, II and IIIA are homogeneous whereas IIIB, IVA and IVB are heterogeneous. The incubations were carried out as described in the General Methods. 10  $\mu$ l enzyme aliquots were used in the assays with epitestosterone. The aliquots of enzymes used for other substrates were adjusted accordingly.

evident with enzymes IIIA and IVB. In this case, the activity of the enzymes toward  $17\alpha$ -estradiol 3-glucuronide is quite different while the rest remains relatively constant. These data may provide evidence for the close identity of the enzymes II and IIIB or IIIA and IVB bearing in mind these enzymes, except II and IIIA, were not homogenous.

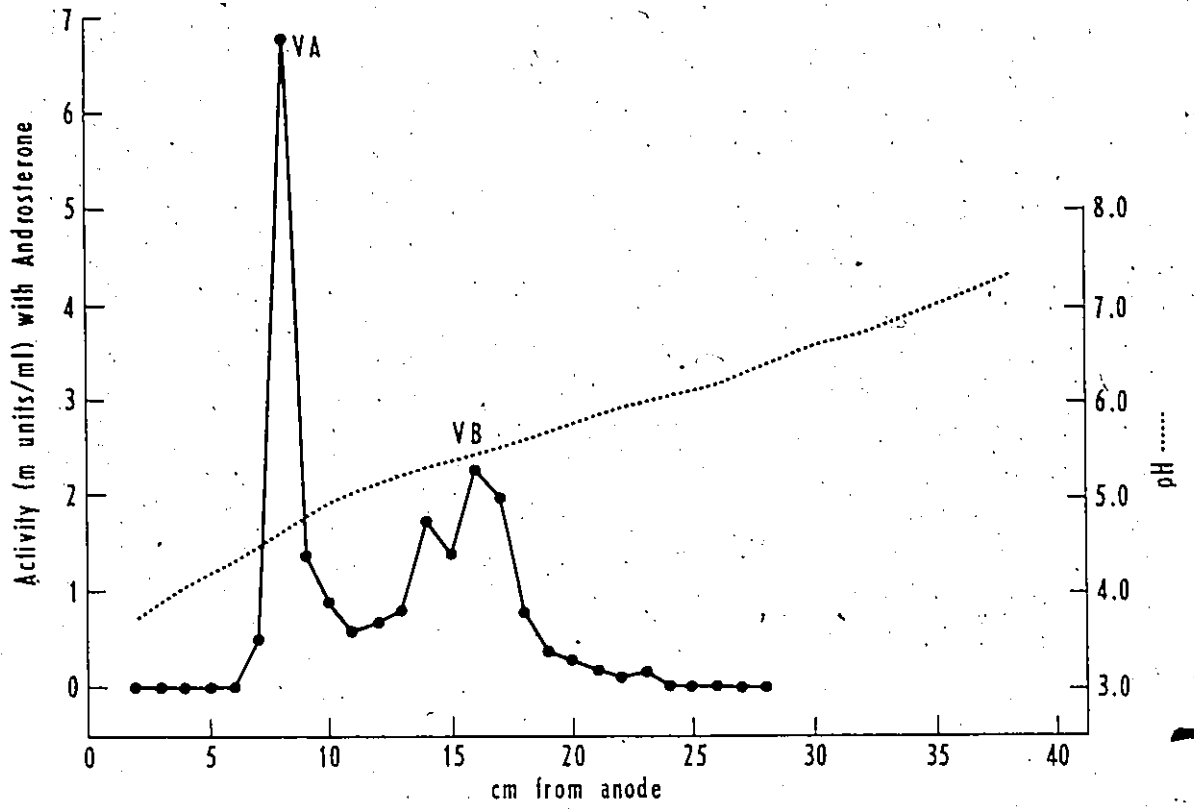
f) Isoelectric focusing of DEAE-column peak V

Isoelectric focusing of DEAE-column peak V produced the enzyme activity profile with androsterone shown in Figure 12. The very acidic form VA has a pI value of 4.61. It is not established whether the two minor peaks with pIs of 5.33 and 5.44 represent additional multiple forms although the more cathodic form is in close proximity with enzyme form IVA (Figure 10) with respect to its pI value. The polyacrylamide gel electrophoresis behaviour of the most acidic peak is of considerable interest. This is discussed in the next chapter.

FIGURE 12

Isoelectric Focusing of DEAE-cellulose Chromatography  
Peak V Enzyme Activity

The gradient was made up of 6 ml pH 5-7 and 6 ml of pH 4-6, ampholine. About 22 mg of protein containing 173 milliunits of androsterone dehydrogenase activity in 3 ml was applied on to the slot allocated in the middle of the gel plate. The preparation of the gel plate was as described in Figure 6. Initial voltage applied was 600V (12 mA) for 20 hours after which the voltage was 610V (2.8 mA). Final focusing was carried out at 1000V (4.6 mA) for another 6½ hours. 8 µl enzyme aliquots was used for assay with androsterone substrate.



## DISCUSSION

The NADP<sup>+</sup>-linked 17 $\alpha$ - and 17 $\beta$ -HSDs of female rabbit kidney can be separated by DEAE-cellulose column chromatography. However, associated with the 17 $\alpha$ -enzymes are the 3 $\alpha$ -HSD activities which have a high preference for 3 $\alpha$ -hydroxysteroids of the 5 $\alpha$ -androstane series. The 3 $\alpha$ -HSD activities of young adult male rabbit has been studied earlier by K $\acute{o}$ chakian and co-workers (Aoshima et al, 1964) to a limited extent. In agreement with the present study, the NADP<sup>+</sup>-dependent 3 $\alpha$ -enzyme activity was localized in the soluble fraction. Although the presence of 17 $\alpha$ -HSD was detected in the adult male rabbit kidney homogenate as early as 1952, (Kochakian et al, 1952) no follow-up studies have been reported.

The bifunctional specificity as well as heterogeneity of the rabbit kidney 3(17) $\alpha$ -HSDs merit some considerations. First of all, is the heterogeneity of the enzyme "real"? Evidence provided so far indicates that it is unlikely that the multiple steroid dehydrogenases are artefacts induced by protein - carrier ampholyte interaction during isoelectric focusing. This phenomenon has been observed in the electrofocusing of acidic wool protein (Frater, 1970), yeast isocitrate dehydrogenase (Illingworth 1972), bovine serum albumin (Wallevik, 1973), tRNA (Galante et al, 1976), certain acidic dyes (Righetti et al, 1977b), heparin (Righetti & Gianazza, 1978) and phosphoprotein of dentin with a high content of strongly acid phosphate groups (Jonsson et al, 1978), among others. A common relationship among most of these proteins or compounds is that they are strong acids or polyanions either phosphorylated or sulphonated and they exhibit apparent pI values less than 4.5. Righetti et al (1977b) suggested that in the pH range 4-9 there is no ampholyte-protein

interaction but with strongly acidic proteins or with unusual structures such as bovine serum albumin which binds to a number of ligands, the interaction with ampholyte is a strong possibility. However, in contrast, results excluding any protein-ampholyte interactions have also been reported for albumin, ferritin and  $\beta$ -glucuronidase (Dean & Messer, 1975) and two peptide hormones (Baumann & Chrambach, 1975). In our studies heterogeneity of the kidney enzyme was apparent even before isoelectric focusing i.e. during DEAE-cellulose chromatography. Moreover, when refocusing of a particular enzyme peak was necessary we did not observe the development of new enzyme forms. It is also unlikely that the multiple 3(17) $\alpha$ -HSDs are artefacts generated during the purification procedures, the same relative amounts of the different forms or the same enzyme activity profiles being obtained from different rabbit kidney preparations. Furthermore we observed multiple forms of the kidney enzyme persisting throughout the whole purification procedure when the various fractions were subjected to polyacrylamide gel electrophoresis and the gels stained for enzyme activities. These data will be presented in the next chapter.

Taking advantage of the flexibility or "openness" of the flat-bed isoelectric focusing system whereby different samples can be compared simultaneously we have ruled out what appeared to be extra multiple forms of the rabbit kidney enzymes. These extra enzyme forms were in fact due to cross-contaminations arising from incomplete separation during DEAE-cellulose chromatography fractionation. The general conclusion is that there are at least four forms of the rabbit kidney hydroxysteroid dehydrogenase active toward both 3 $\alpha$ - and 17 $\alpha$ -hydroxysteroids. In addition there is one very acidic form which has a higher specificity for 3 $\alpha$ -hydroxysteroids than the 17 $\alpha$ -hydroxysteroids. This enzyme also exhibits an appreciable activity toward

17 $\beta$ -hydroxysteroids. Originally, we had focused the various DEAE-cellulose column peaks I-IV individually in separate runs and obtained a total of up to 10 enzyme forms. Because of the poor reproducibility of pI values it was difficult to establish whether they were all distinct enzyme forms. We have also carried out focusing using a narrower pH gradient (pH 5.5-6.1) obtained from the fractionation of the original pH 5-7 ampholyte. But the shallow gradient caused the enzyme peaks to spread more widely making the pI values less discriminating. Perhaps it is worth noting that the pI values reported for the rabbit kidney multiple forms are at best approximate. These values are assigned to the maximum concentration of a focused protein and are therefore average values. In fact, the determination of true isoelectric points of proteins is a major technical difficulty (Righetti & Caravaggio, 1976; Delincee & Radola, 1978). Another problem associated with isoelectric focusing is working with ampholytes of the pH range 5-7. This pH range has the disadvantage of a minimum buffering capacity (Fredriksson, 1977). Apparently, this minimal buffering capacity is not due to the lack of amphoteric species focused in this region but to the fact that the compounds focusing in this interval are "poor" carrier ampholytes (Righetti et al, 1977a).

The identification of 3 $\alpha$ -enzyme activity with the 17 $\alpha$ -HSDs of the rabbit kidney opens up several possibilities. Most importantly, is this coincidence a true identity? The co-purification of the two enzyme activities is highly supportive evidence. A relatively constant ratio of 3 $\alpha$ -enzyme activity (represented by androsterone) to 17 $\alpha$ -enzyme activity (represented by epitestosterone) of 2.2-2.9 : 1 was maintained up to and including the Sephadex filtration step. However, after DEAE-cellulose column chromatography and isoelectric focusing whereby these activities were resolved into multiple forms,

slightly different ratios were obtained. This is expected considering that these multiple forms differ in substrate specificity. The best evidence for the common identity of the  $3\alpha$ - and  $17\alpha$ -HSD enzyme comes from the co-migration of the two activities in polyacrylamide gels. This, as well as evidence from substrate kinetic studies are dealt with in Chapters V and VI.

Hitherto, the presence of  $3(17)\alpha$ -HSD has not been encountered in the literature but several workers have identified the  $3\alpha$ -HSD activity with the purified  $17\beta$ -HSD (or vice versa) of different sources. Thaler-Dao et al (1972) reported the presence of a  $3\alpha,17\beta$ -HSD in the female rabbit liver cytosol. Shen and Kochakian (1978) also found a  $3\alpha$ -HSD activity associated with two of the purified forms of the  $\text{NADP}^+$ -dependent  $17\beta$ -HSD of male guinea pig kidney cytosol. In contrast, a  $17\beta$ -estradiol dehydrogenase activity was found to be associated with a pure  $3\alpha$ -HSD of *Pseudomonas testosteroni* (Battais et al, 1977).

One notable feature apparent from the substrate specificity studies of the kidney enzymes is the high specificity of enzyme IB for  $17\alpha$ -estradiol 3-glucuronide compared to other enzyme forms. This specificity was also observed with one of the rabbit liver enzyme forms (Hasnain, & Williamson, 1975). This reiterates the close relationship between conjugation with glucuronic acid at C-3 of  $17\alpha$ -estradiol, and oxidoreduction at C-17 during the metabolism of estrogens in the rabbit. However, higher activities were generally observed with androgenic substrates than with estrogens. This was also seen with the  $17\alpha$ -HSDs (Hasnain & Williamson, 1977) and  $17\beta$ -HSD (Thaler-Dao et al, 1972) of female rabbit liver. Furthermore, with respect to the androgenic substrates, the  $3\alpha$ -HSD activity of rabbit kidney exceeds that of the  $17\alpha$ -enzyme activity. This is in contrast with the intrinsic  $3\alpha$ -HSD activity of the  $17\beta$ -HSD determined by Thaler-Dao et al (1972) and Shen and Kochakian (1978). In both of these instances, the  $17\beta$ -HSD activity (with testosterone or  $5\alpha$ -dihydrotestosterone) was

higher than the  $3\alpha$ -enzyme activity (with androsterone or etiocholanolone). In addition, the substrate specificity of the rabbit kidney  $3\alpha$ -HSD activity differs from the rabbit liver  $3\alpha$ -enzyme activity determined by Thaler-Dao et al (1972) in that the former shows a preference for  $C_{19}$ -steroids with the A/B ring fused in a trans manner whereas the latter enzyme prefers a cis configuration of the A/B ring. On the other hand, the  $17\beta$ -HSD activity of the male guinea pig kidney cytosol (Shen & Kochakian, 1978) showed the same discrimination as the rabbit kidney enzyme with regard to the A/B ring configuration.

The multiple forms of the rabbit kidney  $3(17)\alpha$ -HSDs are charge isomers since they differ in their pI values during isoelectric focusing or have different elution characteristics from the DEAE-cellulose column. The identical molecular weight of these multiple forms is an evidence against their being size isomers. The majority of the multiple forms of guinea pig kidney (Liu & Kochakian, 1972ab; Shen & Kochakian, 1978) and liver (Kobayashi & Kochakian, 1978)  $17\beta$ -HSDs have also been shown to differ in electrical charge rather than molecular size. The molecular weights of these guinea pig enzymes ranged from 31-34,000, slightly smaller than the average value of 40,700 found in this study for the rabbit kidney  $3(17)\alpha$ -HSDs or the 39,600 reported for six forms of the rabbit liver  $17\alpha$ -HSDs (Hasnain & Williamson, 1977). No apparent subunit structure of the rabbit enzymes was observed in these studies ruling out the possibility that these multiple forms might originate from a combination of non-identical subunits. The  $17\beta$ -HSD of porcine testes also has a monomeric structure with a molecular weight of 35,400 (Inano et al, 1977). However a dimeric subunit structure has been shown for the human placental  $17\beta$ -estradiol dehydrogenase (Burns et al, 1971, 1972) where the subunit molecular weight is about 34,000. A tetrameric composition has been reported for the  $3(17)\beta$ -HSD of Pseudomonas testosteroni

(Schultz et al, 1977), the monomeric molecular weight being 23,500. This enzyme in its native state is believed to be formed by a combination of two subunits of similar molecular weight but different amino acid composition to generate a family of tetrameric proteins. These multiple forms or isoenzymes are also charge isomers. The active forms of these enzymes which possess both the  $3\beta$ - and the  $17\beta$ -HSD activities are the two subunits within the tetrameric structures. In contrast, the active form of the human placental EDH is the dimer (Pons et al, 1977).

CHAPTER V

POLYACRYLAMIDE GEL ELECTROPHORETIC ANALYSES OF THE MULTIPLE FORMS OF 3(17) $\alpha$ -  
HYDROXYSTEROID DEHYDROGENASES OF FEMALE RABBIT KIDNEY

The purification of the rabbit kidney 3(17) $\alpha$ -HSDs was monitored by electrophoresis in 10.5% polyacrylamide disc gels, staining for either 3 $\alpha$ - or 17 $\alpha$ -HSD activities. The results from one particular set of experiments, unless otherwise stated, are presented.

1. Substrate staining of 3(17) $\alpha$ -HSDs present in the crude 105,000 g supernatant of rabbit kidney

Figure 13 shows the complex patterns of enzyme activities of the cytosol obtained with either androsterone or epitestosterone substrates under two experimental conditions. With 2 mM cyanide present in the substrate staining reagent mixture a cluster of three to four bands of lesser mobility stained with androsterone (Figure 13 gel and scan a) or epitestosterone (Figure 13 gel and scan b) are observed. The intensity of the different bands depends on the substrate. However, there exists a very intense band (the most anodic form) which specifically stains for 3 $\alpha$ -HSD activity under the standard conditions. Gel and densitometric scan c shows the staining of the control gel in the absence of either steroid substrate but in the presence of cyanide. The achromatic or colorless band seen near the top of the gel or the trough on the scan is presumably due to the cyanide-insensitive superoxide dismutase (SOD) activity (Weisiger & Fridovich, 1973). This band is also observed in the substrate-stained gels a and b of Figure 13. The action of the SOD activity has been shown to inhibit the reduction of nitroblue tetrazolium (NBT) and thus prevented.

the formation of the purplish-blue diformazan causing instead the formation of achromatic bands (Beauchamp & Fridovich, 1971). The band indicated by an arrowhead is likely an artefact developed on storage of the gels. This blue band in contrast to the normal purplish-blue substrate stained bands, is not observed in the fresh gels and from the densitometric scans which were usually carried out immediately after substrate stainings.

When the polyacrylamide gels containing the same amounts of proteins are stained for  $3\alpha$ - or  $17\alpha$ -enzyme activities in the absence of cyanide (Figure 13 gels and scans d and e) only the most anodic band exhibiting the  $3\alpha$ -HSD activity is observed. The cluster of bands of lesser mobility seen in the presence of cyanide are not obvious in this case but are seriously obscured by achromatic bands due to the SOD activities. These SOD activities are cyanide-sensitive because they are not apparent when the staining reagent contains cyanide (Figure 13ab). About four of these enzyme forms are present and are seen more clearly as troughs in the "blow-up" scan provided in the inset g (Figure 13). This interference by multiple forms of the SOD prevented several earlier attempts to demonstrate on gels the occurrence of multiple forms of the  $17\alpha$ -enzyme in the crude cytosol, after calcium phosphate gel fractionation or after Sephadex gel chromatography. Although the addition of cyanide circumvented the problem, the resolution of the different multiple bands in the gels did not improve. This was partly due to the high protein load required to get enough enzyme activity for substrate staining or due to the closeness in the net charges under the electrophoretic conditions, of the different multiple forms of the steroid dehydrogenases.

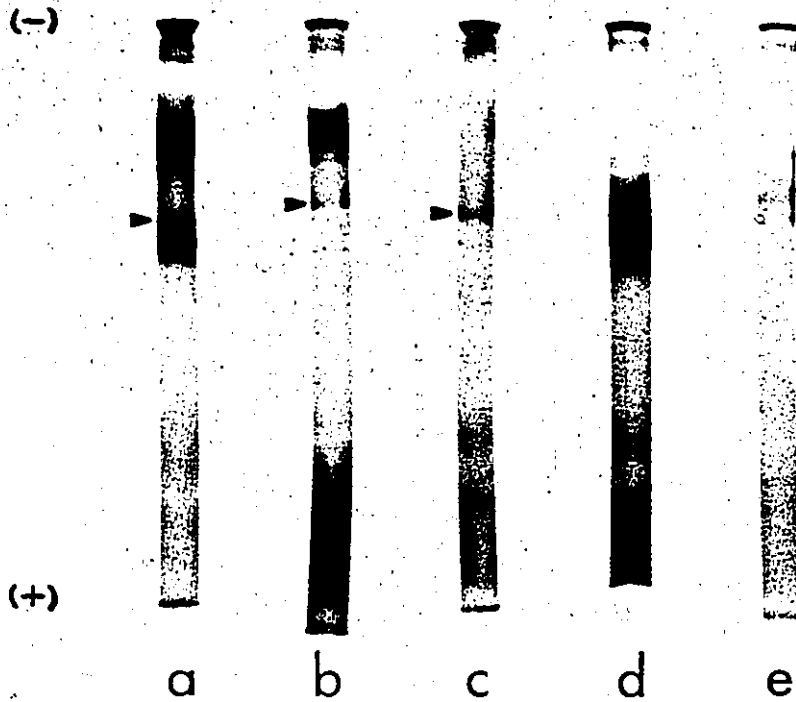
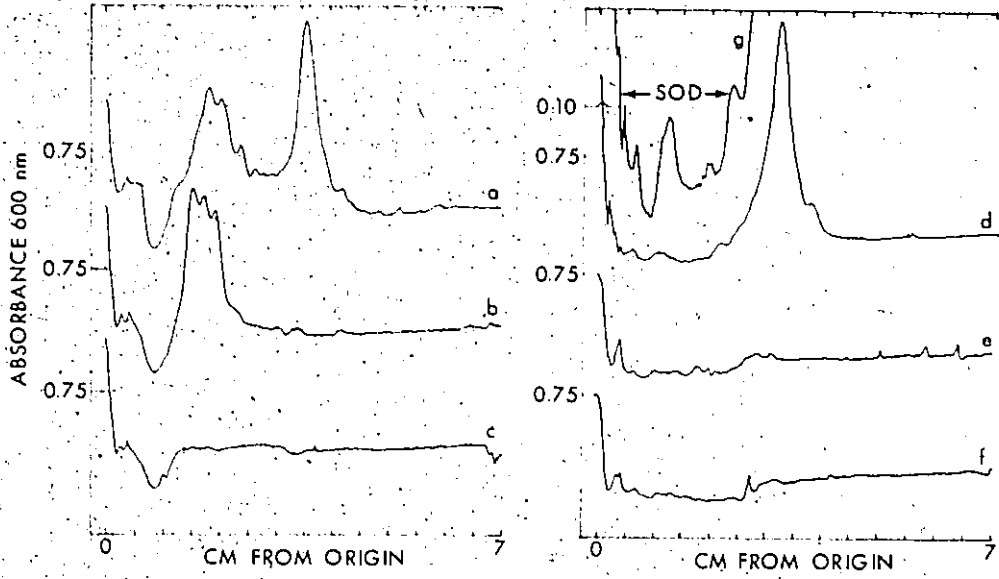
2. Substrate staining of the  $3(17)\alpha$ -HSD activities after calcium phosphate gel fractionation and Sephadex gel filtration

The complex pattern of multiple enzyme activities seen on polyacrylamide

FIGURE 13

Polyacrylamide Gel Electrophoresis of the Crude Soluble 3(17) $\alpha$ -HSDs  
of Rabbit Kidney

Electrophoresis and substrate staining were performed in 10.5% polyacrylamide gels as described in the General Methods (p. 65). 50  $\mu$ l of the crude 105,000 supernatant containing about 1700  $\mu$ units of enzyme activity with androsterone was applied to gels a and d. 100  $\mu$ l containing about 1400  $\mu$ units of enzyme activity with epitestosterone was applied to gels b and e. The control gels c and f (the latter not shown) contained 100  $\mu$ l of enzyme. Gels a, b and c were stained in the presence of 2 mM cyanide whereas gels d, e and f were stained in the absence of cyanide. The absorbance reading as indicated represents the mid-point of the full scale used for each of the densitometric scan. Each gel was scanned from the top (cathode end) for a distance of 7 cm. The band marked with an arrowhead is an artefact (see text).



gels stained with either androsterone or epitestosterone persisted through the calcium phosphate gel fractionation (Figure 14 gels and scans a,b,c) and Sephadex gel filtration (Figure 14 gels and scans d,e,f). The intensity of the individual bands especially those of the more cathodic forms varies from step to step. One peculiar feature of the gel electrophoresis of the calcium phosphate gel is the presence of a very sharp non-specific stained band near the top of all the gels including the control. This non-specific band is removed by Sephadex gel filtration whereas the cyanide-insensitive SOD activity associated with the crude cytosol is eliminated by calcium phosphate gel fractionation. The electrophoresis of the enzyme activities of the Sephadex gel filtration step clearly show co-purification of the  $3\alpha$ - and  $17\alpha$ -proteins. Moreover, at least four enzymatically active bands of lesser mobility are present, active toward both androsterone and epitestosterone. In addition, one major and one minor band of fastest mobilities stain specifically for  $3\alpha$ -HSD activity.

### 3. Substrate staining of the $3(17)\alpha$ -HSD activities after DEAE-cellulose column chromatography

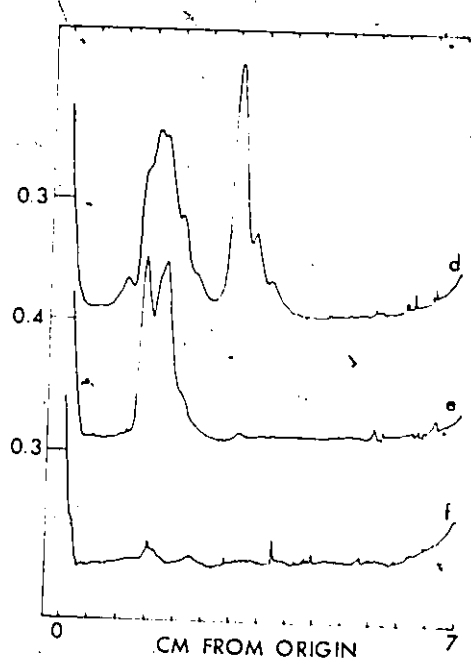
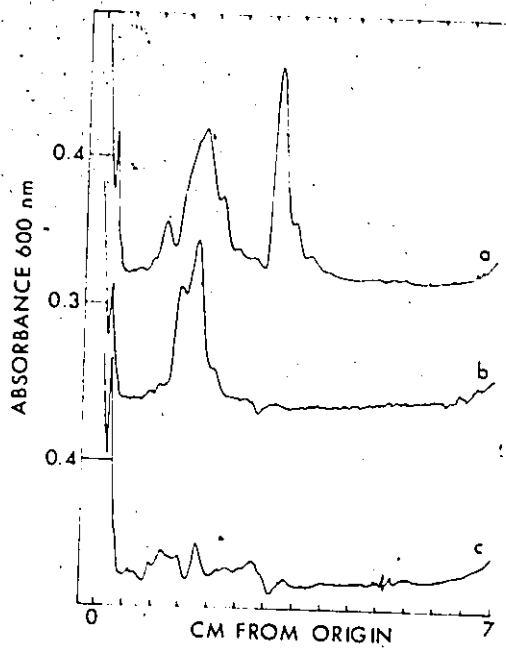
#### a) DEAE-cellulose column peak I to V enzyme activities

The designations of the different DEAE-enzyme peaks I to V of rabbit kidney are as described in Figure 5. The substrate-stained gels and their corresponding densitometric scans of the various DEAE-enzyme peaks are presented in Figure 15A (with androsterone) and Figure 15B (with epitestosterone). Several features are worth noting. First of all, there is gradation of increasing acidity of the different multiple forms which parallels the elution pattern of the enzyme activities from the DEAE-cellulose column. For example, the most basic enzyme form on the polyacrylamide gel corresponds to the enzyme peak I that is eluted from the DEAE-cellulose column at the lowest salt concentration whereas the most

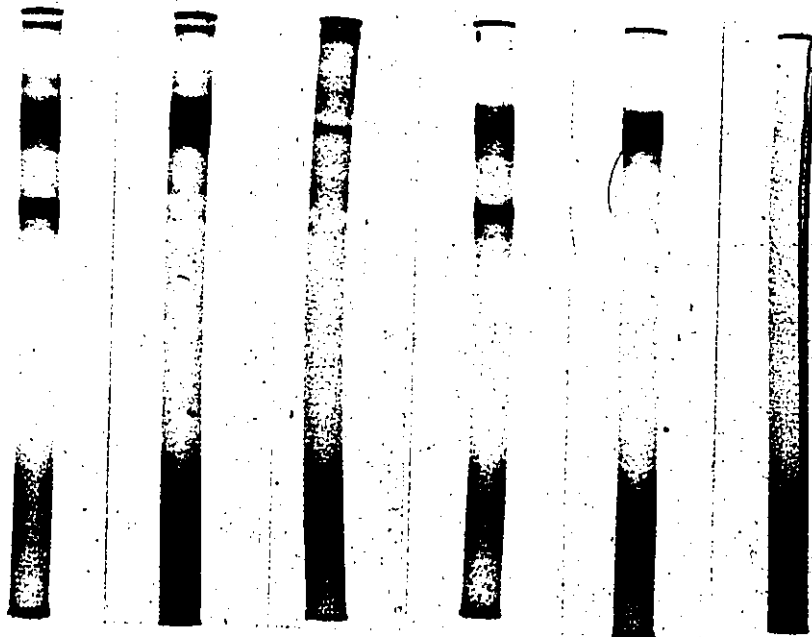
FIGURE 14

Polyacrylamide Gel Electrophoresis of 3(17) $\alpha$ -HSDs in the Calcium Phosphate Gel Fraction and Sephadex Gel Filtration Fraction

Electrophoresis and substrate staining were carried out as described in the General Methods (p. 65). The calcium phosphate gel fraction containing about 1900  $\mu$ units of androsterone dehydrogenase activity and 1700  $\mu$ units of epitestosterone dehydrogenase activity was applied to gels a and b respectively and stained accordingly. Gel c is a control. Gels d and e are of the Sephadex gel fraction containing about 1200  $\mu$ units of activity toward androsterone and 900  $\mu$ units of activity toward epitestosterone respectively. Gel f is a control for the Sephadex gel enzyme fraction. All the gels were stained in the presence of 2 mM cyanide. The scanning conditions are as described in Figure 13.



(-)



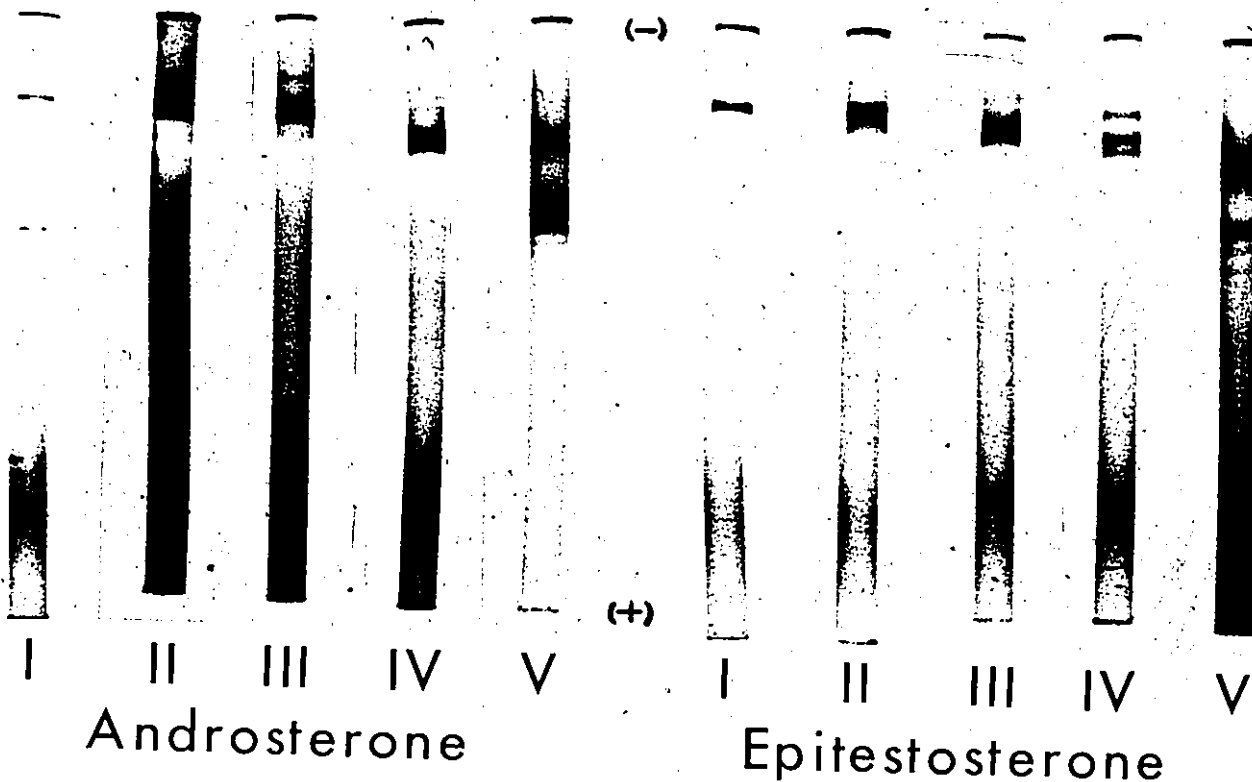
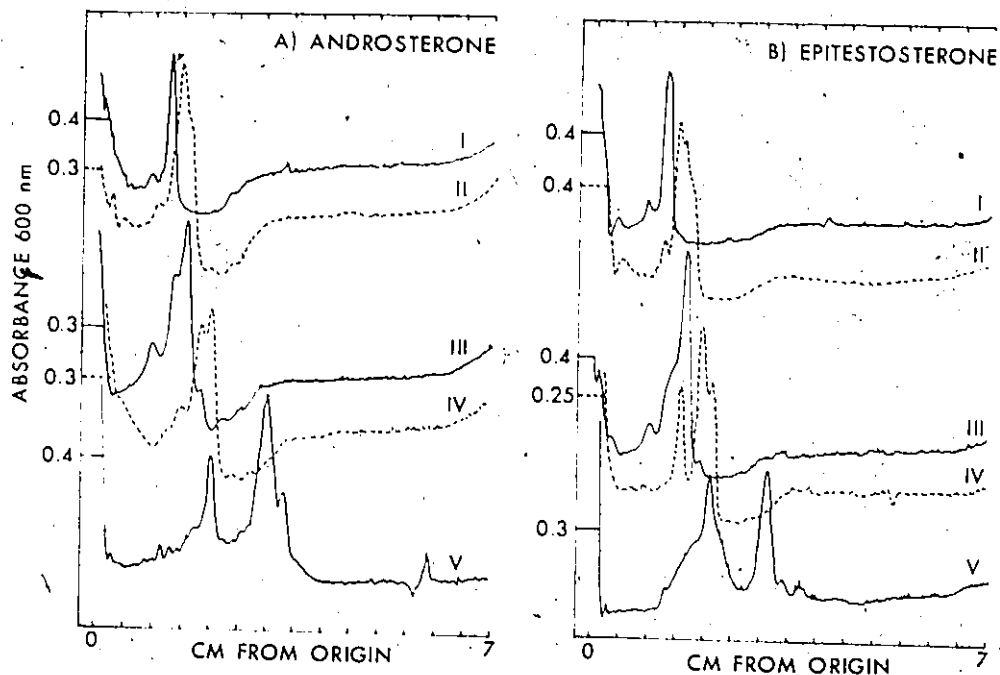
(+)

a b c d e f

FIGURE 15

Polyacrylamide Gel Electrophoresis of 3(17) $\alpha$ -HSDs in the DEAE-cellulose  
Column Chromatography Peaks I to V

Electrophoresis and substrate staining were carried out as described in the General Methods (p. 65). The gels stained with androsterone (A) contained 1200-1700  $\mu$ units of enzyme activity whereas those stained with epitestosterone (B) contained 1100-1400  $\mu$ units. A control gel for each enzyme fraction (I to V) was also run (not shown). Only the control gel for enzyme fraction II stained in the absence of steroid. All the gels were stained in the absence of cyanide. The scanning conditions of the gels are as described in Figure 13.



acidic form on the gel corresponds to the fraction (peak V) retained the longest on the ion-exchange column. Secondly, there is considerable overlap in the mobility of the enzyme activities of the various DEAE-column peaks on the polyacrylamide gels. Thirdly, although it was demonstrated in the earlier purification steps that the most acidic enzyme forms on the gel stained specifically for the  $3\alpha$ -hydroxysteroid (Figure 13 & 14), with an excess of this particular enzyme fraction (DEAE-peak V), dehydrogenation of the  $17\alpha$ -hydroxysteroid is also illustrated (Figure 15B V). This is in accord with the substrate specificity study of the peak V enzyme where it was shown earlier that although this particular enzyme peak exhibited highest activity toward the  $3\alpha$ -hydroxysteroids it could also catalyze the oxidation of  $17\alpha$ -hydroxysteroids (Table 6).

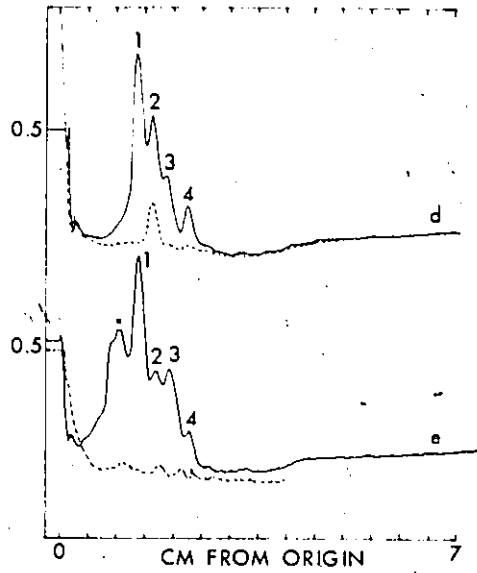
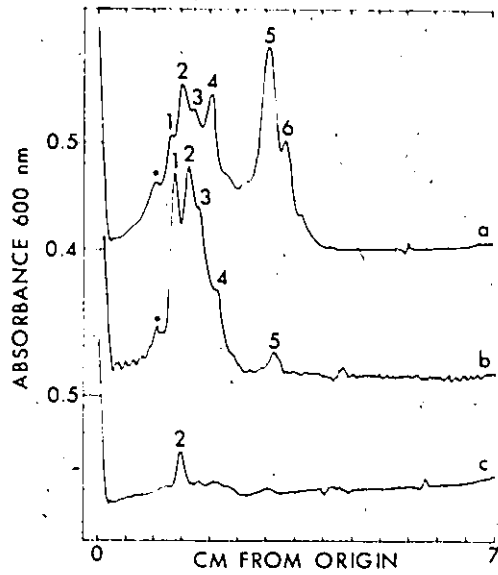
b) Composite profile of DEAE-cellulose column enzyme activities

When various aliquots of enzymes of the DEAE-cellulose column enzyme peaks are combined and subjected to electrophoresis, four bands of lesser mobility (bands 1-4) staining for either  $3\alpha$ - or  $17\alpha$ -hydroxysteroid substrate are obtained (Figure 16 gels and scans a,b). Band 5 which previously stained specifically with  $3\alpha$ -hydroxysteroid (Figure 12, 13) is also active with epitestosterone in this case. However a minor band (band 6) is also obvious with the androsterone substrate. This was also obvious in the Sephadex gel filtration step (Figure 14 gel and scan d). In a different experiment, bands 5 (major) and 6 (minor) were also observed with  $5\alpha$ -androstane- $3\alpha,17\beta$ -diol substrate when the various DEAE-column enzymes were pooled and electrophoresed (not shown). The band marked with an asterisk in scan a and b of Figure 16 is attributed to artefact formation perhaps due to freezing and thawing. This is shown more clearly in Figure 16 scan e. The pattern was obtained from a combined fraction of the four DEAE-enzyme peaks (I-IV) that had been frozen and thawed three times prior to

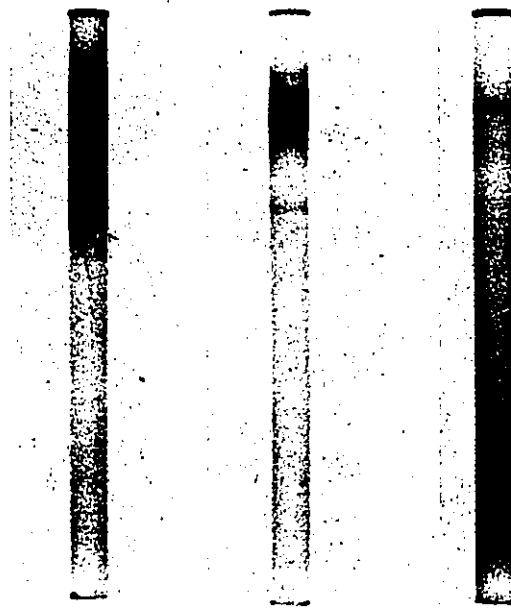
FIGURE 16

Polyacrylamide Gel Electrophoresis of 3(17) $\alpha$ -HSDs in the Combined DEAE-cellulose Column Chromatography Peaks - Effect of Freezing and Thawing

Electrophoresis and substrate staining were carried out as described in the General Methods (p. 65). The various concentrated DEAE-column enzyme fractions (I to V) were pooled and applied to gels a, b and c. Gels a and b were stained with androsterone and epitestosterone respectively whereas gel c is a control. The densitometric scans d and e are from gels (not shown) stained with epitestosterone. The enzyme fractions applied in these gels were the pooled fraction of the concentrated DEAE-column enzyme peaks I to IV from a different preparation as above. The samples in gel d were frozen and thawed once whereas those in gel e were frozen and thawed three times. The dashed tracings represent the control gels. All the above gels were stained in the absence of cyanide. The scanning conditions are as described in Figure 13.



(-)



(+)

a

b

c

electrophoresis. The gel was stained with epitestosterone. While the four enzymatically active bands (bands 1-4) on the gel correspond to those obtained with the fresh enzyme fractions (Figure 16 scan d) the band marked with the asterisk is obviously an artefact.

Another characteristic feature of the gel electrophoresis of the DEAE-column fractions is the presence of a non-steroid staining band in the control gels, (Figure 16 gel and scan c) associated particularly with DEAE-peak II (band 2) enzyme activity. This phenomenon has been described as due to "nothing dehydrogenase" activity (Pearse, 1972). Some of the properties of this activity will be presented in a later section.

#### 4. Substrate staining of 3(17) $\alpha$ -HSDs after isoelectric focusing

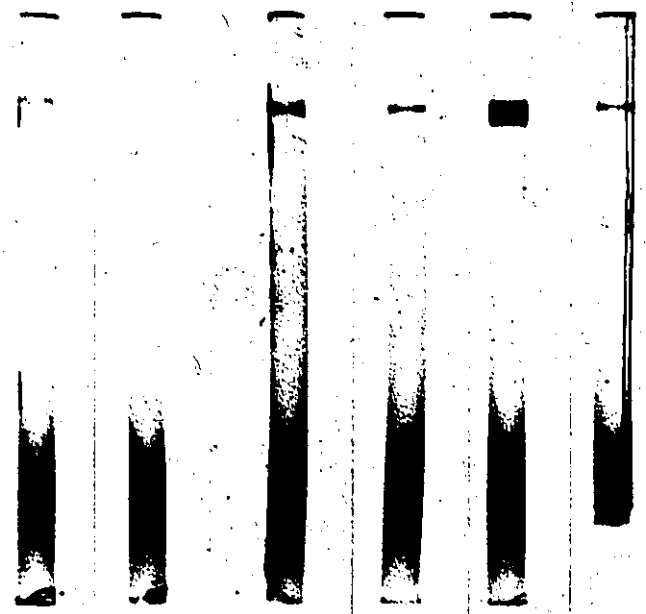
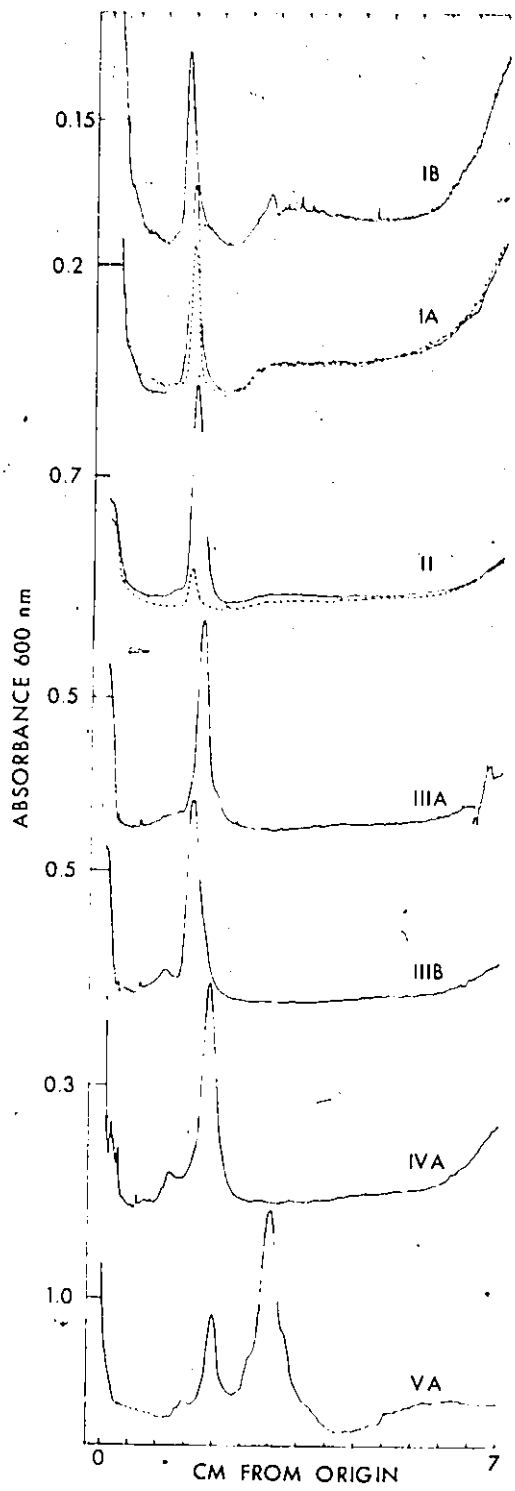
##### a) Individual electrofocused enzyme activities

The densitometric scans and their corresponding androsterone-stained gels of the various focused enzyme fractions of rabbit kidney IA, IB, II, IIIA, IIIB, IVA and VA are presented in Figure 17. The designations of the focused enzyme fractions are as assigned in Figures 6, 7, 8, 9, 10 and 12. One enzymatically active band is observed with each of the focused fractions except VA. Enzyme forms IA and II also show activity, although less intense in the absence of the steroid substrate. This characteristic has been made use of to establish the common identity of the two enzyme forms — IA being a cross contaminant of II. Their relative migration in the polyacrylamide gel provides additional evidence. Both IA and II enzyme forms migrate slightly faster than the enzyme form IB which constitutes the most cathodic form. Enzyme form IIIA which had a lower isoelectric point than IIIB (Figures 9 & 10) migrates accordingly on the polyacrylamide gel, the former enzyme being the faster moving species whereas enzyme IIIB migrates the same distance as enzymes II and IA, all of these having the same apparent isoelectric point. Shifted further toward the anode in accordance with its more

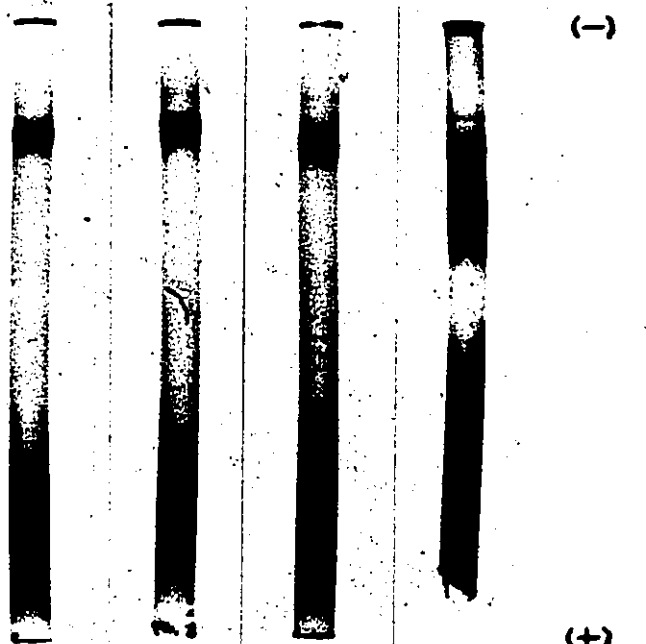
FIGURE 17

Polyacrylamide Gel Electrophoresis of 3(17) $\alpha$ -HSDs after  
Isoelectric Focusing

Electrophoresis and substrate staining were carried out as described in the General Methods (p. 65). The electrofocused fractions applied on the gels were as described in Figure 10 and 12. All the gels except IBC, IAc and IIc were stained with androsterone. These exceptions represent the control experiments stained in the absence of added steroid. All the control gels for enzyme fraction IIIA, IIIB, IVA and VA stained negligibly in the absence of steroid (not shown). The protein stains corresponding to fraction IA, IB, II and IIIA are shown in Figure 11. The scanning conditions are as described in Figure 13.



IB IBc IA IAc II IIc



IIIA IIIB IVA VA (-) (+)

acidic isoelectric point is the enzyme form IVA. However, gel electrophoresis of the enzyme VA which has the lowest isoelectric point produces two distinct enzymatically active bands, a gel pattern similar to that observed after DEAE-cellulose chromatography (Figure 15 gel and scan V). The slower moving band has a mobility similar to that of enzyme IVA but the two forms have quite dissimilar isoelectric points (Figures 10 & 12).

b) Composite profile of electrofocused enzyme activities

Electrophoresis of the combined focused enzyme fractions and staining with androsterone or epitestosterone produced the patterns shown in Figure 18 gels and scans a and b. Again, four forms of lesser mobility staining differentially with androsterone or epitestosterone and a very acidic form staining quite specifically with androsterone are observed. The control<sup>s</sup> gel also shows the characteristic non-specific staining corresponding to enzyme form II or IA (band 2). The reconstituted enzyme profile after isoelectric focusing resembles those electrophoretic patterns obtained with the crude 105,000 g enzyme fraction (Figure 13) or with the fractions after calcium phosphate gel fractionation, Sephadex gel filtration (Figure 14) and DEAE-cellulose column chromatography (Figure 16).

The protein staining with Coomassie brilliant blue of the polyacrylamide gels of the various electrofocused fractions which revealed the homogenous nature of enzyme fractions IB, IA(II) and IIIA (corresponding to bands 1, 2 and 3 respectively on the polyacrylamide gels) have been presented earlier (Figure 11).

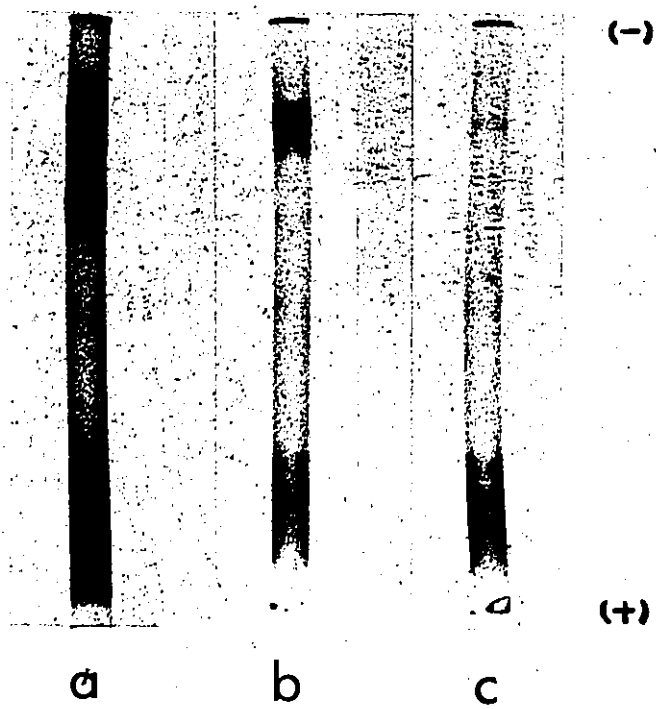
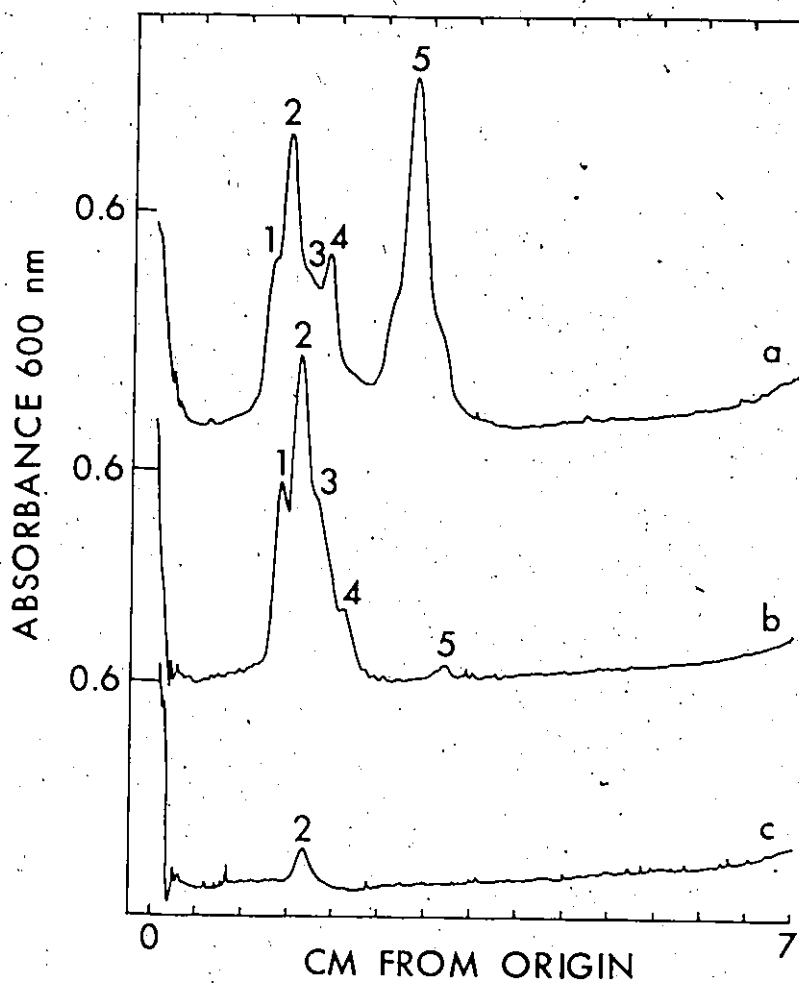
5. Some properties of the "nothing dehydrogenase" activity

As noted earlier, the nonspecific reduction of NBT in polyacrylamide gels in the absence of added steroid substrate was particularly linked to the partially purified DEAE-cellulose column peak II enzyme activities or the homogenous electrofocused II or IA fractions whereas with other enzyme forms,

FIGURE 18

Polyacrylamide Gel Electrophoresis of 3(17) $\alpha$ -HSDs in the  
Combined Fractions after Isoelectric Focusing

Electrophoresis and substrate staining were carried out as described in General Methods (p. 65). Gels a and b were stained with androsterone and epitestosterone respectively. Gel c represents the control experiment stained in the absence of steroid. All the gels contained the same total amount of protein. The scanning conditions are as described in Figure 13.



this phenomenon was less apparent or not present at all. The intensity of the formation of the diformazan band in the absence of the added steroid substrate was invariably lower than the staining in the presence of the steroid (Figures 16, 17 & 18). We have carried out some further studies on this "nothing dehydrogenase" activity in an attempt to identify the source of the endogenous or exogenous substrates. Some properties of this activity are summarized as follows:

1. PCMB at a final concentration of 1 mM inhibited the appearance of the "nothing dehydrogenase" band as well as the steroid-stained band when the sulfhydryl reagent was added to the enzyme prior to electrophoresis or to the staining reagent mixture.
2. Sodium cyanide at a final concentration of 2 mM did not inhibit the non-specific staining.
3. The non-specific staining was equally intense in gels with or without glycerol.
4. When methanol or ethanol was omitted from the staining reagent mixture, the non-specific staining was still observed.

## DISCUSSION

To demonstrate the existence of isozymes or multiple forms of an enzyme it is imperative to establish their presence in the crude tissue extract in order to rule out any artefactual formation which may accompany purification of the enzyme (IUPAC-IUB CNB, 1977). One commonly used technique for resolving multiple forms or isozymes is zone electrophoresis. Once resolved, the isozymes can be identified by various histochemical staining techniques. We have used electrophoresis in polyacrylamide gels and the PMS-NBT staining technique to localize the 3(17) $\alpha$ -HSD activities of rabbit kidney and demonstrate the heterogeneity and co-purification of these enzymes during various stages of the purification procedures. The results in Chapter V showed that the multiplicity of the rabbit kidney 3(17) $\alpha$ -HSDs was indeed present in the crude cytosol. At least four molecular forms active toward both the 17 $\alpha$ - and 3 $\alpha$ -hydroxysteroid and one major acidic form quite specific for the 3 $\alpha$ -hydroxysteroid were apparent when these enzyme fractions, reconstituted or otherwise, at various stages of the purification procedures were subjected to polyacrylamide gel electrophoresis. These observations were in keeping with the isoelectric focusing data presented in Chapter IV. The difference in charges of the multiple forms revealed by polyacrylamide gel electrophoresis in conjunction with the isoelectric focusing profile of these proteins further supported the view that these proteins are charge isomers. Furthermore, the co-purification of the 3 $\alpha$ - and 17 $\alpha$ -HSD activities was illustrated by polyacrylamide gel electrophoresis in addition to the substrate specificity studies and various other protein separation methods employed in the purification of these enzymes.

Although the formation of an artefact due to repeated freezing and thawing of the 3(17) $\alpha$ -HSD enzymes was noted in an isolated case, this could not account for the extent of heterogeneity of these enzymes. The possible cause(s) of the heterogeneity of these kidney steroid dehydrogenases will be discussed in a later chapter. However, storage in the cold of the cytosol or the presence of 2-mercaptoethanol has been shown to affect the electrophoretic pattern of the 17 $\beta$ -HSDs of the adult male guinea pig kidney (Liu & Kochakian, 1972b; Shen & Kochakian, 1978), liver (Kobayashi & Kochakian, 1978) or those of the female guinea pig kidney (Shen & Kochakian, 1979). More molecular forms of these enzymes were apparent in the fresh sample in the absence of mercaptoethanol than in its presence. But, upon storage at 4 $^{\circ}$ C or -20 $^{\circ}$ C more forms were generated both in the absence or presence of the sulfhydryl reagent. Recent results from our laboratory (unpublished) have shown that immediate polyacrylamide gel electrophoresis of the fresh cytosolic 3(17) $\alpha$ -HSD of the rabbit kidney yielded essentially the same number of enzyme bands as the sample which was stored overnight or longer at -20 $^{\circ}$ C. However, we did not study the effect of the absence of sulfhydryl reagent on the electrophoretic pattern of our enzymes.

As mentioned earlier in the text, several initial attempts to demonstrate the multiplicity of the rabbit kidney 3(17) $\alpha$ -HSD activities in the crude cytosol, the fractions after calcium phosphate gel fractionation or Sephadex gel filtration, in polyacrylamide gels proved unsuccessful. The problem was due to the interference by multiple forms or isozymes of superoxide dismutase (SOD) in these crude extracts. These activities appeared as achromatic bands on the gels in the PMS-NBT staining system and unfortunately coincided with or were localized near the sites of the hydroxysteroid dehydrogenase activities (except the most acidic enzyme form). To circumvent this problem we made use of the inhibitory

action of cyanide on the SOD activities (Beauchamp & Fridovich, 1971). Apparently, cyanide inhibits the cytochrome c peroxidases which might interfere by causing a peroxidation of the diformazan. However cyanide-insensitive SOD activities also exist (Weisiger & Fridovich, 1973). The latter activity was first demonstrated in chicken liver extracts and was purified to homogeneity. This cyanide-insensitive enzyme was of mitochondrial origin with a molecular weight of 80,000 composed of four subunits of equal size and it contained manganese. In contrast, the faster moving cyanide-sensitive SOD activities (at least three) of the chicken liver preparations were of cytosolic location with a molecular weight of about 31,000 composed of two subunits of equal size and they contained copper and zinc. The pig heart was also shown to contain two electrophoretically distinct bands of SOD activity one in the cytosol inhibited by cyanide and the other in the mitochondria insensitive to cyanide (Weisiger & Fridovich, 1973). However, more recently both cyanide-sensitive and cyanide-insensitive SOD activities have been reported in the liver mitochondria of rat, mouse and chicken (de Rosa et al, 1979). These workers used 2 mM sodium cyanide to completely inhibit the cyanide-sensitive enzyme. Cyanide did not affect the mitochondrial activity even at concentrations as high as 8 mM.

We have adopted a sodium cyanide concentration of 2 mM in our PMS-NBT assay system and demonstrated the presence of multiple forms or isozymes of both the cyanide-insensitive SOD and cyanide-sensitive SOD activities in the rabbit kidney cytosol. We were fortunate that the cyanide-insensitive SOD species did not occupy the sites of the slower moving group of HSD activities. Hitherto, these SOD activities have not been reported in the rabbit tissues although they have been isolated from a wide range of eukaryotes and procaryotes (Fridovich, 1978). In view of the wide occurrence of the SOD in tissue extracts

and its inhibitory action on NBT reduction under aerobic conditions it may be recommended (with caution) that cyanide be included in the standard PMS-NBT staining mixture if reliable steroid dehydrogenase reactions were to be demonstrated, especially in crude extracts. Moreover, oxygen has been shown to strongly interfere with the reduction of NBT under aerobic conditions (Worsfold et al, 1977; Ponti et al, 1978). Worsfold et al (1977) studying the lactate dehydrogenase isozyme pattern of mouse muscle observed that a vacuum-evacuated PMS-NBT staining mixture enhanced the diformazan considerably while the sample stained in air displayed weak bands or no bands at all. Although staining in vacuo improved the picture this may not be very practical especially when the reaction has to be carried out in the dark and at elevated temperature.

During the course of our electrophoretic studies of the multiple forms of the rabbit kidney 3(17) $\alpha$ -HSDs we have also encountered the so-called "nothing dehydrogenase" activity (NDH). Pearse (1972) described this activity as the NBT reducing ability in the absence of added substrate but in systems containing NAD(P)<sup>+</sup>. However the NDH activity has also been shown in systems not involving the PMS-NBT reaction mixture but demonstrated by spectrophotometric reduction of NAD<sup>+</sup> (Mezey et al, 1969) or in a system without the participation of NAD(P)<sup>+</sup> (de Ligny et al, 1975). Restricting our discussion of NDH to the PMS-NBT system in gel electrophoresis the occurrence of this activity has been variable. These include the study of alcohol dehydrogenase in starch gel (Shaw & Koen, 1965) aldehyde dehydrogenase in polyacrylamide gel (Robbins, 1966), galactose dehydrogenase in starch gel (Beutler, 1967), lactate dehydrogenase in polyacrylamide gel, cellulose acetate or starch gel (Falkenberg et al, 1969; Ferguson, 1971) and malate dehydrogenase (Silverstein & Geller, 1974) and/or glutamate dehydrogenase in starch gel or polyacrylamide gels (Ressler & Stitzer, 1967; Gill, 1978). Explanations for the discrete non-specific staining include

the possible presence of substrate as an impurity in the chemicals used in staining (Beutler, 1967) or the presence of some reactive groups in the gel matrix similar to the substrate (Falkenberg et al, 1969). The potential substrate might also be a non-dialyzable species closely attached to the enzyme macromolecule (Silverstein & Geller, 1974; Gill, 1978). The possibility that the buffer system might act as substrate was also considered. Shaw & Koen (1965) detected the NDH band in both the Tris and phosphate buffers whereas Ressler and Stitzer (1967) observed the NDH band in both veronal and glycine buffers. Recently, Gill (1978) investigated twelve enzymes in the snail Cepaea nemoralis using four different buffer/gel systems. The NDH activity was detected in only two systems one of which was the discontinuous Tris-glycine (pH 8.5)/Tris-HCl (pH 8.9) system. Moreover, the NDH activity was attributed to either glutamate or malate dehydrogenase activity.

The rabbit kidney NDH in this study was inhibited by the addition of PCMB to the enzyme sample prior to electrophoresis or to the staining reagent mixture. This made it likely as earlier suggested by Pearse (1972) that protein bound sulfhydryl compounds might be responsible for the reduction of NBT in the absence of the added substrate. There is evidence that autoxidation of thiols (dithiothreitol, 2-mercaptoethanol, reduced glutathione and ethyl mercaptan) generate superoxide radicals which reduce the NBT to its diformazan (Misra, 1974). We have observed the reduction of NBT in the staining reagent mixture by the addition of dithiothreitol but this was not demonstrable in polyacrylamide gels. The possibility that the NDH reaction might involve cytochrome c and cytochrome c oxidase was investigated by the addition of cyanide to the PMS-NBT reaction mixture. No inhibition was observed. We also ruled out the participation of methanol (solvent for the steroid substrate) or glycerol (mixture for enzyme sample for loading on to the gel surface) as

substrates. Alcohol dehydrogenase being the major NDH activity reported by Shaw and Koen (1965) was not detected with our staining system or with the recipe of Shaw and Koen (1965).

Although the NDH activity was observed in gel electrophoresis of several other steroid dehydrogenase studies (Liu & Kochakian, 1972ab; Pollow et al, 1976b; Shen & Kochakian 1978, 1979) these authors made no comments on it. Often, the phenomenon of the NDH reaction was viewed as a serious source of error in the histochemical demonstration of dehydrogenases in tissue sections (Pearse, 1972; Anderson & Hoyer, 1974) or in steroid dehydrogenase localization on polyacrylamide gels (O'Connor et al, 1977). On the contrary in our study the NDH activity was considered an intrinsic property of the particular steroid dehydrogenase enzyme and we further made use of this property as a marker in the identification or separation of this protein from another.

CHAPTER VI

COMPARATIVE STUDIES ON THE PROPERTIES OF 3(17) $\alpha$ -HYDROXYSTEROID DEHYDROGENASES  
OF FEMALE RABBIT KIDNEY AND LIVER

1. Comparison of the substrate specificities of the kidney and liver NADP<sup>+</sup> dependent 17-hydroxysteroid dehydrogenases in the crude cytosol

Two characteristic differences are observed among the 17 $\alpha$ - and 17 $\beta$ -HSD activities present in rabbit liver and kidney (Table 8). First of all, while the specific activities of 17 $\alpha$ -estradiol dehydrogenase and the epitestosterone dehydrogenase are very similar in both tissues, the 17 $\alpha$ -estradiol 3-glucuronide dehydrogenase activity is about three times higher in the kidney than the liver. Secondly, the 17 $\beta$ -HSD is consistently higher in the liver, the highest activity being exhibited with testosterone which is 94 times greater than the corresponding kidney enzyme. With free estrogens, the activity toward 17 $\alpha$ -estradiol is about twice that of the 17 $\beta$ -estradiol in the kidney whereas this situation is reversed in the liver. With androgens, the activity ratio of epitestosterone to testosterone in the kidney is 29 : 1 whereas that of the liver is 0.29 : 1. One common feature shared by the two tissues is the preference of both the 17 $\alpha$ - and 17 $\beta$ -enzymes for androgenic substrates.

The 3 $\alpha$ -HSD activities were also localized in the crude cytosol of the rabbit kidney and liver. At two separate occasions when these activities were measured the ratio of androsterone to epitestosterone dehydrogenase activities in the kidney was found to be 2.5-3.0 : 1. The liver 3 $\alpha$ -HSD activities was also consistently higher than the 17 $\alpha$ -enzyme activities; the ratio of androsterone : epitestosterone activity being 5.4 : 1.

TABLE 8

Substrate Specificities of the Female Rabbit Kidney and Liver  
17-Hydroxysteroid Dehydrogenases in the 105,000 g supernatant

Substrate	Specific Activity (picomole product/min/mg protein)	
	Kidney*	Liver**
17 $\alpha$ -Estradiol	40.7 $\pm$ 4.1	38.9 $\pm$ 11.7
17 $\beta$ -Estradiol	19.6 $\pm$ 2.4	57.2 $\pm$ 10.7
17 $\alpha$ -Estradiol 3-glucuronide	488.7 $\pm$ 69.0	132.0 $\pm$ 7.0
17 $\beta$ -Estradiol 3-glucuronide	14.2 $\pm$ 1.7	24.0 $\pm$ 5.5
Epitestosterone	825.2 $\pm$ 111.5	787.7 $\pm$ 136.7
Testosterone	28.8 $\pm$ 5.8	2712.5 $\pm$ 225.0

\*The values are means of 8 experiments  $\pm$  standard deviation.

\*\*The values are means of 2 experiments  $\pm$   $\frac{1}{2}$  range ( difference between two values )

2. Isoelectric focusing pattern of partially purified 17 $\alpha$ -hydroxysteroid dehydrogenases of rabbit kidney and liver

At least four molecular enzyme forms of the epitestosterone dehydrogenase of rabbit kidney were apparent after isoelectric focusing of the enzyme fraction previously partially purified by calcium phosphate fractionation and Sephadex gel filtration. The apparent pI values were 5.41, 5.60, 5.76 to 5.80 and 6.04 (Figure 19). In contrast, three distinct enzyme forms of the liver fraction prepared in the same manner as the kidney sample were obtained after isoelectric focusing. These liver enzyme forms were focused at pH 5.76, 5.90 and 6.17. Thus the most anodic enzyme form of the kidney and the most cathodic enzyme form of the liver appear to be unique to each tissue whereas the other forms have essentially overlapping isoelectric points.

3. Comparison of isoelectric focusing patterns of rabbit kidney and liver 17 $\alpha$ -hydroxysteroid dehydrogenases present in peak I of the DEAE-cellulose column chromatography

It was previously shown that when the soluble 17 $\alpha$ -hydroxysteroid dehydrogenases of female rabbit liver, partially purified by calcium phosphate gel fractionation and Sephadex gel filtration were subjected to DEAE-cellulose chromatography (2.4 litre salt gradient, 25-70 mM NaCl) four peaks of enzyme activity were separated. These peaks were monitored with 17 $\alpha$ -estradiol and its glucuronide derivative (Hasnain & Williamson, 1975). The activity profile obtained with epitestosterone is shown in Figure 20.

Isoelectric focusing of the DEAE-column peak I enzyme activity of the liver (Figure 20) resolved the enzyme further into two peaks, IA and IB shown in Figure 21. The figure also compares the focusing profile of the kidney DEAE-peak I enzyme activity (Figure 6) carried out simultaneously with the liver

FIGURE 19

Isoelectric Focusing of Rabbit Kidney and Liver 17 $\alpha$ -Hydroxysteroid  
Dehydrogenase Activities after Sephadex Gel Filtration

Both the kidney and liver enzyme samples were partially purified by calcium phosphate gel fractionation and Sephadex gel filtration. After concentration of the active fractions, a 1 ml aliquot of each sample containing approximately 275 milliunits of activity toward epitestosterone was applied to separate 1 x 4 cm troughs allocated in the middle of the gel plate. The composition of the gel slurry was as follows: 300 ml distilled water; 12 g Sephadex G-200 superfine; Ampholine 5-7, 12.0 ml; Ampholine pH 3.5-10, 1.2 ml; DTT 23 mg. Initial voltage applied was 600V (15 mA). After 17 hours, the voltage was 680V at 2 mA which was then stepped up to 1000V at 3.4 mA for an additional 8 hours. After focusing the gel slurries were scraped into individual test tubes and 2 ml of distilled water added to each tube. 5-10  $\mu$ l Aliquots of the gel suspension were assayed for enzyme activity with epitestosterone. The same gel suspension was also used for pH gradient measurement.

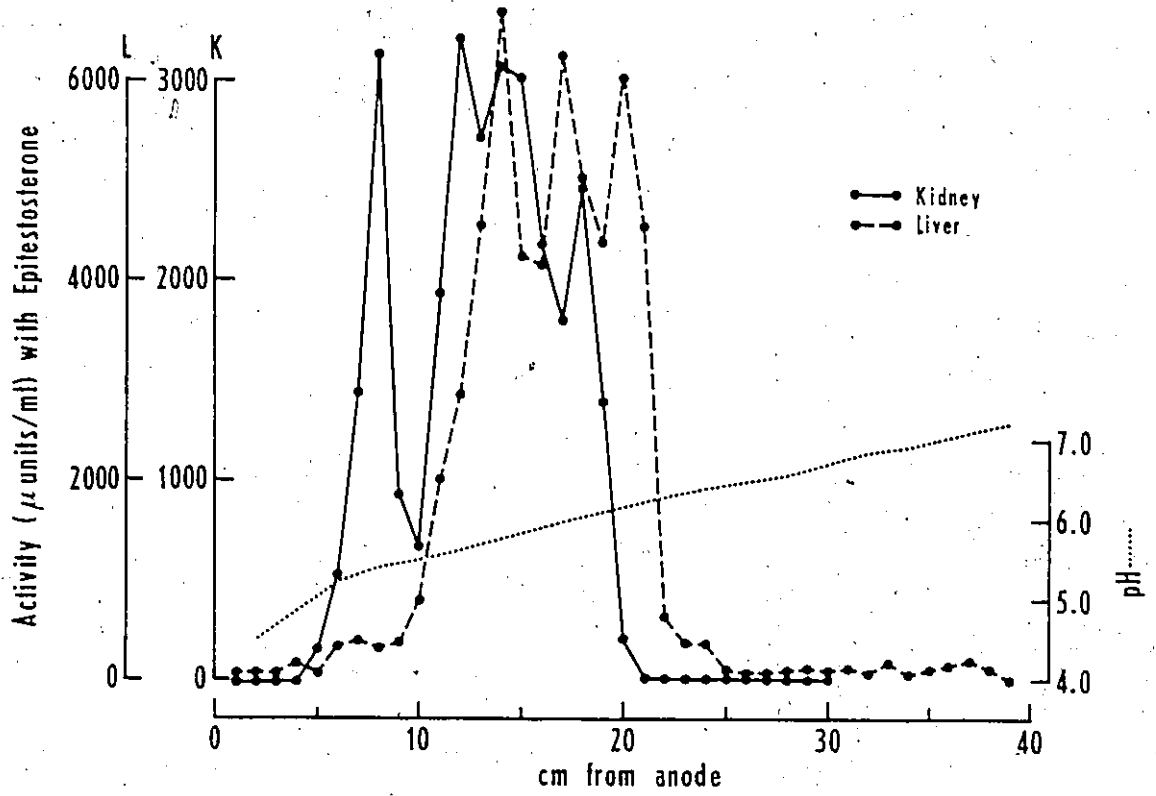
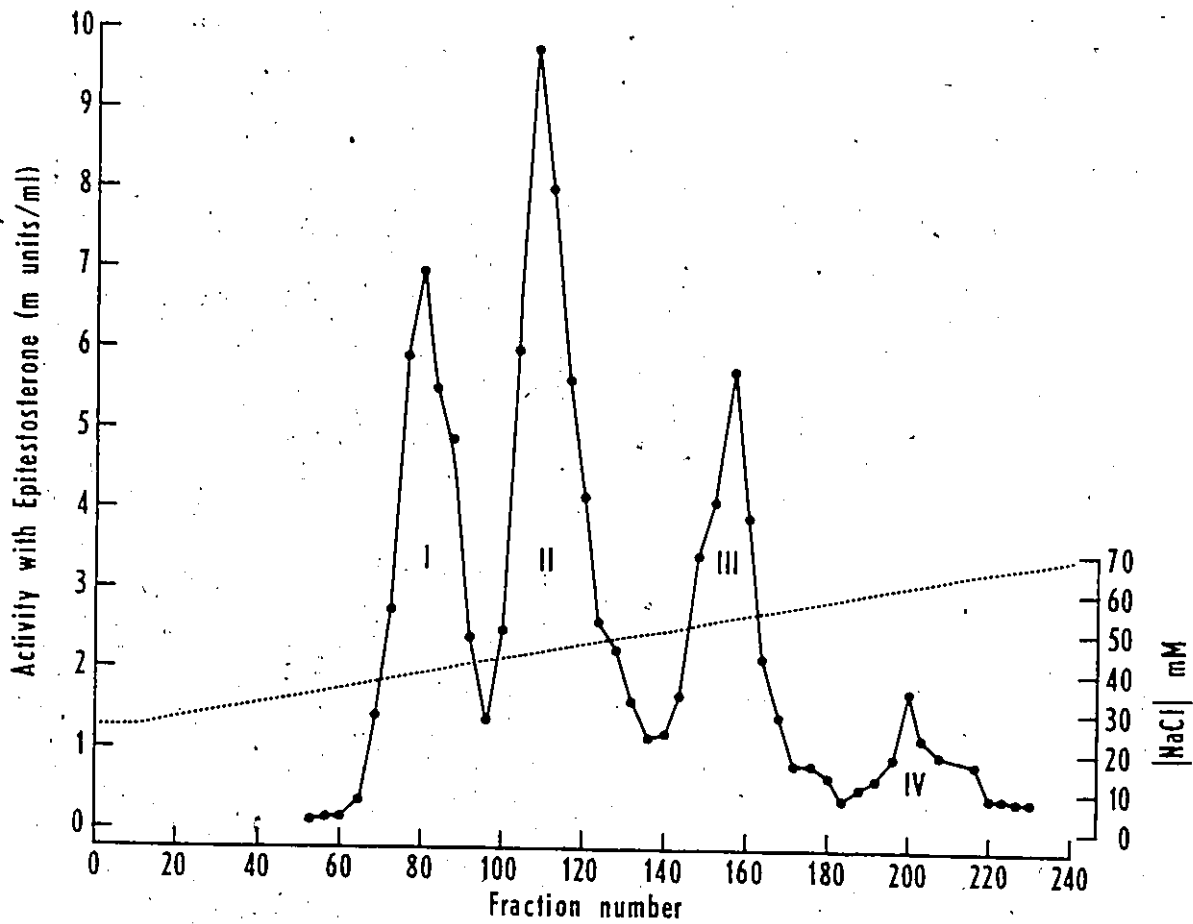


FIGURE 20

DEAE-cellulose Column Chromatography of Female Rabbit Liver  
17 $\alpha$ -Hydroxysteroid Dehydrogenases

The 17 $\alpha$ -hydroxysteroid dehydrogenase activity previously partially purified by calcium phosphate gel fractionation and Sephadex gel filtration was chromatographed on a DEAE-cellulose column (2.5 x 40 cm). The equilibrating buffer was 10 mM Tris-HCl at pH 8.0 containing 25 mM NaCl and 0.5 mM DTT. The 17 $\alpha$ -enzyme activities were eluted with a 2.4-litre linear gradient of 25-70 mM NaCl in the Tris-buffer. 5  $\mu$ l aliquots of the fractions collected (about 10 ml) were assayed with epitestosterone. The protein absorbance readings at 280 nm were not monitored.



enzymes. The activities with  $17\alpha$ -estradiol 3-glucuronide are shown in Figure 21a and with epitestosterone in Figure 21b. Liver IA enzyme displays a higher dehydrogenase activity with epitestosterone than with  $17\alpha$ -estradiol 3-glucuronide. This order is reversed with liver IB enzyme. This latter enzyme, high in  $17\alpha$ -estradiol 3-glucuronide dehydrogenase activity is similar to the kidney enzyme IB which exhibits the same preference and has essentially the same isoelectric point. Kidney enzyme IA (which represented enzyme form II) also co-focused with liver enzyme IA.

The purity of the liver IA and IB enzyme forms was monitored by polyacrylamide gel electrophoresis. Gels were stained for protein and for enzyme activity. Figure 22 shows the homogeneous nature of the two proteins. The single bands of protein also stain for enzyme activity with epitestosterone substrate. However in the control gels, liver enzyme IA also stains nonspecifically in the absence of its substrate as seen previously with the kidney IA or II enzyme forms. The control gel of the liver IB enzyme deliberately left longer in the staining reagent mixture did not show the nonspecific nothing dehydrogenase activity.

Polyacrylamide gel electrophoresis of the liver IA, IB enzymes in the presence of SDS also shows one protein band (Figure 22, gels g & h). The mobilities of the two proteins correspond to a molecular weight of 41,000 for liver IA and 42,500 for liver IB enzyme.

The purity of the kidney IB and II enzymes has been established previously (Chapter IV). The following studies are aimed at further comparisons of the two pure enzyme forms from each of the two tissues.

FIGURE 21

Comparison of Isoelectric Focusing of Rabbit Liver and Kidney  
DEAE-cellulose Column Peak I  $17\alpha$ -Hydroxysteroid Dehydrogenase Activities

Fractions from peak I of the DEAE-column chromatography of the kidney or liver enzymes were pooled, concentrated, dialyzed and further concentrated. 1.2 ml of the kidney enzyme containing 170 and 231 m units of enzyme activities toward epitestosterone and  $17\alpha$ -estradiol 3-glucuronide respectively were subjected to isoelectric focusing. The liver sample consisted of 1.5 ml containing 662 and 321 m units of activities toward epitestosterone and  $17\alpha$ -estradiol 3-glucuronide respectively. Focusing was carried in the Ultrodex gel system in 2.0% ampholyte for 17 hours at 600V and final focusing at 1000V for an additional 6½ hour. After focusing each fraction was scraped into separate test tubes and eluted with 2 ml of distilled water. For the assay for enzyme activity, the following aliquots were used:

Liver fraction: 1-2  $\mu$ l for epitestosterone and 2-4  $\mu$ l for  $17\alpha$ -estradiol 3-glucuronide.

Kidney fraction: 5  $\mu$ l for epitestosterone and 5-10  $\mu$ l for  $17\alpha$ -estradiol 3 glucuronide.

The pH gradient was determined by the use of a combination pH electrode.

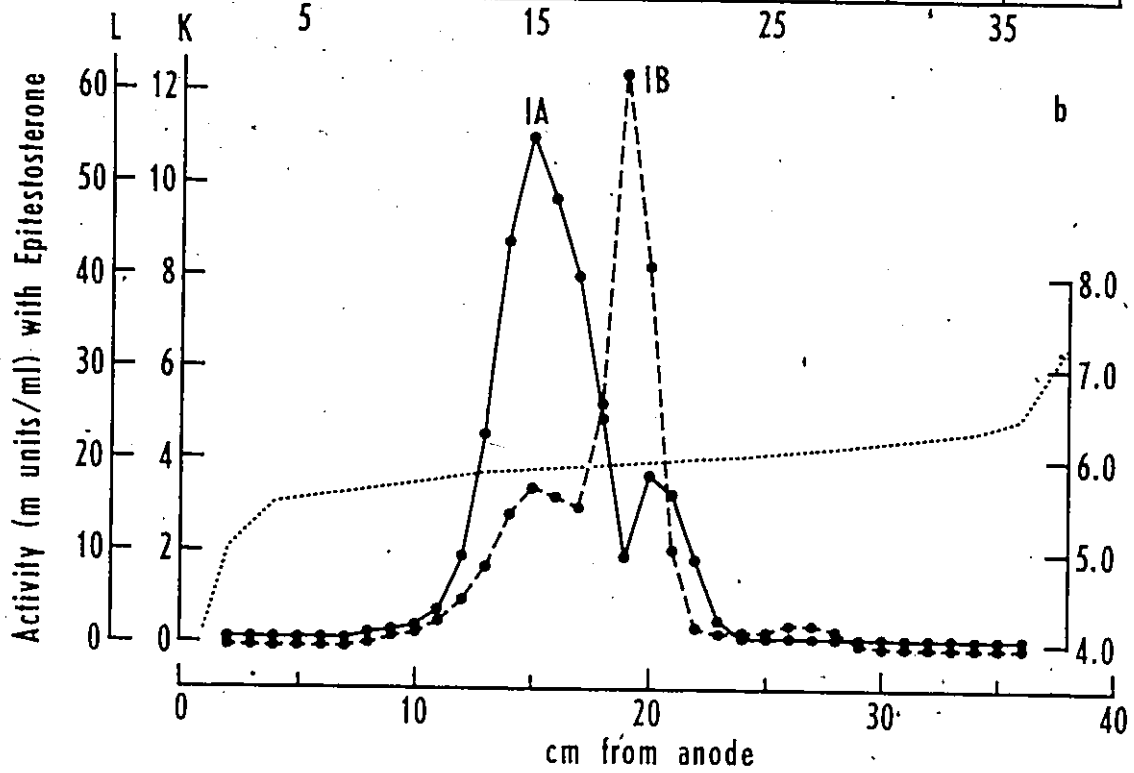
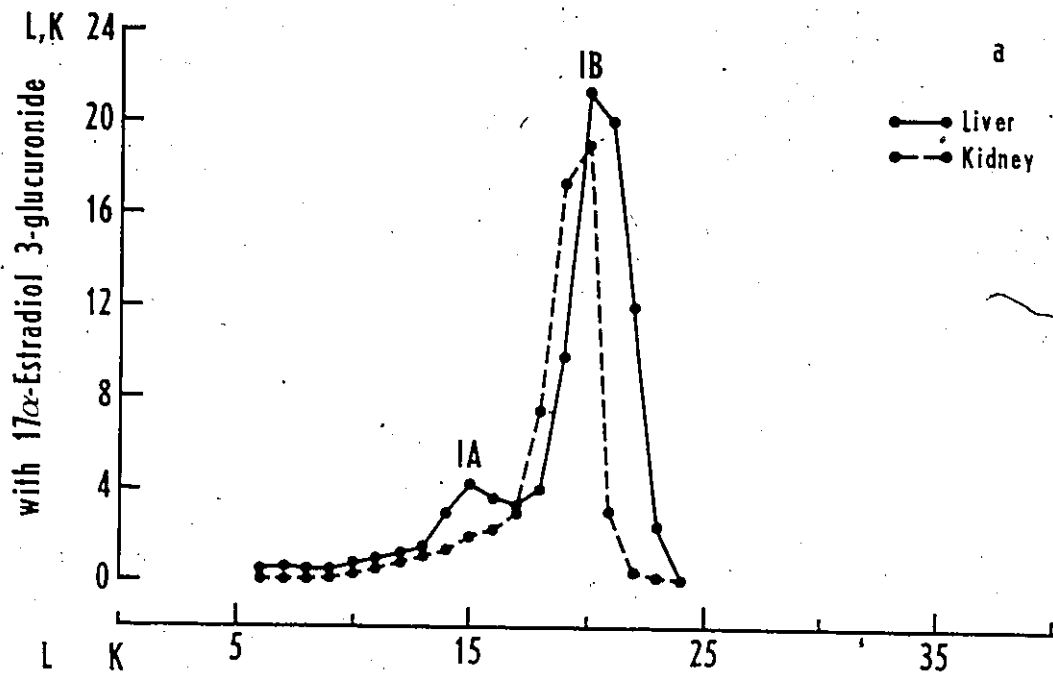


FIGURE 22

(SDS) Polyacrylamide Gel Electrophoresis of Rabbit Liver IA and IB  
17 $\alpha$ -HSD after Isoelectric Focusing

Electrophoresis in 10.5% polyacrylamide gel was carried out as described in the General Methods (p. 65). 10-20  $\mu$ g of protein fractions IA and IB were applied to gels a and d respectively. Gels b and e containing enzyme IA and IB respectively were stained with epitestosterone. Gels c and f are their respective control experiments. Gels g and h containing IA (10  $\mu$ g) and IB (2  $\mu$ g) proteins respectively were electrophoresed in the presence of SDS.

(-)



g

h

(+)

a

b

c

d

e

f

4. Substrate specificities of the purified enzyme forms of rabbit liver and kidney

a) Oxidative reactions

The relative activities of the kidney 3(17) $\alpha$ -HSDs toward various substrates have been shown in Table 7 and part of these are reproduced in Table 9 to enable comparison with the two corresponding liver enzyme forms. Like the kidney enzymes, the purified liver enzymes also exhibit higher oxidative rates of the hydroxyl group at C-3 than at C-17, androsterone also being the better substrate among the two 5 $\alpha$ -androstane steroids. With estrogenic substrates, kidney enzyme II and liver enzyme IA are very similar in having a low 17 $\alpha$ -estradiol 3-glucuronide dehydrogenase activity in contrast with the kidney and liver IB enzymes which have a high activity toward the 3-glucuronide derivative of 17 $\alpha$ -estradiol.

We have tested a series of steroids as possible inhibitors to the oxidation of epitestosterone catalyzed by the purified enzyme forms of the liver and kidney. The inhibition by various non radioactive C<sub>18</sub>-, C<sub>19</sub>- and C<sub>21</sub>-steroids are presented in Tables 10a, 10b and 10c.

All four epitestosterone dehydrogenase activities are weakly inhibited by 17 $\alpha$ -estradiol present at five times the concentration of the epitestosterone substrate (1  $\mu$ M) (Table 10a). 17 $\alpha$ -Estradiol-3-acetate consistently shows a higher inhibition than 17 $\alpha$ -estradiol with all the four enzyme forms especially with kidney and liver IB enzymes. However, with 17 $\alpha$ -estradiol-3-trimethylacetate and 6-keto-17 $\alpha$ -estradiol the inhibitory effects of the two steroids vary among the different enzyme forms. In general they are weak inhibitors. Both 17-epiestriol and 16,17-epiestriol also inhibit the oxidation of epitestosterone poorly.

TABLE 9

Oxidation of Various Steroids by the Purified Rabbit Kidney and Liver  
3(17) $\alpha$ -Hydroxysteroid Dehydrogenases

		Activity Relative to 17 $\alpha$ -Estradiol			
		Oxidation at C-17	Oxidation at C-3	Oxidation at C-3	Oxidation at C-3
Substrate:	17 $\alpha$ -Estradiol	17 $\alpha$ -Estradiol 3-glucuronide	Epitestosterone	Androsterone	5 $\alpha$ -Androstan-3 $\alpha$ ,17 $\beta$ -diol
Enzyme					
Kidney	I	33	28	120	72
	II	4	16	71	65
Liver	IA	2	83	458	156
	IB	41	32	103	49

The assay conditions were as described in the General Methods (p. 56).

TABLE 10a

Inhibition of Oxidation of Epitestosterone by Various Estrogens

Compound	Enzyme -	% Inhibition			
		K IB	K II	L IA	L IB
17 $\alpha$ -Estradiol		10	8	9	5
17 $\alpha$ -Estradiol-3-acetate		28	16	25	30
17 $\alpha$ -Estradiol-3-trimethylacetate		6	14	25	10
6-Keto-17 $\alpha$ -Estradiol		15	9	18	17
17-Epiestriol		7	10	NA	4
16,17-Epiestriol		10	13	11	6

30  $\mu$ l of methanol containing 15 nmoles of nonradioactive steroids were dispensed into separate test tubes and the contents were dried down under nitrogen. The nonradioactive steroids were then re-dissolved in 30  $\mu$ l methanol containing 3 nmole of radioactive epitestosterone substrate. The reaction mixtures in a final volume of 3 ml each were made up accordingly in glycine buffer pH 9.5 containing 0.5  $\mu$ mole NADP<sup>+</sup> as described in the General Methods. The reactions were started by the addition of aliquots of the purified enzymes. The control experiment was carried out without any inhibitor present but including all other ingredients. A reaction blank was also done consisting of radioactive substrate and nucleotide but in the absence of enzyme or inhibitor. All the values given were the average of duplicate samples. NA = not available.

In contrast to the estrogens, androgens of the 5 $\alpha$ -androstane series with 3 $\alpha$ -hydroxyl or 17 $\alpha$ -hydroxyl functions strongly inhibit the oxidation of epitestosterone (Table 10b). For example, in the case of liver IB enzyme, 5 $\alpha$ -androstan-3 $\alpha$ -ol-17-one (androsterone) at a concentration equal to that of the epitestosterone substrate (1  $\mu$ M) inhibits the reaction by 97%. However, the different enzymes respond differently to the various 5 $\alpha$ -androstane inhibitors present at different concentrations. Generally, androsterone is more potent than the 5 $\alpha$ -androstan-3 $\alpha$ ,17 $\beta$ -diol, a result in keeping with the activity observed when these steroids were used as substrates (Tables 6 & 7). Although 5 $\alpha$ -androstan-17 $\alpha$ -ol-3-one is not available as a radioactive substrate, the strong inhibition by this steroid suggests that it could be as good a substrate as androsterone for all the enzymes especially liver IA enzyme. It is also evident that the 5 $\beta$ -androstane steroids are poor inhibitors and possibly poor substrates as previously shown with etiocholanolone (5 $\beta$ -androstan-3 $\alpha$ -ol-17-one) (Tables 6 & 7). Similarly, 5 $\alpha$ -androstan-3 $\beta$ ,17 $\beta$ -diol also shown to be utilized negligibly by the kidney enzymes is seen here to be a weak inhibitor with the liver enzymes. However, 4-androstene-3 $\beta$ ,17 $\alpha$ -diol is a good inhibitor.

The inhibitory patterns obtained with C<sub>21</sub>-steroids are shown in Table 10c. With 5 $\alpha$ -pregnane steroids strong inhibition is exerted by 5 $\alpha$ -pregnan-3 $\alpha$ -ol-20-one and 5 $\alpha$ -pregnan-3 $\alpha$ ,20 $\beta$ -diol with all the four enzymes. However, only with liver IA enzyme is the oxidation of epitestosterone significantly inhibited by 5 $\alpha$ -pregnan-20 $\alpha$ -ol-3-one. Among the weak inhibitors are 5 $\alpha$ -pregnan-20 $\beta$ -ol-3-one, 5 $\alpha$ -pregnan-3 $\alpha$ ,20 $\alpha$ -diol, and the 5 $\beta$ -pregnane steroids (Table 10c). One apparent difference between the inhibitory actions of C<sub>19</sub> and C<sub>21</sub>-steroids is the effect of the presence of double bonds in these structures. While the 4-androstene-3 $\beta$ ,

TABLE 10b

Inhibition of Oxidation of Epitestosterone by Various C<sub>19</sub>-steroids

Compound	[conc.]	% Inhibition			
		Enzyme			
		KIB	KII,	LIA	LIB
5 $\alpha$ -Androstan-3 $\alpha$ -ol-17-one (Androsterone)	1 $\mu$ M	22	19	24	97
	5 $\mu$ M	61	52	62	97
5 $\alpha$ -Androstan-17 $\alpha$ -ol-3-one	1 $\mu$ M	--	--	44	34
	5 $\mu$ M	62	61	89	73
5 $\alpha$ -Androstan-3 $\alpha$ ,17 $\beta$ -diol	1 $\mu$ M	--	--	9	39
	5 $\mu$ M	28	29	36	48
5 $\alpha$ -Androstan-3 $\beta$ ,17 $\beta$ -diol	10 $\mu$ M	--	--	5	8
5 $\beta$ -Androstan-3 $\alpha$ -ol-17-one (Etiocholanolone)	5 $\mu$ M	9	28	8	18
5 $\beta$ -Androstan-3 $\alpha$ ,17 $\beta$ -diol	5 $\mu$ M	2	6	--	3
	10 $\mu$ M	--	--	7	--
4-Androstene-3 $\beta$ ,17 $\alpha$ -diol	1 $\mu$ M	--	--	17	27
	5 $\mu$ M	52	47	38	59

The assay conditions were as described in Table 10a.

TABLE 10c

Inhibition of Oxidation of Epitestosterone by Various C<sub>21</sub>-steroids

Compound	% Inhibition			
	KIB	KII	LIA	LIB
5 $\alpha$ -Pregnan-3 $\alpha$ -ol-20-one	66	51	72	83
5 $\alpha$ -Pregnan-20 $\alpha$ -ol-3-one	8	6	48	12
5 $\alpha$ -Pregnan-20 $\beta$ -ol-3-one	14	7	11	13
5 $\alpha$ -Pregnan-3 $\alpha$ ,20 $\alpha$ -diol	0	10	22	12
5 $\alpha$ -Pregnan-3 $\alpha$ ,20 $\beta$ -diol	42	30	43	59
5 $\beta$ -Pregnan-3 $\alpha$ -ol-20-one	22	28	23	18
5 $\beta$ -Pregnan-20 $\beta$ -ol-3-one	14	19	18	--
5 $\beta$ -Pregnan-3 $\alpha$ ,20 $\alpha$ -diol (Pregnanediol)	17	17	10	13
5 $\beta$ -Pregnan-3 $\alpha$ ,20 $\beta$ -diol	5	19	9	24
4-Pregnen-17 $\alpha$ -ol-3,20-dione (17 $\alpha$ -hydroxyprogesterone)	5	3	1	30
5-Pregnen-3 $\beta$ ,20 $\alpha$ -diol	14	16	9	30
5-Pregnen-3 $\beta$ ,20 $\beta$ -diol	13	15	2	--

The assay conditions were as described in Table 10a.

17 $\alpha$ -diol is a good inhibitor (Table 10b), both  $\Delta^4$ - and  $\Delta^5$ -pregnene steroids tested are generally weak inhibitors except with liver IB enzyme where 17 $\alpha$ -hydroxyprogesterone and 5-pregnen-3 $\beta$ ,20 $\alpha$ -diol showed an appreciable inhibition.

b) Reductive reactions

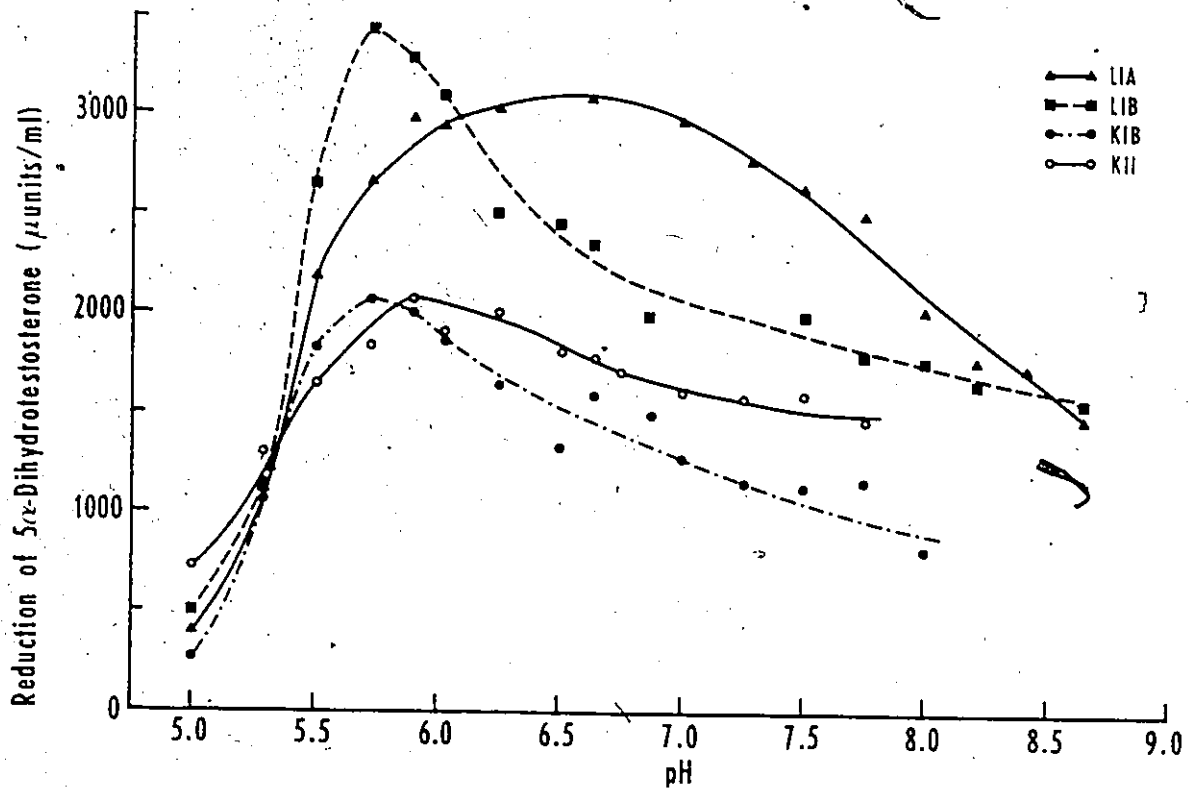
Since dehydrogenase reactions are usually reversible we decided to study the reduction of various ketosteroids in the presence of NADPH. In many cases, the pH optimum of the reductive reaction is lower than the oxidative reaction. For example, the reduction of estrone by the human placental 17 $\beta$ -estradiol dehydrogenase was found to be optimal at pH 6.2 whereas the pH optimum of the oxidative reaction was near 10 (Langer & Engel, 1958). Similarly the human ovarian 17 $\beta$ -estradiol dehydrogenase has pH optima of 6.9 and 8.1 for the reductive and oxidative reactions respectively (Pittaway, et al, 1977). Murono and Payne (1976) reported a pH optimum of 5.6 for the reduction of androstenedione catalyzed by rat testicular 17 $\beta$ -HSD. The rates of the forward and reverse reactions catalyzed by 3 $\alpha$ -HSD of Pseudomonas testosteroni are also strongly pH-dependent (Talalay & Marcus, 1956). The rate of oxidation of androsterone rises logarithmically with the pH from 6 to 10 whereas the rate of reduction of 5 $\alpha$ -androstan-3,17-dione increases as the pH is lowered from 7.5 to 5.8. The reduction of 5 $\alpha$ -dihydrotestosterone by mouse kidney 3 $\alpha$ -HSD was found to have a pH optimum between 6 and 8 (Mowszowicz & Bardin, 1974).

We have determined the pH optima of the four purified liver and kidney enzymes using 5 $\alpha$ -dihydrotestosterone as substrate (Figure 23). Both kidney II and liver IA enzymes show a broad pH profile with an optimum at pH 6.5 for the liver IA enzyme and pH 6.0 for the kidney II enzyme. In contrast, both the kidney and liver IB enzymes show a sharper pH profile with an optimum near pH 5.75.

FIGURE 23

pH Activity Curves of Reduction of 5 $\alpha$ -Dihydrotestosterone

The Tris-maleate buffer (0.05 M) was prepared by the method of Gomori (1955). The assay mixture consisted of 0.5  $\mu$ mole of NADPH, 2 ml of buffer at the indicated pH, 3 nmole of radioactive 5 $\alpha$ -dihydrotestosterone in 30  $\mu$ l methanol. The final volume was made up to 3 ml by the addition of 0.15 M KCl. The reactions were started by the addition of 3, 8, 10 and 20  $\mu$ l of LIA, LIB, KII and KIB enzymes respectively.



We have determined the reductive rates of various ketosteroids with the four purified enzyme forms employing 0.1 M Tris-maleate buffer at pH 5.85. Table 11 summarizes some of these results, expressed relative to the reduction of estrone. Essentially the same trend of substrate specificity as the oxidative direction is observed in the reductive direction. With estrogens, both the kidney and liver IB enzymes prefer the glucuronide derivative of estrone to the free estrone whereas in the case of the kidney II and liver IA enzymes, the preference is not as marked. The high specificity of these enzymes for the glucuronic acid moiety of the steroid apparently can not be replaced by a sulfate group as seen with estrone sulfate. When the reduction of the carbonyl function at C-17 of androstenedione is compared with the reduction of estrone glucuronide, the two steroids are either equally reduced by the kidney and liver IB enzymes or the reduction of the androgenic steroid is considerably greater than the estrogen as in the case of kidney II and liver IA enzymes. This is especially obvious with the liver IA enzyme. The liver IA enzyme is also highly specific toward the reduction of the carbonyl group of androsterone. Even greater rates of reduction of carbonyl function at C-3 of 5 $\alpha$ -dihydrotestosterone are observed among the four enzymes. Apparently, the presence of a double-bond in ring-A of androstenedione impedes the reduction of the carbonyl group at C-3, since no other radioactive metabolite other than epitestosterone is detected. This is supported by the observation that when either radioactive epitestosterone or testosterone is used as a substrate under the reductive conditions and in the presence of excess enzymes, no distinct metabolite is detectable.

The reduction of androstenedione at C-17 is strongly inhibited by the addition of nonradioactive androstenedione or 5 $\alpha$ -androstan-17 $\alpha$ -ol-3-one, at

TABLE II

Reduction of Various Steroids by the Purified Forms of Kidney and Liver  
3(17) $\alpha$ -Hydroxysteroid Dehydrogenases

		Activity Relative to Estrone						
		Reduction at C-17			Reduction at C-3			
Substrate:	Enzyme	Estrone 3-sulfate	Estrone 3-glucuronide	Androstenedione	Androsterone*	5 $\alpha$ -Dihydrotestosterone		
Kidney	IB	1	9	48	47	36	128	
	II	1	5	7	17	14	74	
Liver	IA	1	5	2	57	117	134	
	IB	1	5	23	23	20	60	

\*The product was presumed to be 5 $\alpha$ -androstan-3 $\alpha$ ,17 $\alpha$ -diol. This standard compound was not available but the radioactivity was found at a spot more polar than 5 $\alpha$ -androstan-3 $\beta$ ,17 $\beta$ -diol which in turn trailed the corresponding 3 $\alpha$ ,17 $\beta$ -diol standard in the benzene:acetone (4:1, v/v) system.

equimolar or higher concentrations (Table 12). It is possible that these saturated steroids are good substrates.

We have also examined the ability of the purified 3(17) $\alpha$ -HSDs of rabbit kidney and liver to reduce the carbonyl function at C-20 of radioactive pregnenolone. This was prompted by the observations of the ability of human placental 17 $\beta$ -estradiol dehydrogenase to catalyze the reduction of progesterone at C-20 (Purdy et al, 1964) or the ability of cortisone reductase (20 $\beta$ -HSD) of Streptomyces hydrogenans to act as a 3 $\alpha$ -HSD (Gibb & Jeffrey, 1973). Moreover some C<sub>21</sub>-steroids with 20 $\alpha$ - or 20 $\beta$ -hydroxyl groups were shown to be inhibitors to the oxidation of epitestosterone (Table 10c). Significant activity was observed with all the four enzymes, the ratios to estrone activity being 5 : 1 for kidney IB, 11 : 1 for liver IA and 2 : 1 for both kidney II and liver IB enzymes. Both 20 $\alpha$ - and 20 $\beta$ -metabolites were presumed to be the products of the reduction at C-20, but it was not established whether there was a preferential formation of either of the reduced metabolites. These metabolites run very closely with each other in either the benzene : acetone (4 : 1, v/v) system (see Chapter II, p.58) or in the benzene : ethyl acetate (7 : 3, v/v) system.

## 5. Kinetic studies of rabbit liver and kidney 3(17) $\alpha$ -hydroxysteroid dehydrogenases

### a) Effect of substrate concentration

Estimations of the Michaelis constants ( $K_m$ ) and maximum velocities ( $V_{max}$ ) of the purified enzymes for the substrates epitestosterone and androsterone are presented in Table 13. The  $K_m$  and  $V_{max}$  values were obtained by Lineweaver-Burk double-reciprocal plots, four of which together with the Michaelis-Menten plots are given in Figure 24ab and Figure 25ab. The Michaelis-Menten plot for liver

TABLE 12

Inhibition of the Reduction of Androstenedione by 5 $\alpha$ -Androstane Steroids

Inhibitor Concentration	Enzyme -	% Inhibition	
		LIA	LIB
Androstenedione	1 $\mu$ M	55	90
	5 $\mu$ M	98	98
	10 $\mu$ M	100	99
5 $\alpha$ -Androstan-17 $\alpha$ -ol-3-one	1 $\mu$ M	79	74
	5 $\mu$ M	100	95

30  $\mu$ l of methanol containing 3, 15 or 30 nmoles of nonradioactive steroids were dispensed into separate test tubes and the contents dried down under nitrogen. The nonradioactive steroids were then re-dissolved in 30  $\mu$ l methanol containing 3 nmole of radioactive androstenedione substrate. The reaction mixtures in a final volume of 3 ml each were made up accordingly in 0.1 M Tris-maleate buffer pH 5.85 containing 0.5  $\mu$ mole NADPH. The reactions were started by the addition of aliquots of the purified enzymes. The control experiment consisted of all components except the inhibitors. A reaction blank containing radioactive substrate and nucleotide but no enzyme or inhibitor was also incubated.

TABLE 13

Kinetic Parameters of the Purified Multiple Forms of Rabbit Liver and Kidney  
3(17) $\alpha$ -Hydroxysteroid Dehydrogenases

Enzyme	Epitestosterone		Androsterone	
	Km ( $\mu$ M)	Vmax (nmole/min per mg protein)	Km ( $\mu$ M)	Vmax (nmole/min per mg protein)
Kidney IB	2.91 $\pm$ 0.15	12.0 $\pm$ 1.3	2.1 $\pm$ 0.2	24.5 $\pm$ 4.2
	0.61 $\pm$ 0.05	3.8 $\pm$ 0.0	4.0 $\pm$ 1.3	33.7 $\pm$ 8.7
Liver IA	0.53 $\pm$ 0.20	11.3 $\pm$ 0.6	4.7 $\pm$ 0.3	400 $\pm$ 110
	2.34 $\pm$ 0.36	26.1 $\pm$ 3.1	1.2 $\pm$ 0.4	45.7 $\pm$ 15.0

The Km and Vmax values were obtained at two incubation time intervals, 15 minutes and 30 minutes. Prior to the addition of enzymes, the incubation mixtures were pre-incubated at 37°C for 10 minutes. The numbers presented were the average values  $\pm$  range.

FIGURE 24 ab

Lineweaver-Burk Plots for Kidney IB and Liver IA Epitestosterone  
Dehydrogenase Activities

Each point on the graph is an average of three incubations, but the value obtained from each incubation was considered in the linear regression analyses. The slopes ( $K_m/V_{max}$ ) and intercepts on the Y-axis ( $1/V_{max}$ ) obtained from the linear regression analyses were used to calculate the  $K_m$  and  $V_{max}$  values. The units of  $v$  and  $\frac{1}{v}$  are  $\text{nmole product} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$  and  $\text{min} \cdot \text{mg protein} \cdot \text{nmole}^{-1}$  respectively.

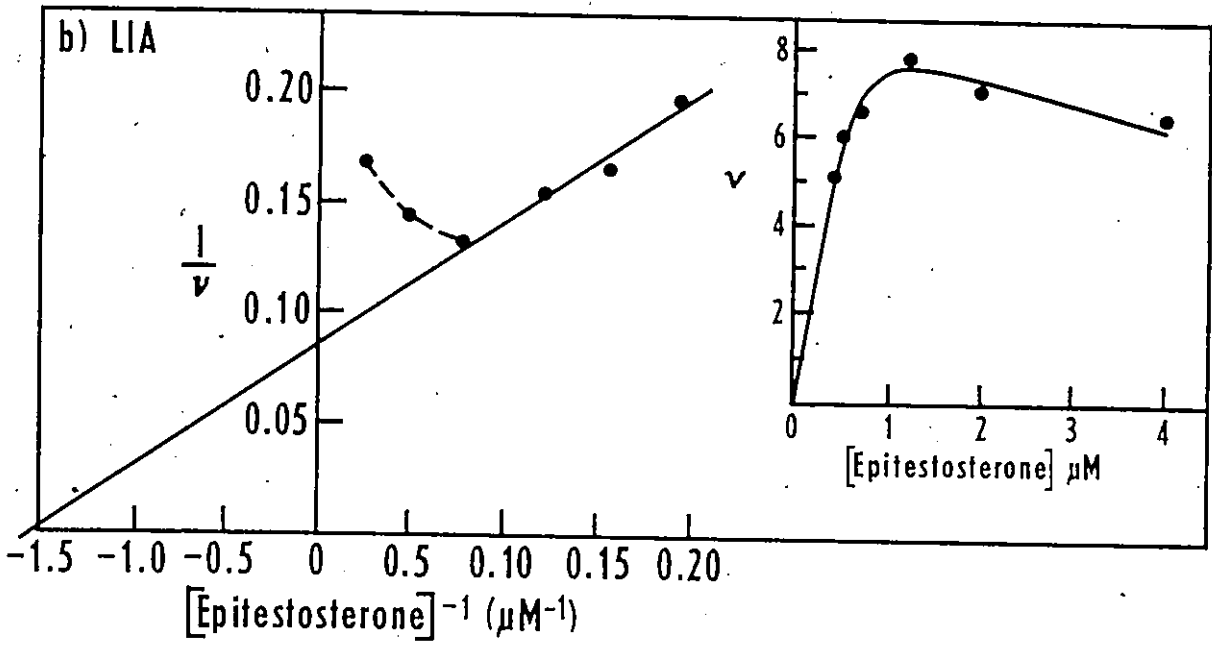
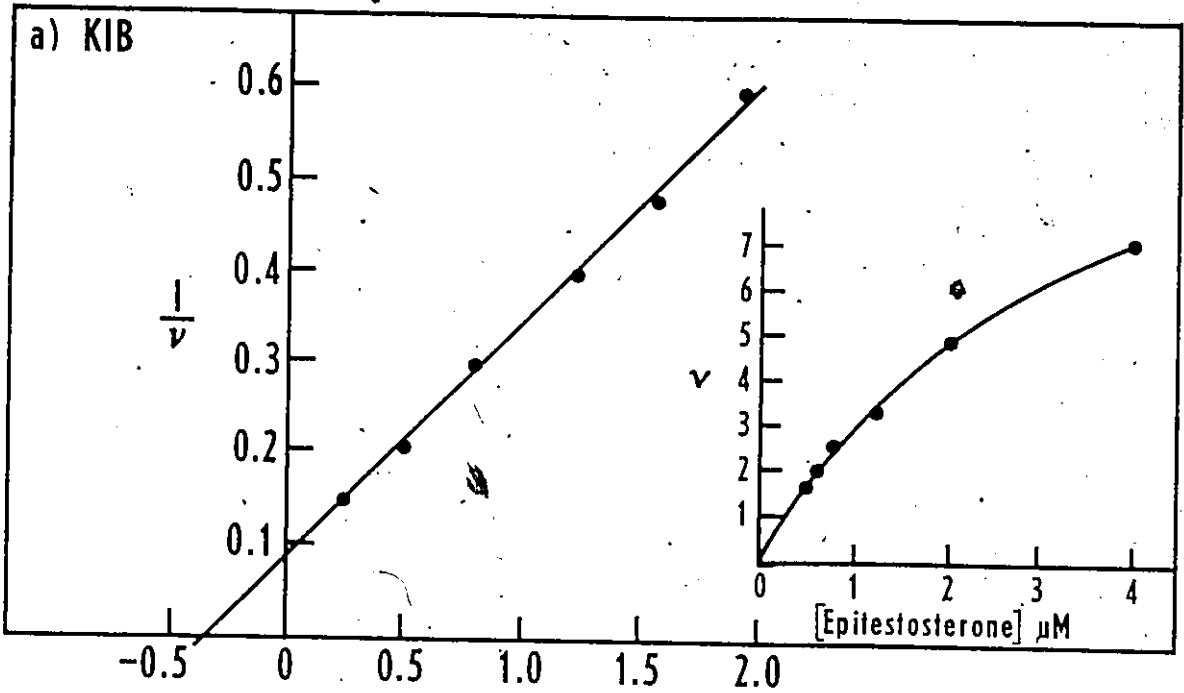
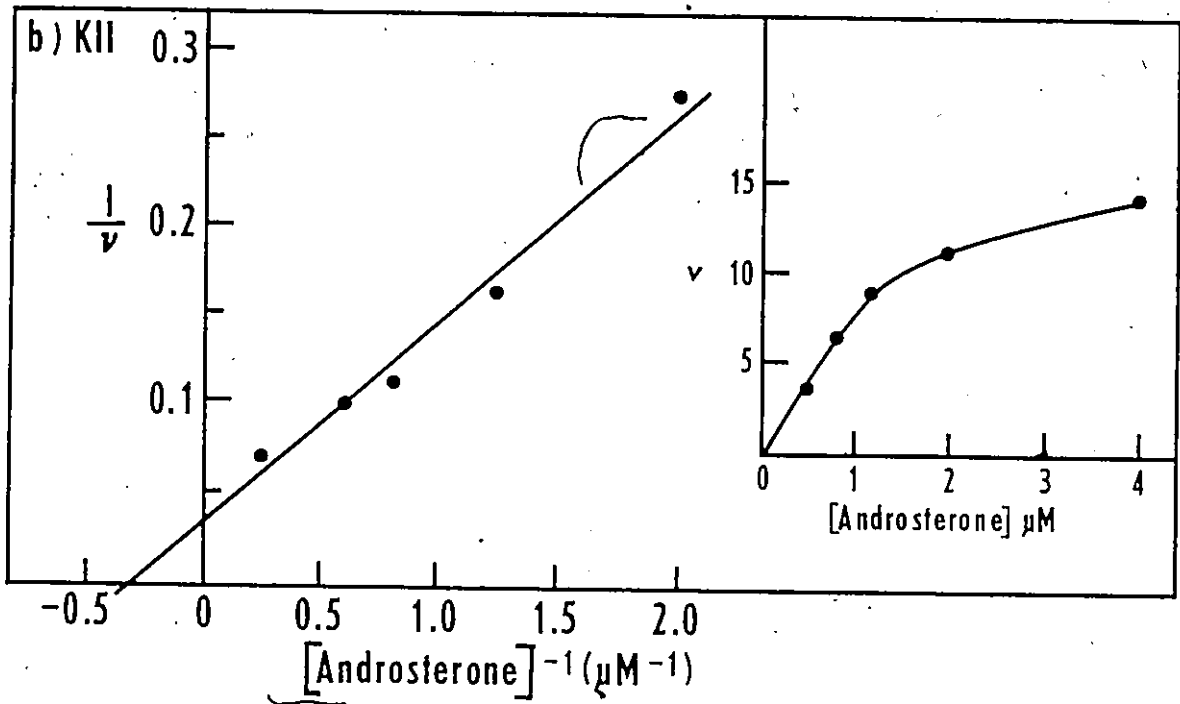
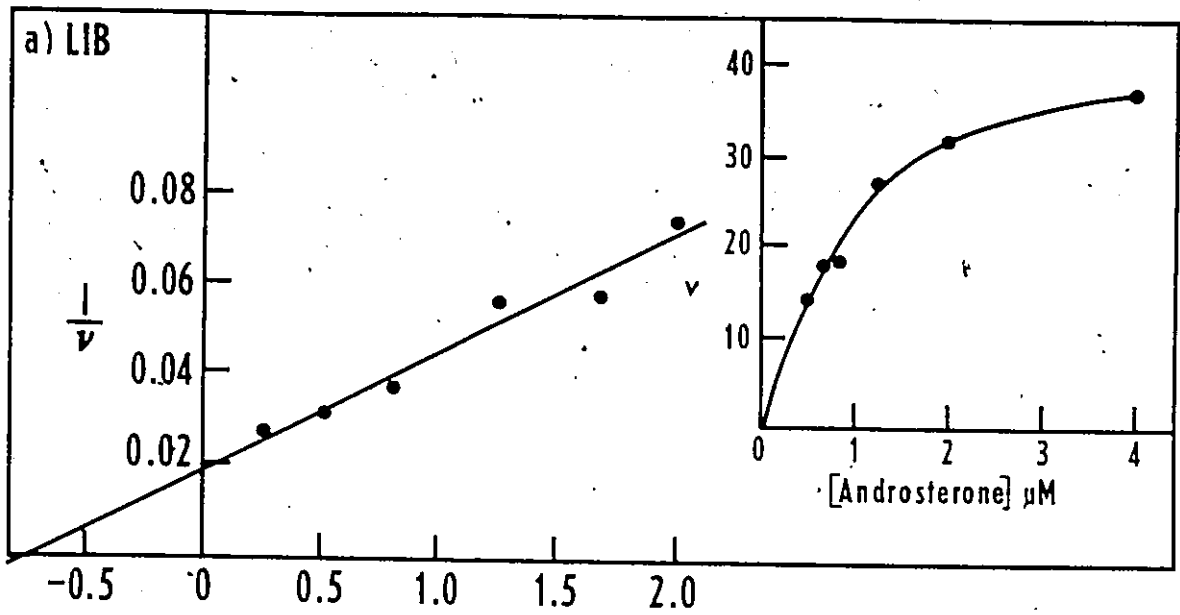


FIGURE 25 ab

Lineweaver-Burk Plots for Liver IB and Kidney II Androsterone  
Dehydrogenase Activities

Each point on the graph is an average of three incubations, but the value obtained from each incubation was considered in the linear regression analyses. The slopes ( $K_m/V_{max}$ ) and intercepts on the Y-axis ( $1/V_{max}$ ) obtained from the linear regression analyses were used to calculate the  $K_m$  and  $V_{max}$  values. The units of  $v$  and  $\frac{1}{v}$  are  $\text{nmole product}\cdot\text{min}^{-1}\cdot\text{mg protein}^{-1}$  and  $\text{min}\cdot\text{mg protein}\cdot\text{nmole}^{-1}$  respectively.



enzyme IA, when incubated with epitestosterone shows a characteristic substrate inhibition at steroid concentrations greater than 2  $\mu\text{M}$  (Figure 24b). With other enzyme forms, this inhibition was not apparent up to the concentration limit of 4  $\mu\text{M}$  tested.

Considering the  $K_m$  values of the various enzymes obtained with epitestosterone and androsterone, the kidney and liver IB enzymes appear to have very similar affinity for the two substrates examined (Table 13). Similar  $K_m$  values are also obtained for enzymes KII and LIA with epitestosterone but these are about 5-fold lower than those obtained with kidney and liver IB enzymes for the same substrate. In contrast, with androsterone, KII and LIA enzymes exhibit a 7-9 fold higher  $K_m$  values compared to those obtained with these enzymes for epitestosterone.

In most cases the  $V_{max}$  values differ considerably, especially the LIA androsterone dehydrogenase activity which is about 9-fold higher than the corresponding LIB androsterone dehydrogenase activity. In all instances, androsterone gives a higher  $V_{max}$  than epitestosterone. Overall, it appears that the better substrate, androsterone, has a high  $V_{max}$  rather than a low  $K_m$ .

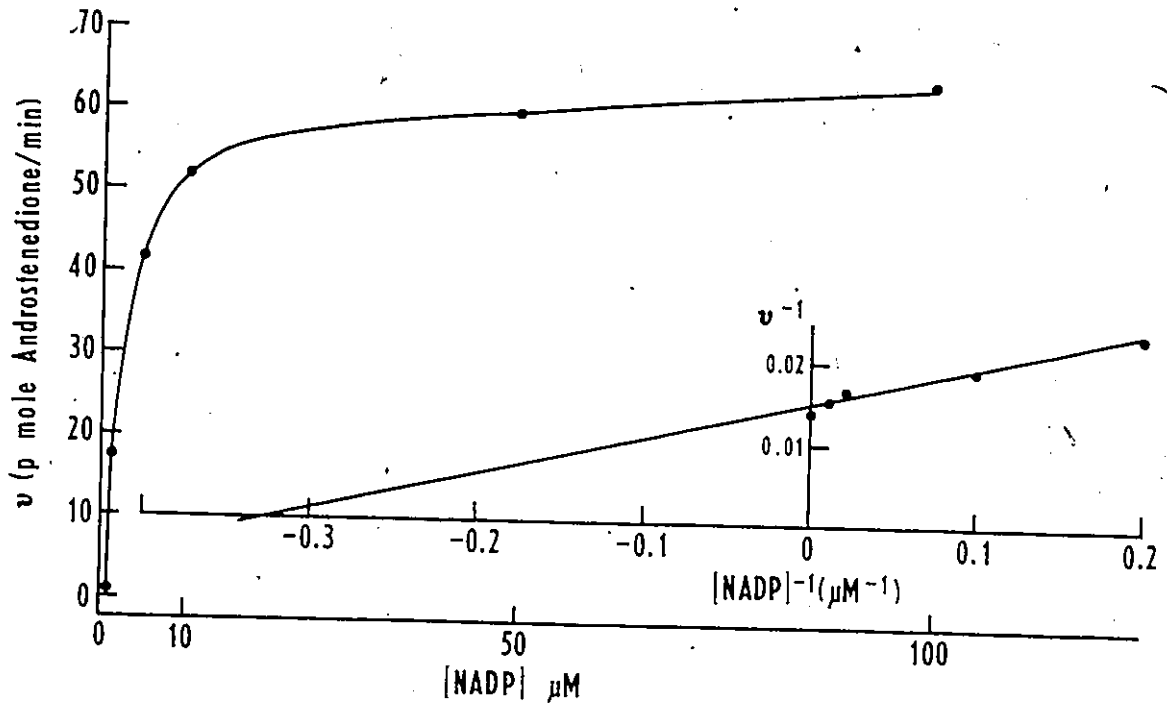
b) Effect of nucleotide concentration

The dependence of the purified liver IA epitestosterone dehydrogenase activity on  $\text{NADP}^+$  concentrations is shown in Figure 26. A  $K_m$  value of 3.1  $\mu\text{M}$  for  $\text{NADP}^+$  was obtained with epitestosterone as the second substrate. The effects of  $\text{NADP}^+$  concentration on the other three purified enzyme activities toward androsterone and epitestosterone were also very similar (not shown). The nucleotide saturation levels in all cases were low. The concentration of

FIGURE 26

Dependence of 17 $\alpha$ -Hydroxysteroid Dehydrogenase Activity of Liver IA  
on NADP<sup>+</sup>

The 17 $\alpha$ -hydroxysteroid dehydrogenase activity of liver IA enzyme toward epitestosterone (final concentration, 4  $\mu$ M) was assayed under varied NADP<sup>+</sup> concentrations. The assay conditions were as described in the General Methods (p.54). The unit of  $v^{-1}$  = min.pmole product.



nucleotide employed in the determination of the kinetic constants shown in Table 13 was in the order of 1 mM. This value far exceeded that required for enzyme saturation. Also in our routine assays we used 167  $\mu\text{M}$   $\text{NADP}^+$  which again exceeded the saturating conditions.

c) Inhibition of 17 $\alpha$ -hydroxysteroid dehydrogenase activities by androsterone

The inhibition of the oxidation of epitestosterone by androsterone is shown in Figures 27-30. The inhibition patterns of the Dixon plots (reciprocal of rate against inhibitor concentration) for liver IA enzyme (Figure 27a) liver IB enzyme (Figure 28a) and kidney II enzyme (Figure 29a) are all of the linear competitive type (Dixon, 1953). The inhibitor constants,  $K_i$ , are 0.4, 0.84 and 0.45  $\mu\text{M}$  respectively. The slopes of the  $[\text{S}]/v$  against [inhibitor] plots (Figures 27b, 28b and 29b) are essentially parallel in each case which is characteristic of a linear competitive type of inhibition (Cornish-Bowden, 1974). However, the inhibitory pattern with KIB enzyme (Figure 30ab) is less straightforward because at high inhibitor concentrations (at or above 2  $\mu\text{M}$ ) both the Dixon and the  $[\text{S}]/v$  versus  $[\text{I}]$  plots become non-linear (parabolic?). In addition, at low inhibitor concentrations the plots do not clearly indicate whether inhibition is of the competitive or mixed type.

6. Effects of sulfhydryl reagents and metal ions

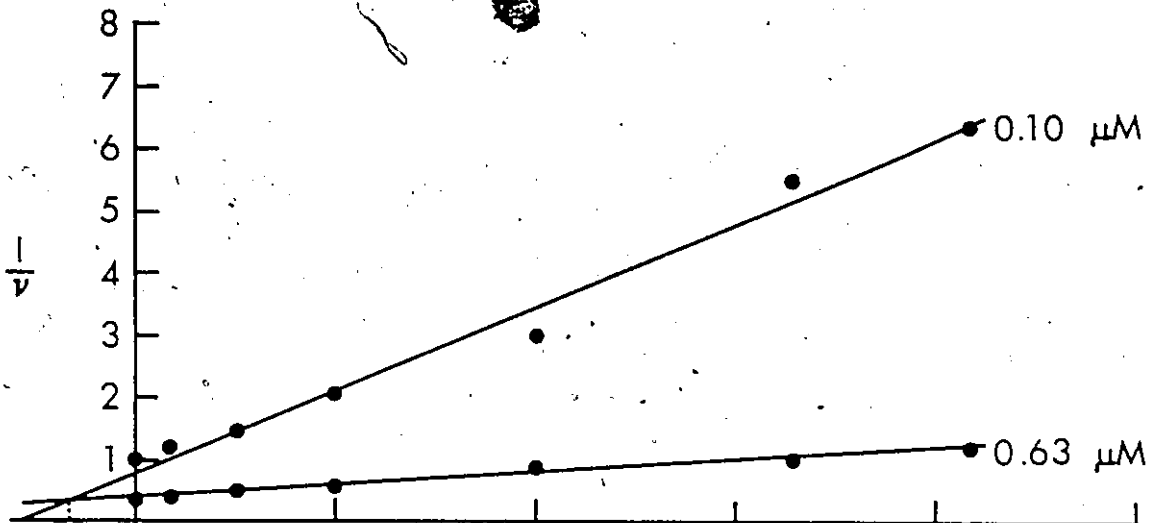
Table 14 shows the effects of two metal ions and two sulfhydryl reagents on the oxidation of epitestosterone catalyzed by the purified liver and kidney enzymes. Both of the metal ions tested are potent inhibitors abolishing greater than 48% of the enzyme activities even at 1  $\mu\text{M}$  concentration. Para-chloromercuribenzoate at 1  $\mu\text{M}$  concentration also inhibits greater than 70% of

FIGURE 27

Androsterone inhibition of the Oxidation of Epitestosterone by the Liver  
IA Enzyme

The initial velocities  $v$  were measured in the presence of various concentrations of inhibitor (androsterone), at two or three fixed substrate (epitestosterone) concentrations as indicated. The units are  $1/v = \text{min. mg protein. mmole}^{-1}$  and  $[S]/v = \text{min. mg protein. ml}^{-1}$ . The linear regression method was used to plot the lines. Each point on the graphs is an average of two readings but all experimental points were considered in the linear regression analyses.

a



b

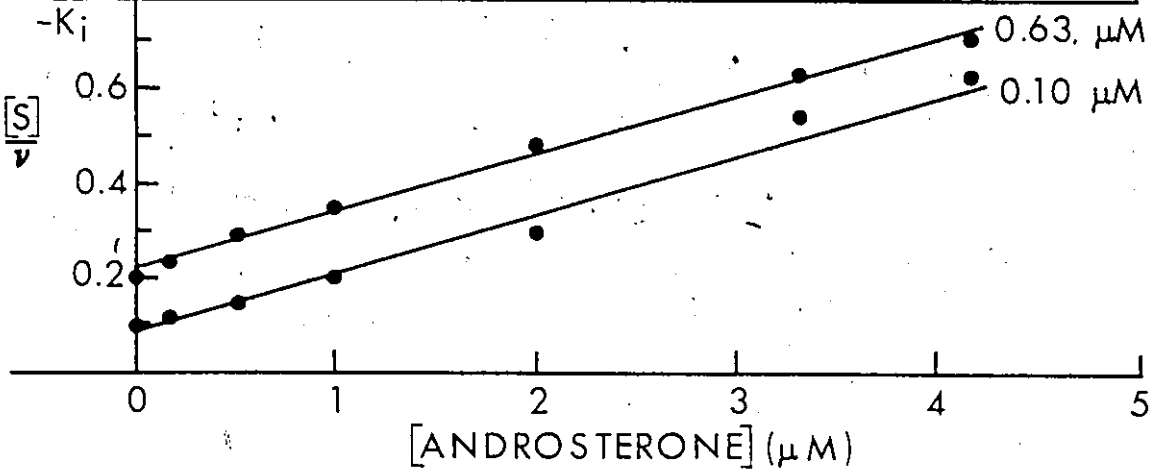


FIGURE 28

Androsterone inhibition of the Oxidation of Epitestosterone by the Liver  
IB Enzyme

See legend in Figure 27.

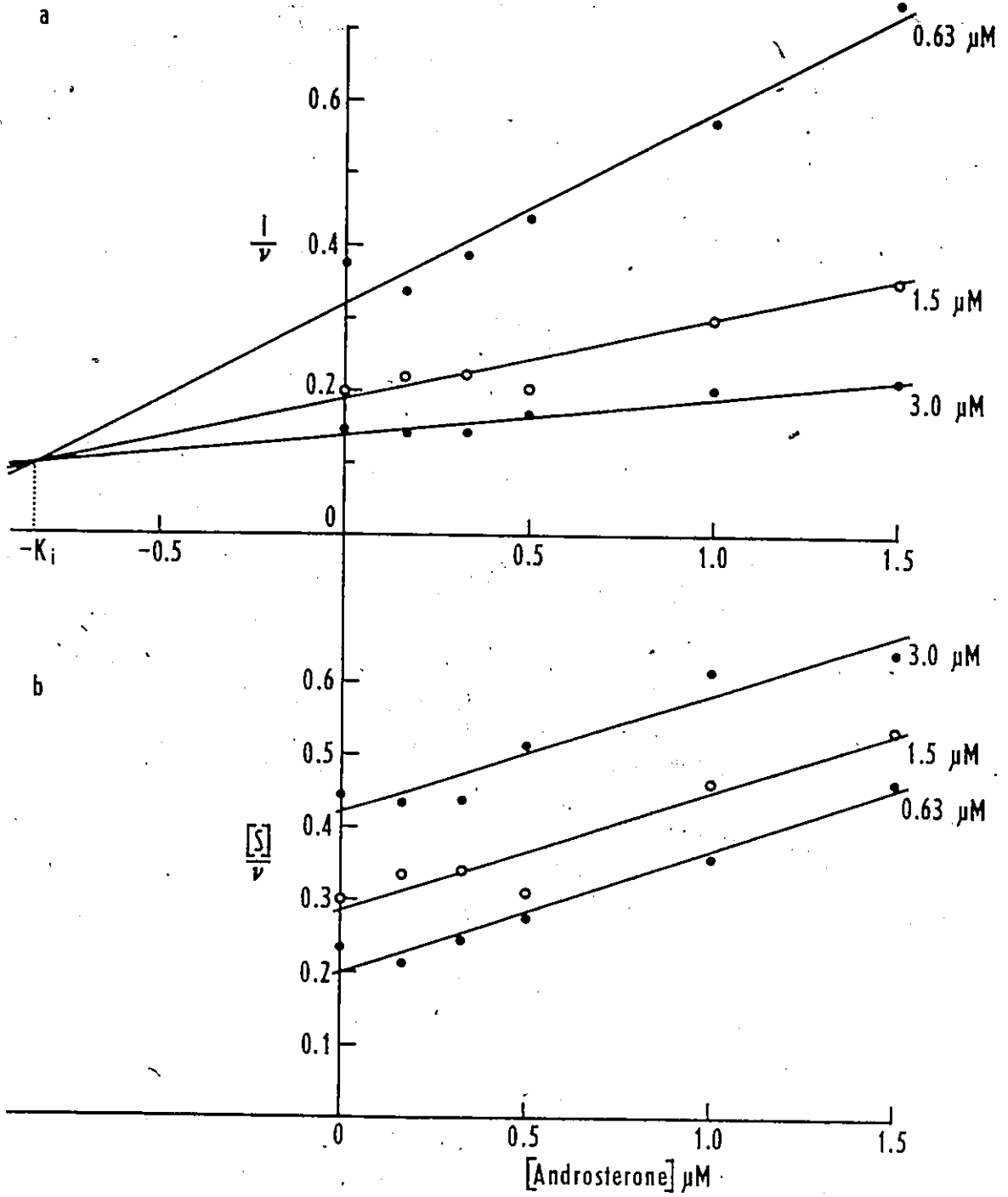


FIGURE 29

Androsterone inhibition of the Oxidation of Epitestosterone by the Kidney  
II Enzyme

See legend in Figure 27.

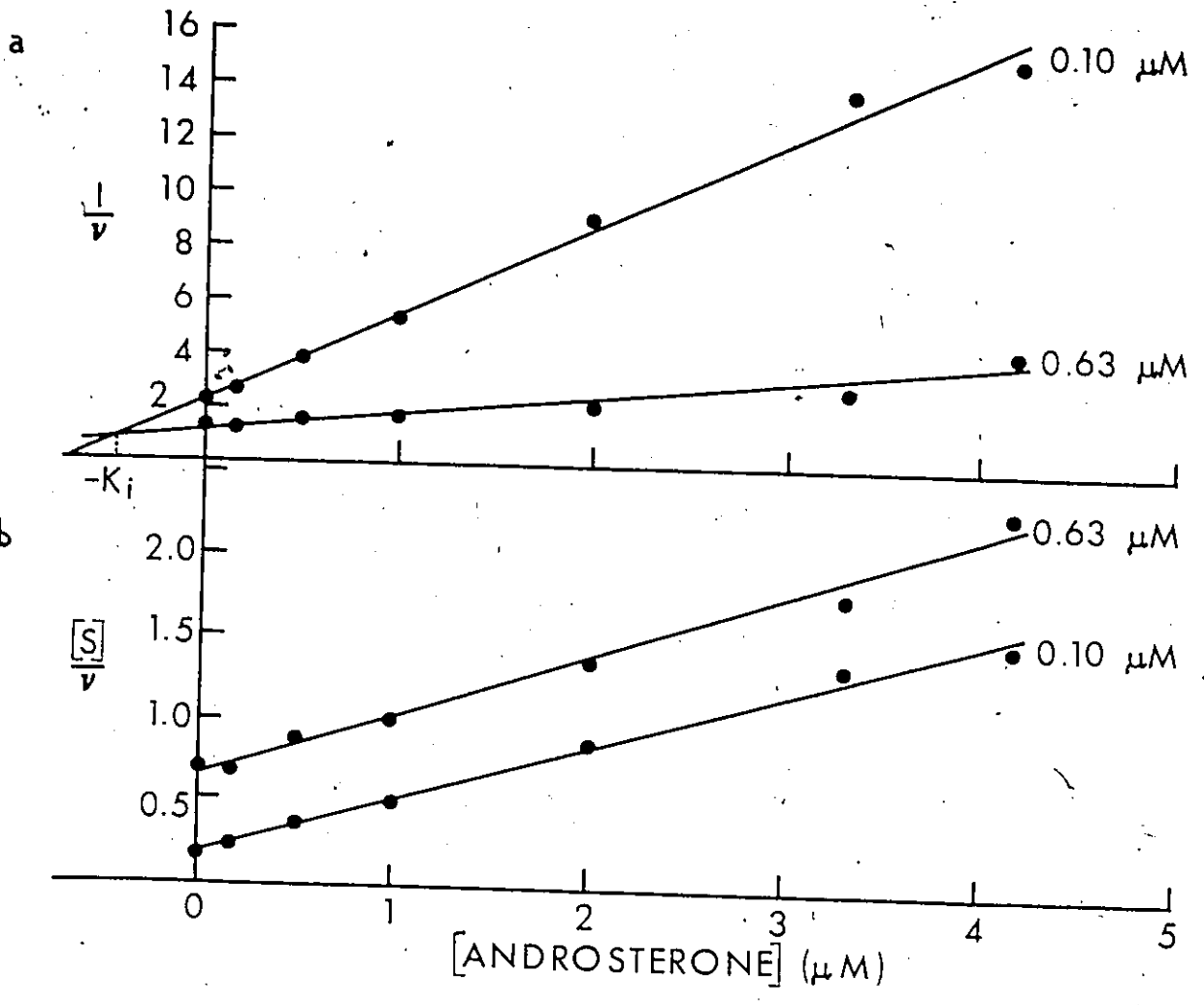


FIGURE 30

Androsterone inhibition of the Oxidation of Epitestosterone by the Kidney  
IB Enzyme

See legend in Figure 27.

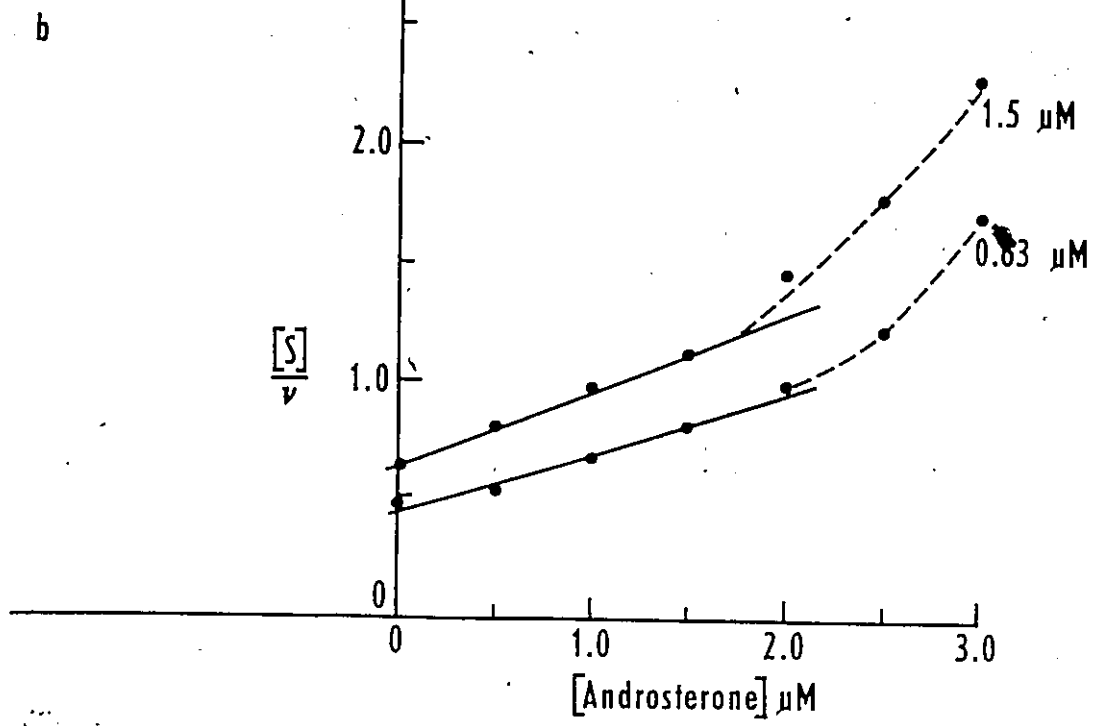
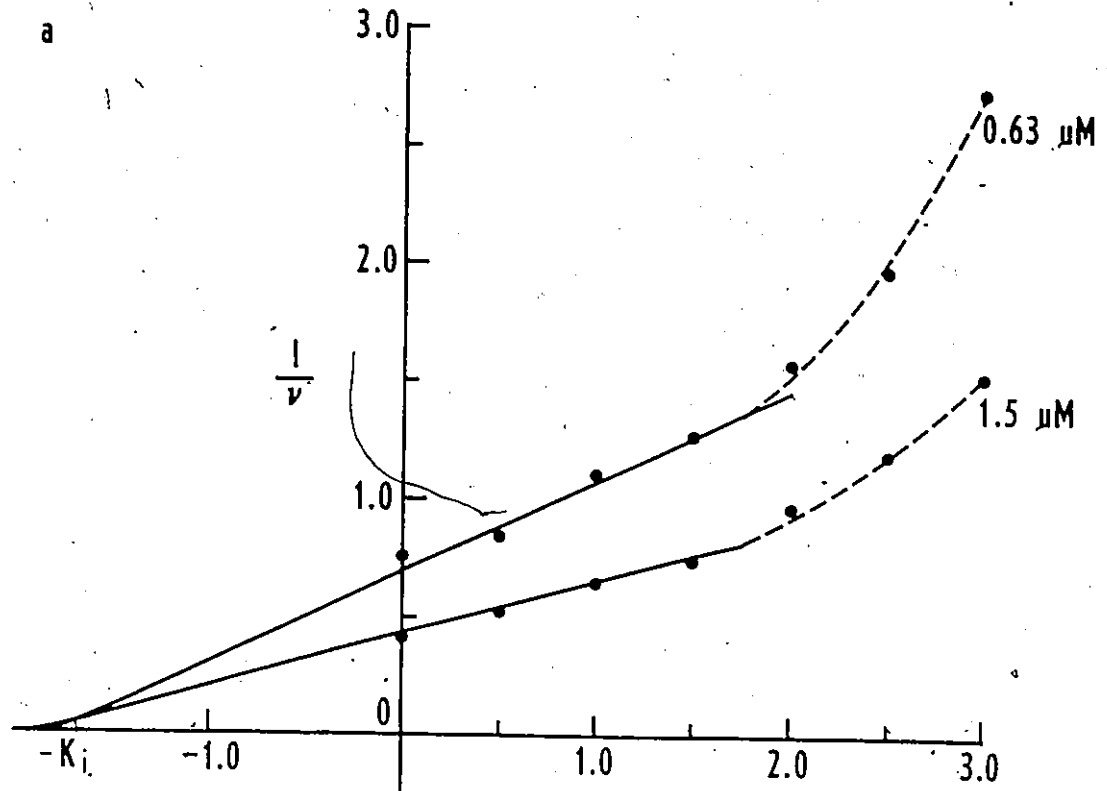


TABLE 14

Effects of Sulfhydryl Reagents and Metal Ions on Epitestosterone  
Dehydrogenase Activity

Compound	Final [conc.]	% Inhibition			
		Enzyme			
		KIB	KII	LIA	LIB
HgCl <sub>2</sub>	1 μM	65	74	84	80
	1 mM	100	100	87	88
CuCl <sub>2</sub>	1 μM	52	48	63	80
	1 mM	60	47	76	83
pCMB	1 μM	71	74	75	82
	1 mM	100	92	97	98
Iodoacetate	1 mM	0	9	4	8

The incubation mixtures were as described in the General Methods (p.56) except that the sulfhydryl reagents and the metal ions were added at the final concentration as indicated. The control experiments were carried out in the absence of the enzymes or in the absence of the sulfhydryl reagents and metal ions.

the enzyme activity. In contrast, another sulfhydryl inhibitor, iodoacetate even at a higher concentration shows very little or no inhibition.

7. Chemical modification of rabbit liver and kidney 3(17) $\alpha$ -hydroxysteroid dehydrogenases

The similarities of the physico-chemical properties of the purified 3(17) $\alpha$ -HSDs of the rabbit liver and kidney described so far suggested that these multiple forms might be closely related in structure. To unravel the possible similarities or differences in the primary structures of these proteins we employed a "finger-printing" method involving acetylation of these proteins with  $^{14}\text{C}$ -acetic anhydride. Upon proteolytic digestion, radioactive peptides were separated by high-voltage electrophoresis and the peptide patterns were visualized by autoradiography. It was necessary to use radioactive labeling because of the micro-quantity of proteins available. The yield of each pure kidney enzyme from eight kidneys (about 56 gm) was about 300  $\mu\text{g}$  for IB and 400  $\mu\text{g}$  for fraction II. Acetylation of amino groups, viz. the  $\epsilon$ -amino groups of lysine residues and the  $\alpha$ -amino group of the amino-terminus (if any) was selected as the method of choice because of the following reasons: a) lysine is one of the major constituents of proteins and is usually exposed on the "surface" of the molecule and therefore readily accessible to chemical reagents; b) acetylation of amino groups is rapid, mild and relatively group-specific. Acetic anhydride was used by Kaplan *et al* (1971) to determine the relative reactivities of the amino groups of elastase by a competitive labeling technique. We have adopted the trace-labeling procedure to obtain autoradiograms of the rabbit kidney and liver 3(17) $\alpha$ -HSD proteins. An acetylated ampholyte sample of pH 5-7 range was also obtained.

Figure 31 shows an autoradiogram of the four acetylated proteins and ampholyte, all digested by papain and electrophoresed at pH 6.5. Comparing the liver IA and IB peptide patterns, both the acidic and basic peptides are very similar. However, differences are also apparent especially among the basic peptides. For example, the basic peptide spots at 6 and 17 units from the origin are distinct to the liver IB protein. Similarly, the basic spots at position 16 and 18 are unique to the liver IA protein. While the kidney II protein reveal close resemblances (except for the acidic spot at position 21) to the liver proteins, the kidney IB protein behaves abnormally in that no discrete spots are discernible but instead smear throughout the length of the electrophoresis. Eluting the radioactivity off the paper and re-electrophoresis at the same pH did not improve the resolution. In fact, the radioactive smear is present in the peptide maps of the other three proteins but to a less extent. The smear is not due to ampholyte which is present in the final purification step of the proteins. The acetylated species of the ampholyte display a characteristic acidic behaviour at pH 6.5. The second dimensional electrophoresis of the ampholyte at pH 2.1 (Figure 32) confirms that no interaction with any of the dehydrogenase proteins is evident. Perhaps it should also be pointed out that an unfractionated ampholyte of pH 5-7 range was used in the acetylation and if there were any amphoteric species associated with the proteins they would be in the vicinity of pH 5.9-6.1 and of negligible amounts.

The second dimensional electrophoresis of the radioactive strips at pH 2.1 of the liver IA, IB and kidney II samples is shown in Figures 33, 34 and 35 respectively. All three proteins generate very similar peptide maps at this pH.

FIGURE 31

Autoradiogram of Papain Digests of  $^{14}\text{C}$ -acetylated Proteins of Rabbit Liver IA and IB, Kidney IB and II and a Sample of Carrier Ampholyte of pH Range 5-7

The papain digests of the  $^{14}\text{C}$ -acetylated proteins or amphoteric species of the ampholyte were subjected to high-voltage electrophoresis at 3000 V for 1 hour at pH 6.5. B and Y marked the positions of the midpoints of the migration of the blue dye marker (xylene cyanol FF) and yellow dye marker ( $\epsilon$ -DNP-L-amino-lysine) respectively.



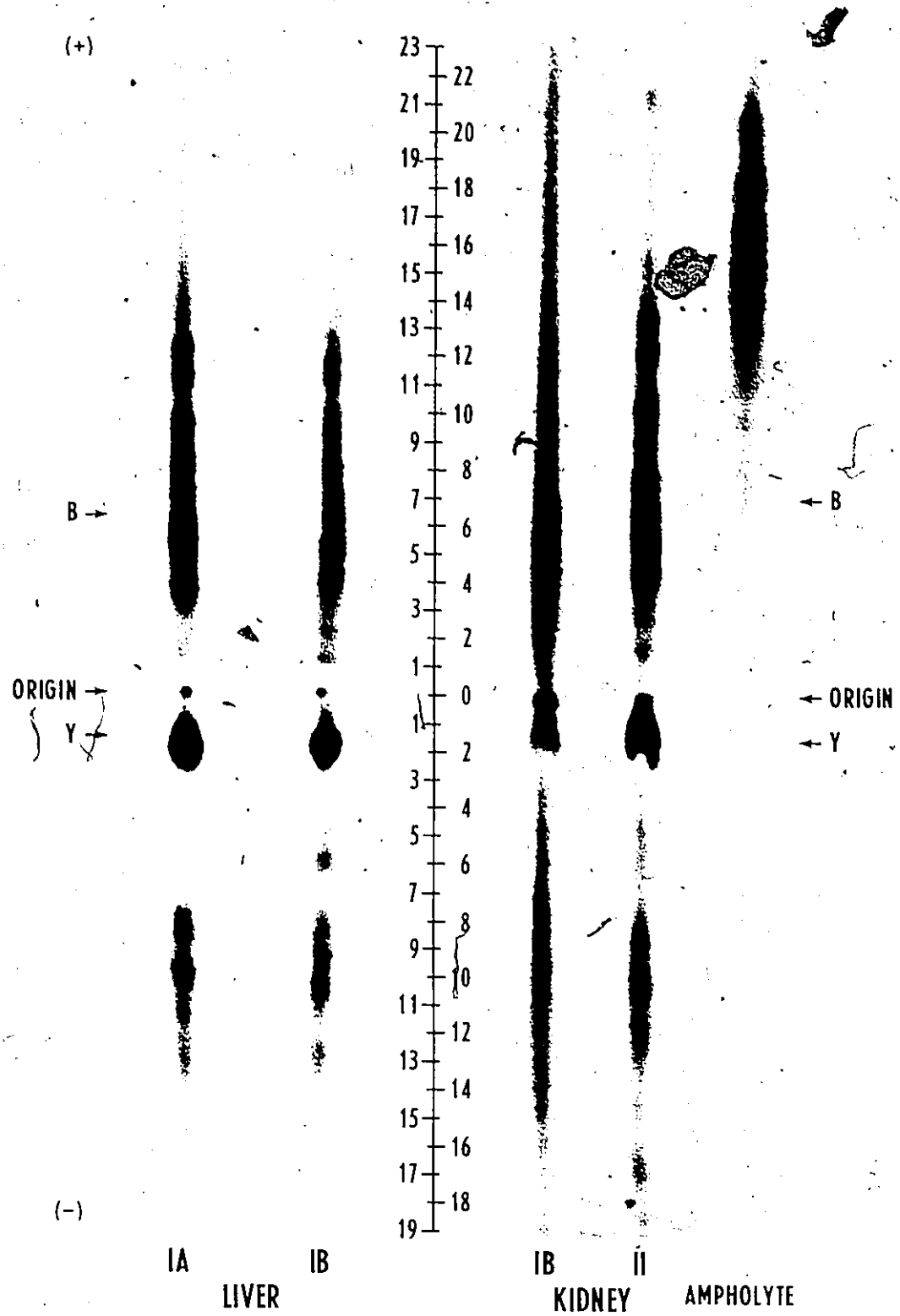
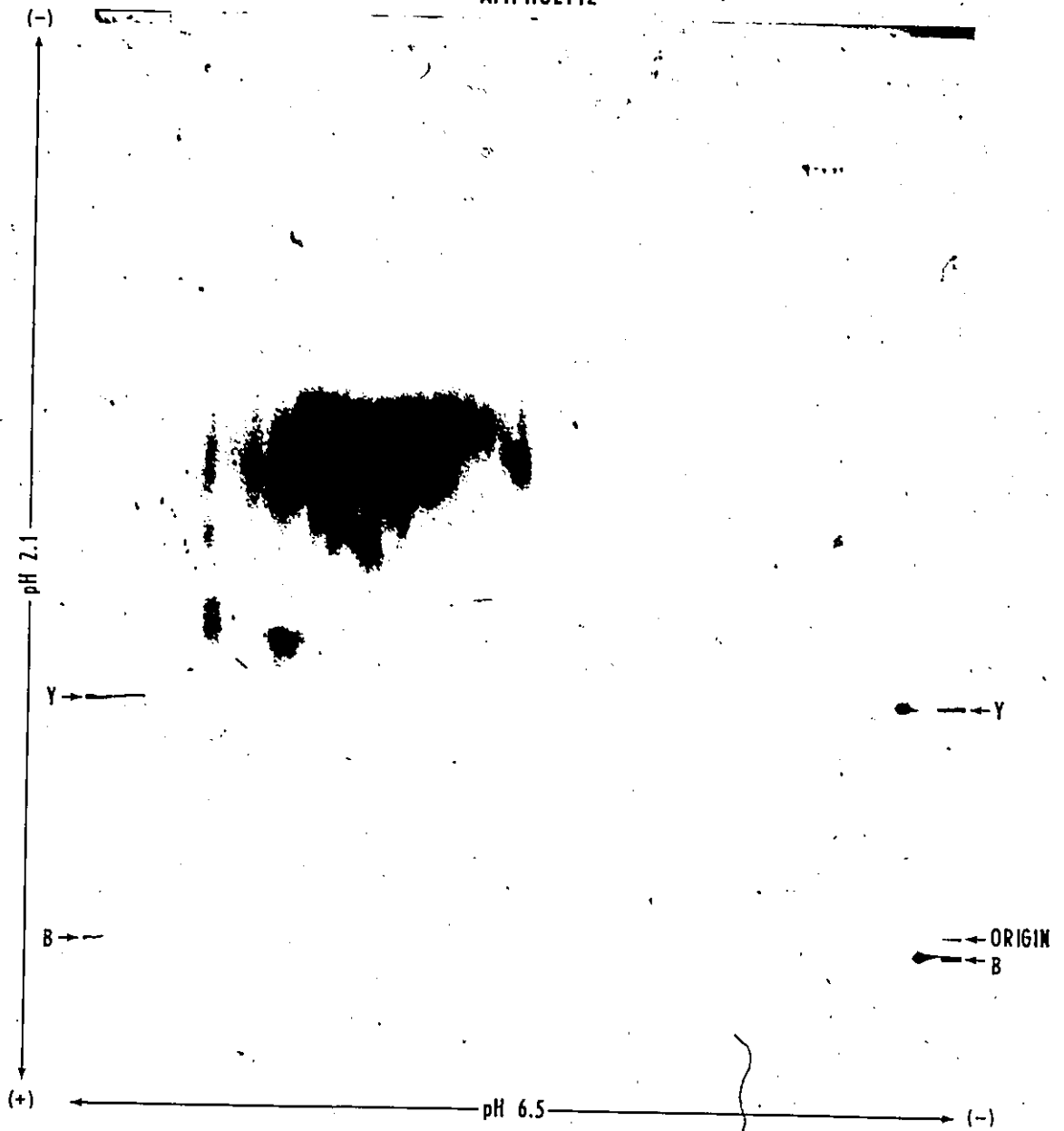


FIGURE 32

Autoradiogram of the Second Dimensional Electrophoresis at pH 2.1  
of Carrier Ampholyte

The papain digest of the  $^{14}\text{C}$ -acetylated carrier ampholyte was electrophoresed first at pH 6.5 and then at pH 2.1. Electrophoresis was carried out at 3000V for one hour. The symbols are as described in Figure 31.

AMPHOLYTE



For the ease of discussion of the maps, those peptides that are acidic, basic and neutral at pH 6.5 will be referred to as region A, B and N respectively. Thus, in region A of the liver IA protein, there are two peptides, marked as "a" and "c", unique to this protein whereas in the same region of the liver IB protein, peptide "b" is distinctive. Comparing the region B peptide patterns of the two liver proteins, spot "d" is unique to the liver IB sample. In other unmarked cases, the differences apparently reside in the intensity of the radioactivity present. Fewer peptides are resolved in the pH 2.1 electrophoresis of the kidney II protein (Figure 35). This was partly due to the relatively shorter running time at pH 6.5 of this particular electrophoresis. But the general peptide pattern resembles those of the liver tissue.

The amino-terminal peptides of region A of all the three proteins are of considerable interest. These are acidic at pH 6.5 (Figure 31) and neutral at pH 2.1 (Figures 33, 34 & 35). These properties exclude the presence of arginyl or histidyl residues in these peptides. A autoradiogram of these N-terminal peptides is shown in Figure 36 when the radioactivity in this region for each of the three proteins shown in Figures 33, 34 and 35 was eluted off and re-electrophoresed at pH 6.5. Common to all the three proteins, viz. the liver IA and IB and kidney II, was the peptide having an  $R_f$  between 0.56 and 0.60. But the migration of other spots were quite distinct although relatively close to each other. Again, the autoradiogram of the kidney IB sample was unsuccessful because of low radioactivity.

Although the region N of the three protein samples at pH 2.1 showed no discrete peptide spots other than smearing toward the cathode, the electrophoreses of these radioactive strips at pH 3.5 revealed some distinctive

FIGURE 33

Autoradiogram of the Second Dimensional Electrophoresis at pH 2.1  
of Liver IA Protein

The papain digest of the  $^{14}\text{C}$ -acetylated liver IA protein was electrophoresed first at pH 6.5 and then at pH 2.1. Electrophoresis was carried out at 3000V for one hour. The symbols are as described in Figure 31. The spot located on the bottom right-hand corner in between the marks, origin and Y, was due to the radioactive identification mark.

LIVER IA



FIGURE 34

Autoradiogram of the Second Dimensional Electrophoresis at pH 2.1  
of Liver IB Protein

The conditions are as described in Figure 33 and the symbols in Figure  
31.

LIVER IB



FIGURE 35

Autoradiogram of the Second Dimensional Electrophoresis at pH 2.1  
of Kidney II Protein

The conditions are as described in Figure 33 and the symbols in  
Figure 31.

2

KIDNEY II

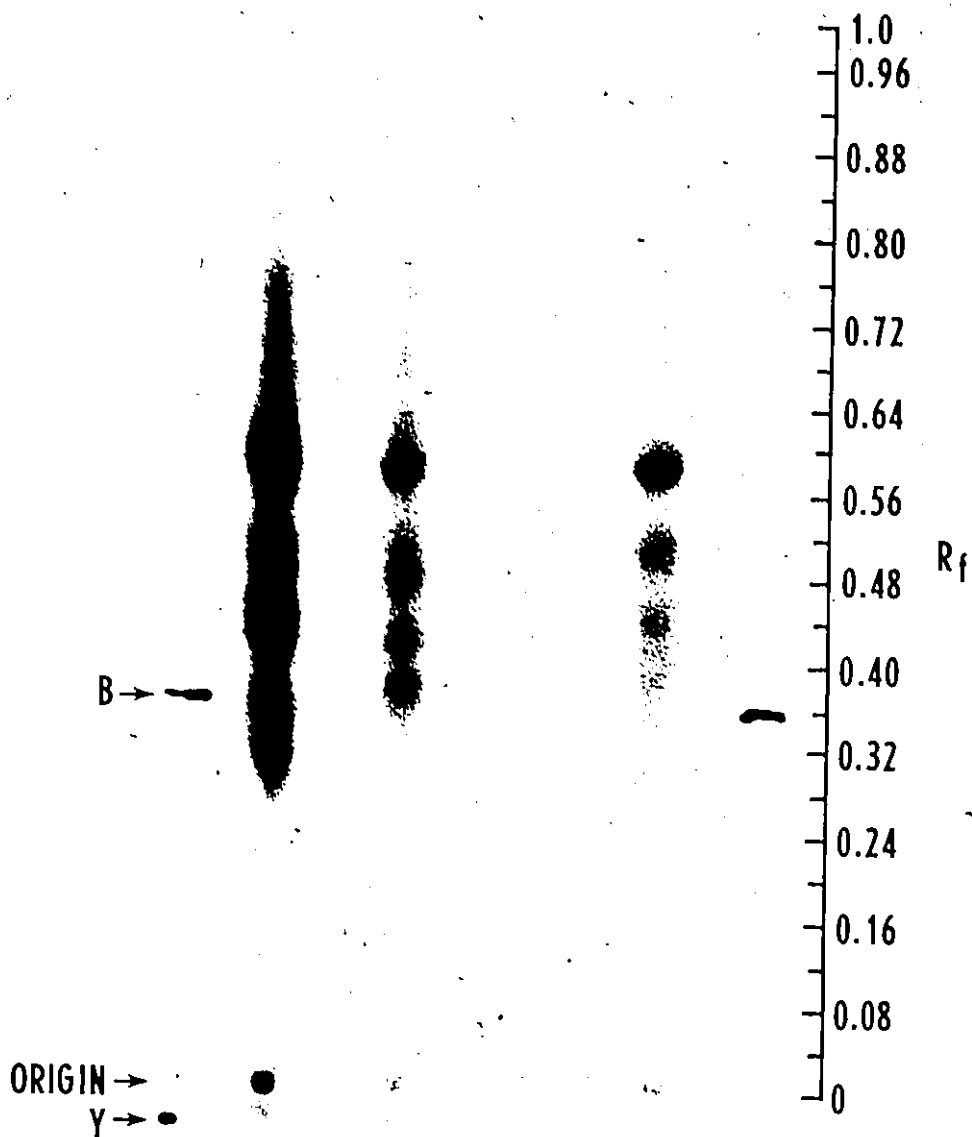


FIGURE 36

Autoradiogram of the Amino-Terminal Peptides of Liver IA, IB and  
Kidney IB and II Proteins at pH 6.5

The radioactivity strips of the N-terminal peptides (i.e. materials at origin) at pH 2.1 of the liver IA, IB and kidney IB (not shown) and II proteins were eluted separately and re-electrophoresed at pH 6.5. The kidney IB sample contained too little radioactivity to produce a visible radioautogram. The  $R_f$  scale was an arbitrary one, 25 cm from the origin being assigned as 1 unit.

(+)



(-)

IA IB  
LIVER

IB II  
KIDNEY

features. These autoradiograms are shown in Figures 37, 38 and 39 for the liver IA and IB and kidney II proteins respectively. First, comparing the liver IA and IB patterns, a very similar distribution of the peptides is observed. An unique feature of the two patterns is the presence of the radioactive spot marked "a", migrating closely with the yellow dye marker. The peptide spots "b" and "c" are unique to the liver IA protein. However, two similar spots also marked "b" and "c" are observed with the kidney II protein (Figure 39), but spot "a" common to the two liver proteins, is clearly absent in the kidney sample. At the moment other differences or similarities among the three proteins remain unresolved.

FIGURE 37

Autoradiogram of the Neutral Peptides of Liver IA Protein at pH 3.5

The neutral peptides of liver IA protein at pH 6.5 were first subjected to a second dimensional electrophoresis at pH 2.1 seen in Figure 33 and then at pH 3.5. Electrophoresis was carried out at 3000V for 2 hours. The blue dye marker was at the bottom of the chromatogram and not included in the autoradiogram.

LIVER IA

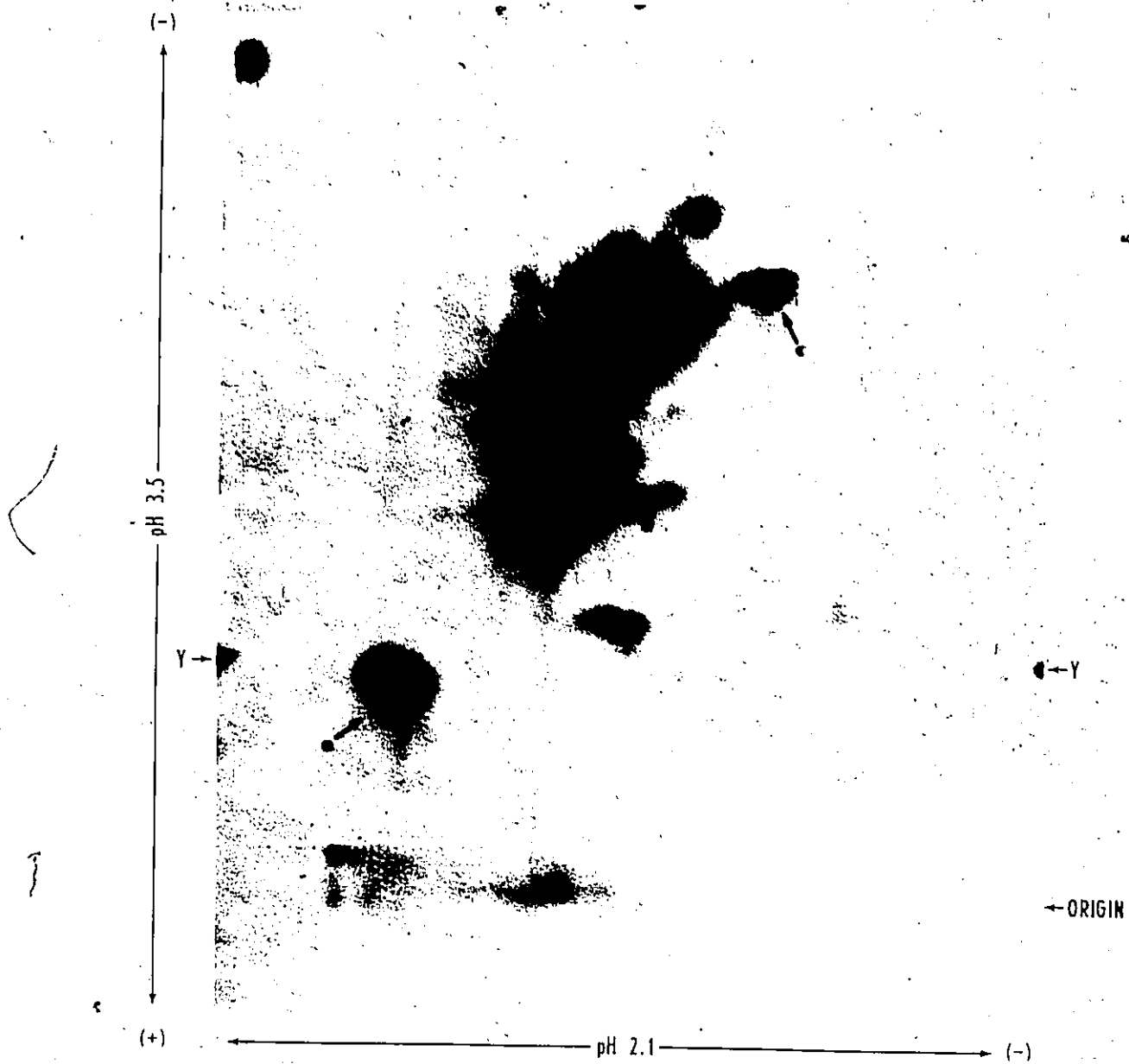


FIGURE 38

Autoradiogram of the Neutral Peptides of Liver IB Protein at pH 3.5

The neutral peptides of liver IB protein at pH 6.5 were first subjected to a second dimensional electrophoresis at pH 2.1 seen in Figure 34 and then at pH 3.5. The conditions are described in Figure 37.

LIVER IB

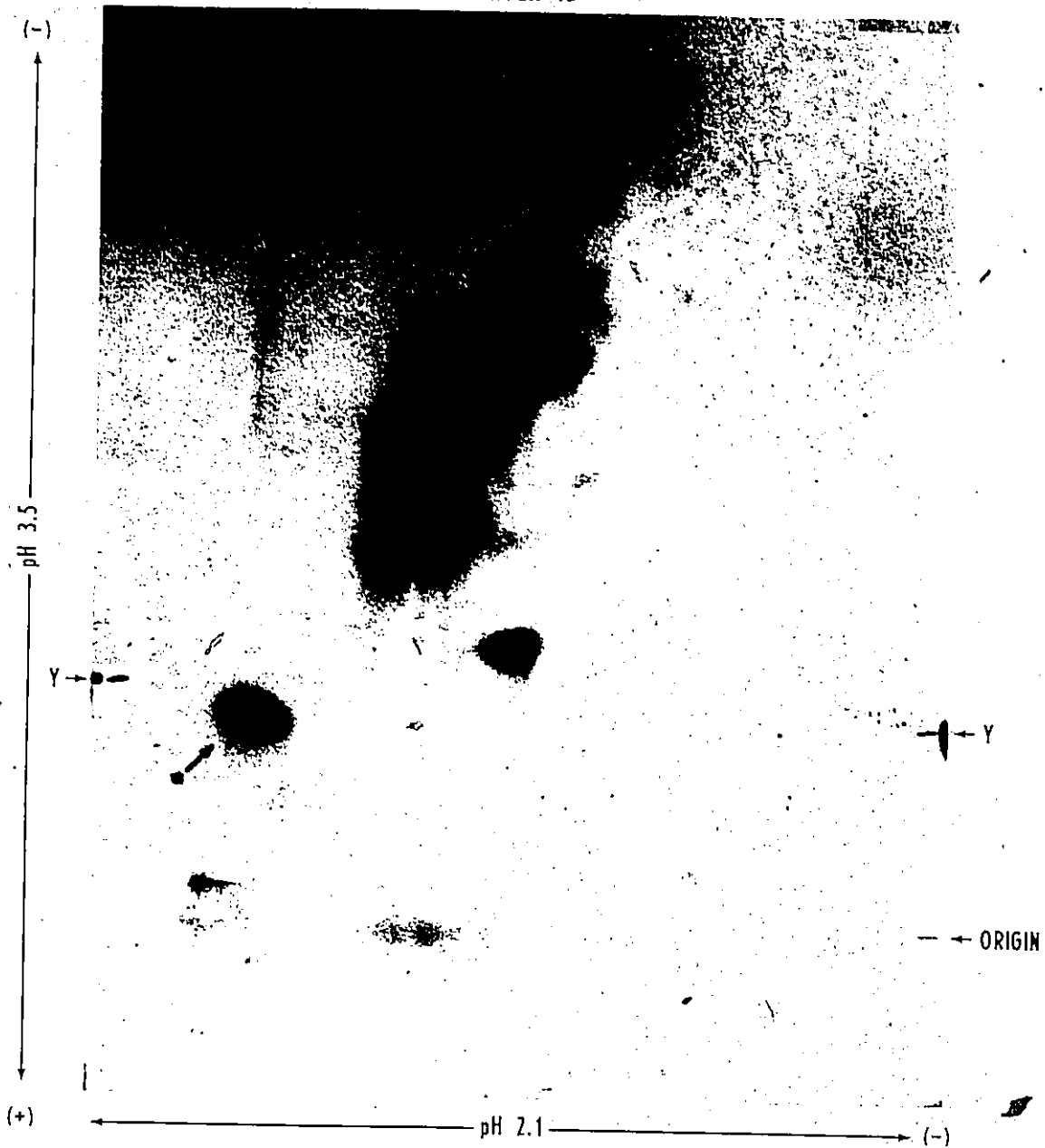


FIGURE 39

Autoradiogram of the Neutral Peptides of Kidney II Protein at pH 3.5

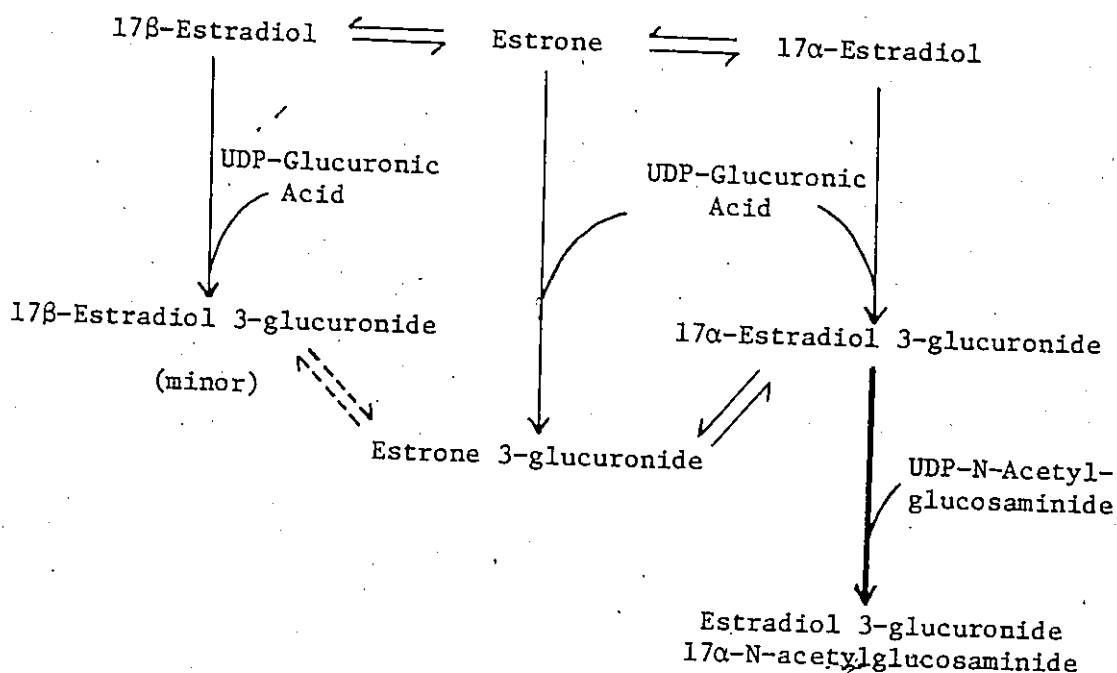
The neutral peptides of kidney II protein at pH 6.5 were first subjected to a second dimensional electrophoresis at pH 2.1 seen in Figure 35 and then at pH 3.5. The conditions are as described in Figure 38. Note the position of the blue dye marker, B.

KIDNEY II



## DISCUSSION

The relatively high level of 17 $\alpha$ -hydroxysteroid dehydrogenase in the kidney and especially the presence of an enzyme form highly specific for the glucuronide derivative of 17 $\alpha$ -estradiol or estrone, suggests that the kidney plays an active role in extrahepatic estrogen metabolism in the rabbit. The following metabolic pathway has been proposed (Layne et al, 1975):



With unconjugated estrogens, the predominant 17 $\beta$ -HSD activity of liver catalyzes the interconversion of 17 $\beta$ -estradiol and estrone (Hasnain & Williamson, 1974). However, after formation of the 3-glucuronide derivative conversion into the 17 $\alpha$ -epimer predominates due to the presence of the 17 $\alpha$ -HSD specific for the glucuronide derivative (Hasnain & Williamson, 1975, 1977).

17 $\alpha$ -Estradiol 3-glucuronide is the only substrate for N-acetylglucosaminyl transferase (Collins et al, 1968) which transfers N-acetylglucosamine from the nucleotide donor, UDP-acetylglucosamine to the 17 $\alpha$ -position of the steroid to form the double conjugate which is released into the blood (Quamme et al, 1972) and subsequently excreted in the urine (Layne et al, 1964; Layne, 1965). However, the 3-glucuronide derivative of estrone and 17 $\alpha$ -estradiol are also found in blood but not excreted in the urine (Quamme et al, 1972). The presence in the kidney of an additional enzyme form highly specific for estrone 3-glucuronide or 17 $\alpha$ -estradiol 3-glucuronide is therefore advantageous. The kidney is also abundant in N-acetylglucosaminyl transferase (Collins et al, 1968). The kidney therefore serves as a final site to complete the detoxification of estrogens which either escape full conjugation with respect to N-acetylglucosaminide formation within the liver or are deconjugated after leaving the liver. In this connection, enzymatic hydrolysis of steroid N-acetylglucosaminides has been demonstrated in the rabbit tissues in in vitro studies (Whittemore & Layne, 1965; Hoffman, 1978). The present study reiterates the close relationship between conjugation with glucuronic acid at C-3 and oxidoreduction at C-17 of the steroid in the rabbit liver and kidney.

The high specificity of the rabbit liver 17 $\alpha$ -enzyme for 17 $\alpha$ -estradiol 3-glucuronide has been studied, the high activity being lost if the glucuronic moiety was removed or replaced by glucose or galacturonic acid (Hasnain & Williamson, 1977). When compared to the oxidation of 17 $\alpha$ -estradiol, the higher activity with the 17 $\alpha$ -estradiol 3-glucuronide was due to a higher V<sub>max</sub>, rather than a difference in the K<sub>m</sub> value. In the present study it was shown that the 3-glucuronide of estrone as well as of 17 $\alpha$ -estradiol are good substrates for one

of the liver or kidney enzyme forms. Moreover, the presence of a sulfate group at C-3 of estrone did not result in the high activity observed with the glucuronide derivative. This stressed that in the interaction with the enzyme the carboxylic group as well as the configuration of the glucuronide substrate was important. The apparent absence of an enzyme specific for estrone sulfate comparable to that of  $17\alpha$ -estradiol 3-glucuronide may be related to the nature of the estrogenic steroids found in the urine. These conjugates are the glucuronides or N-acetylglucosaminides but not sulfate (Layne, 1970).

The substrate specificity studies of the purified rabbit liver enzymes further revealed that like the kidney enzymes, they also catalyzed the oxidation of  $3\alpha$ -hydroxysteroids in addition to the  $17\alpha$ -hydroxysteroids. Previous reports on the rabbit liver  $17\alpha$ -HSDs (Hasnain & Williamson, 1974, 1975, 1977) did not identify this  $3\alpha$ -HSD activity. However, Thaler-Dao et al (1972) had shown a  $3\alpha$ -HSD activity associated with the purified  $17\beta$ -HSD of the female rabbit liver cytosol. This particular protein had a very anionic character and a molecular weight of 36000 by SDS-polyacrylamide gel electrophoresis. Also in the rabbit liver cytosol Takenoshita and Toki (1978) have identified a 3-hydroxyhexobarbital dehydrogenase which catalyzed the dehydrogenation of  $5\beta$ -androstan- $3\alpha,17\beta$ -diol to  $5\beta$ -androstan- $3\alpha,-ol-17-one$ .

In the previous chapter (Chapter V) it was stated that the  $3\alpha$ -HSD activity of the rabbit kidney  $17\alpha$ -HSD showed a high preference for  $3\alpha$ -hydroxysteroids of the  $5\alpha$ -androstane series as opposed to those of the  $5\beta$ -androstane series. Data from inhibition studies presented in Chapter VI indicated that the substrate specificities of both the rabbit liver and kidney  $3\alpha$ -HSDs may be extended to the

oxidation of some 3 $\alpha$ -hydroxyl groups of 5 $\alpha$ -pregnane steroids. But the 20 $\alpha$ - or 20 $\beta$ -hydroxyl groups of these steroids may also be responsible for the inhibitory actions in view of the ability of the 3(17) $\alpha$ -HSDs to catalyze the reduction of pregnenolone to its 20-reduced metabolites. This wide spectrum of substrate specificities of the rabbit hydroxysteroid dehydrogenases is not completely unprecedented, the multiple specificity of hydroxysteroid dehydrogenases has been demonstrated with other systems. The 3 $\alpha$ -HSD activity of cortisone reductase (20 $\beta$ -HSD) of Streptomyces hydrogenans (Gibb & Jeffrey, 1971; Edwards & Orr, 1978) is a pertinent example. The 17 $\beta$ -HSD of monkey liver (Fan et al, 1978) or rabbit ovary (Rodway & Rahman, 1978) and the 17 $\beta$ -estradiol dehydrogenase of human endometrium (Tseng & Gurpide, 1979) may all function as 20 $\alpha$ -HSDs. Earlier, the 17 $\beta$ -estradiol dehydrogenase of human placenta had been shown to have 20 $\alpha$ -HSD activity and competitive inhibition of the two activities was indicated by kinetic analyses (Purdy et al, 1964), but other workers found no measurable 20 $\alpha$ -HSD activity in a pure preparation of the 17 $\beta$ -estradiol dehydrogenase (Karavolas & Engel, 1971). The purified porcine testicular 17 $\beta$ -HSD has also been shown to exhibit 20 $\alpha$ -HSD activity to the extent of one seventh of the 17 $\beta$ -enzyme activity (Inano & Tamaoki, 1974).

The structural requirements of the steroid molecules for 3 $\alpha$ - or 17 $\alpha$ -HSD activity of the rabbit kidney and liver warrant further discussion. The reduction of 3-ketosteroids to 3 $\alpha$ -hydroxysteroids occurs only with those compounds lacking a  $\Delta^4$  double bond. Presumably either 5 $\alpha$  or 5 $\beta$  reduction of the  $\Delta^4$ , 3-ketosteroids must precede the reduction of the ketone group at C-3.<sup>#</sup> Such a restriction appears to be a common characteristic among the 3 $\alpha$ - or 3 $\beta$ -HSDs isolated from other sources. These examples include the soluble 3 $\alpha$ -HSD of the liver (Tomkins, 1956) or the ventral prostate (Taurog et al, 1975) of rat and

(<sup>#</sup> Although  $\Delta^4$ -3 ketone reduction may not go to an appreciable extent, the reverse reaction could be quite rapid (Orr, J.C. personal communication).

the  $3\alpha$ -HSD and  $3(17)\beta$ -HSD of *Pseudomonas testosteroni* (Talalay, 1963). However several contrasting points are also featured among these enzymes. First, the bacterial  $3\alpha$ -HSD is entirely specific for  $\text{NAD}^+$  and for  $3\alpha$ -hydroxyl groups of  $\text{C}_{19}$ -,  $\text{C}_{21}$ -,  $\text{C}_{24}$ -steroids. The A/B ring fusion of the steroid may be trans or cis although a change in configuration of the A/B ring from trans to cis reduces the maximum rate of oxidation to about 60% (Talalay, 1963). The rat ventral prostatic  $3\alpha$ -HSD also favors the  $5\alpha$ -reduced steroid (Taurog et al, 1975). On the other hand, the rat liver enzyme reacts with a variety of  $3\alpha$ -hydroxysteroids of the  $\text{C}_{19}$  and  $\text{C}_{21}$  series but the  $5\beta$  analogs are reduced more rapidly than the  $5\alpha$ -reduced steroids (Tomkins, 1956). In contrast, the  $3\beta$ - and  $17\beta$ -HSDs of *Pseudomonas testosteroni*, also  $\text{NAD}^+$ -specific, promote the reversible oxidation of  $3\beta$ -hydroxyl groups of  $\text{C}_{19}$ - and  $\text{C}_{21}$ -steroids in which the A/B ring fusions are trans or cis and the oxidation of  $17\beta$ -hydroxyl groups of the  $\text{C}_{18}$ - and  $\text{C}_{19}$ -steroids. The discrimination shown by the enzymes with regard to the A/B ring fusion of the  $5\alpha$ - or  $5\beta$  structures can be explained by their conformational differences. Conformational analysis of  $5\alpha$ -steroids such as androsterone or  $5\alpha$ -androstan- $3\alpha,17\beta$ -diol (Duax & Norton, 1975) shows that they have a planar structure while the  $5\beta$ -steroids, for example  $5\beta$ -androstan- $3\alpha,17\beta$ -diol, are characterized by a marked bowing of the A-ring about the C-5,10 bond relative to the remainder of the steroid molecule. The present studies have shown that the  $5\alpha$ -structure is important for interaction with the  $3\alpha$ -HSDs of the rabbit kidney. With respect to the  $17\alpha$ -enzyme activity the reactivity decreases with increasing unsaturation of the A-ring as indicated by the relative activities of the enzymes toward  $17\alpha$ -estradiol and epitestosterone. The presence of an aromatic A-ring may therefore be an impeding factor.

The rabbit liver and kidney enzymes are strongly inhibited by low concentrations of  $Hg^{2+}$ ,  $Cu^{2+}$  and PCMB but not iodoacetate. Hasnain (1975) has shown that  $Mg^{2+}$ ,  $Ca^{2+}$ ,  $Mn^{2+}$ ,  $Fe^{2+}$  and iodoacetate have no inhibitory effect on two liver  $17\alpha$ -HSD enzymes. The resistance of the rabbit kidney and liver  $3(17)\alpha$ -HSDs to iodoacetate cannot be explained on the basis of inaccessibility of the reagent to sulfhydryl groups since PCMB, a larger molecule than iodoacetate, readily reacts. One plausible explanation may be the slow reactivity of iodoacetate compared to PCMB. Webb (1963) noted that protein sulfhydryl groups are frequently resistant to iodoacetate and that their reactivity can be increased by denaturation. Nevertheless the inhibition of the rabbit kidney and liver  $3(17)\alpha$ -HSDs by PCMB as well as by heavy metal ions suggests the free sulfhydryl groups must be present for enzyme activity. A variety of steroid dehydrogenases have also been shown to be sulfhydryl enzymes, examples being the  $3\alpha$ -HSDs of rat liver (Tomkins, 1956) or of Pseudomonas testosteroni (Talalay, 1963) the human placental  $17\beta$ -estradiol dehydrogenase (Langer & Engel, 1958) the  $3\alpha$ ,  $17\beta$ -HSD of female rabbit liver (Thaler-Dao et al, 1972) and the guinea pig kidney  $17\beta$ -HSDs (Liu & Kochakian, 1972a; Shen & Kochakian, 1978).

In addition to the data reported in Chapter IV and V indicating the common identity of the  $3\alpha$ - and  $17\alpha$ -HSDs of rabbit kidney and liver we have obtained information from kinetic analyses that this is so. Kinetic competition studies of the four enzyme forms of the rabbit kidney and liver  $3(17)\alpha$ -HSDs revealed competitive inhibition of the oxidation of the  $17\alpha$ -hydroxysteroid substrate by a  $3\alpha$ -hydroxysteroid. This means that these hydroxysteroids were competing for the same binding site in the enzyme. A linear competitive type of inhibition was observed for three enzymes whereas the fourth enzyme displayed a parabolic-type competitive inhibition. This latter phenomenon was also observed by

Blomquist et al (1978 ) in their inhibition studies of human placental  $17\beta$ -HSD by cyclohexanol. This situation could arise when the combination of a molecule of inhibitor at the active site of the enzyme allowed the binding of a second molecule of the inhibitor so that two molecules of inhibitor contributed to the exclusion of the substrate (Roberts, 1977). This explanation is analogous to the one offered for substrate inhibition where it is possible for a second substrate(S) molecule to bind to the enzyme-substrate complex(ES) to produce an inactive complex(SES). Substrate inhibition at high steroid concentration was observed in this study with epitestosterone with LIA enzyme (Figure 24b) as was previously reported by Hasnain and Williamson (1977) for the rabbit liver  $17\alpha$ -HSD. Talalay (1963) also reported that the kinetic behaviour of the majority of the  $3\beta$ - and  $17\beta$ -hydroxysteroids acted upon by the  $3(17)\beta$ -HSD of Pseudomonas testosteroni exhibited inhibition at excess substrate concentration.

We have emphasized the importance of the  $17\alpha$ -HSD, particularly the enzyme form quite specific toward estrone glucuronide or  $17\alpha$ -estradiol 3-glucuronide, in the inactivation of estrogens in the rabbit. But the intrinsic  $3\alpha$ -HSD activity as well as the  $17\alpha$ -HSD toward the androgens suggest that these multiple forms are also important in the metabolism of androgens in this animal. Unfortunately, there is a paucity of information concerning this aspect. Although it was reported that the metabolites of testosterone were rapidly and almost completely excreted (70-90% in 24 hours) in the urine of intact male rabbits (Taylor, 1971) as glucuronides (major) or sulfates (minor), the identity of the actual metabolites were not discussed. However, it is known that the sheep and the rabbit share a peculiar urinary and biliary excretion pattern of estrogens in that one of the major metabolites is the

17 $\alpha$ -epimer (Velle, 1963). Recently, androgen metabolism in sheep has been studied (Yamamoto et al, 1978). It was found that when radioactive testosterone, androstenedione or epitestosterone were administered to the animal, epitestosterone was the major metabolite detected in the urine or bile. In the urine, epitestosterone was excreted both as a glucuronide and sulfate but in bile it was present only as the glucuronide. Other metabolites also as glucuronides or sulfates that were identified include androsterone, etiocholanolone and 5 $\beta$ -androstan-3 $\alpha$ ,17 $\beta$ -diol. If this excretion pattern of androgens holds true for the rabbit a high activity of the 3(17) $\alpha$ -HSD toward androgens, may be of great relevance to the animal. In the rabbit kidney where the 17 $\beta$ -HSD is low, the formation of epitestosterone which is biologically inactive may have a regulatory role in preventing the presence of a large amount of biologically active androgens in the circulatory system. These active androgens may include testosterone or its reduced metabolites, 5 $\alpha$ -dihydrotestosterone or 5 $\alpha$ -androstan-3 $\alpha$ ,17 $\beta$ -diol. These steroids have been shown to mediate androgenic action in various androgen-sensitive tissues (Liao, 1977). In view of the biological potency of 5 $\alpha$ -dihydrotestosterone and 5 $\alpha$ -androstan-3 $\alpha$ ,17 $\beta$ -diol and considering the high activity of the 3 $\alpha$ -HSD which effects the interconversions of these two steroids, it is speculated that this activity is physiologically important. However, it has yet to be established whether testosterone metabolism is a step necessary for the expression of its full biological activity. This also necessitates the study of the 5 $\alpha$ - or the 5 $\beta$ -reductase enzymes. At this time, the information on the androgen metabolism in the rabbit remains fragmentary.

It is of interest to compare the 3(17) $\alpha$ -HSD of rabbit liver and kidney with the sex steroid-binding protein (SBP) of immature rabbit serum. This SBP has been purified to homogeneity (Mickelson & Petra, 1978) and it is a glycoprotein with a 30% carbohydrate content. The molecular weight is variable depending on the method of analysis but the value of 36,475 obtained by equilibrium sedimentation ultra-centrifugation in 6M guanidine-HCl containing 0.1M mercaptoethanol is generally accepted the molecular weight of the native SBP. This approximates the molecular weight of the rabbit kidney and liver enzymes of our study. But a comparison of the amino acid composition of the SBP (Mickelson & Petra, 1978) and the rabbit liver 17 $\alpha$ -HSDs (Hasnain & Williamson, 1977) reveal significant differences. The former is a more acidic protein as indicated by a higher ratio of Asx + Glx/His + Arg + Lys compared to any of the multiple forms of the rabbit liver enzymes. Hansson *et al*, (1974) indicated a pI value of 5.2 for the SBP whereas the rabbit liver enzymes electrofocused above pH 5.7 (Hasnain & Williamson, 1975; also this thesis). However one interesting feature that is shared by the SBP and the rabbit kidney and liver 3(17) $\alpha$ -HSDs is their high affinity for androgens. Studies on the binding of various steroid hormones to the rabbit SBP show that this protein is mainly an androgen binding protein, the binding specificity decreases in the order: 5 $\alpha$ -dihydrotestosterone, 5 $\alpha$ -androstan-3 $\alpha$ ,17 $\beta$ -diol, testosterone, estrone, 17 $\beta$ -estradiol, progesterone, androstenedione and cortisol (Hansson *et al*, 1974; Mickelson & Petra, 1978). We have shown in this study that the multiple forms of the 3(17) $\alpha$ -HSDs of rabbit kidney and liver are essentially androgen metabolizing enzymes. Another characteristic common to the SBP and 3(17) $\alpha$ -HSD is their multiple nature. This property has not been

completely substantiated for the rabbit SBP although the carbohydrate content has been implicated to be responsible for some of the observations such as the polydispersity of the SBP on ultracentrifugation (Mickelson & Petra, 1978) or its heterogeneous elution pattern from the ion exchange column chromatography (Hansson et al, 1974).

The peptide maps of the papain digests of the purified rabbit liver and kidney 3(17) $\alpha$ -HSDs revealed small differences in the structural composition between the two forms in each of the tissues. The peptide patterns also revealed small differences but significant homology between the corresponding enzymes from the two tissues. The apparent variation in the peptide pattern of the two forms from the same tissue provides an argument against the occurrence of these proteins as conformers (Kaplan, 1968) which otherwise have the same sequence but exist in varying conformations. Instead, multiple similar genes may be involved for the synthesis of these proteins, these genes themselves may stem from gene duplication during the course of evolution (Kaplan, 1968; Williamson et al, 1973). Duplicate enzymes differ from a closely related process, alleles of the same gene, in that the former are present in all individuals of a species and also present in varying amounts in different tissues. These criteria for duplicate enzymes are fulfilled by the multiple forms of the rabbit kidney and liver 3(17) $\alpha$ -HSDs. From the substrate specificity studies and peptide mapping data it appears that the kidney II and liver IA enzymes have a similar gene distinct from the gene(s) for the kidney and liver IB enzymes. The unsuccessful peptide mapping of the kidney IB enzyme prevented its comparison with the liver IB enzyme. However from substrate specificity studies and other characteristics it is likely that

these two proteins come from a similar gene. Shen and Kochakian (1979) have reported that the male and female kidney of the guinea pig contained an identical gene for the 17 $\beta$ -HSD.

Yet another explanation for the microheterogeneity of the rabbit kidney and liver 3(17) $\alpha$ -HSDs could be the process of deamidation, a post-synthetic modification to the polypeptide chain whereby asparagine and glutamine are spontaneously converted to their corresponding acids in vivo or in vitro (Robinson & Rudd, 1974). In this study of the rabbit kidney and liver enzymes it is possible that successive deamidations could endow the proteins with a progressively more negative charge. The high contents of Asx + Glx of the rabbit liver enzymes indicated by their amino acid compositions (Hassan & Williamson, 1977) and presumably in the kidney enzymes (since they have similar peptide patterns) make the occurrence of deamidation probable. The trend of increasing acidity of the rabbit kidney enzymes was observed by three techniques viz. polyacrylamide gel electrophoresis, isoelectric focusing, and DEAE-cellulose column chromatography. The isoelectric points or the electrophoretic mobilities in polyacrylamide gels of these multiple forms revealed small charge differences, except for the most acidic species. It is unlikely that deamidation occurred during the purification procedures of these enzymes since the same multiple forms were observed in the crude cytosol, unless the process occurred as soon as the animals were sacrificed. Nevertheless, deamidation of proteins or peptides has been considered as a real and significant in vivo modification step (Robinson & Rudd, 1974; Uy & Wold, 1977). Rabbit muscle aldolase, a tetramer, offers a well characterized example in which deamidation of a particular asparaginyl residue (the fourth amino acid from the carboxyl-terminal end) of each subunit gave rise to five enzyme forms

(Midelfort & Mehler, 1972). The hydrolysis was found to occur in vivo with a half-life of 8 days. Cytochrome c of bovine heart has also been shown to undergo stepwise deamidation in vivo (Flatmark, 1967). Moreover, the in vitro spontaneous conversion of asparagine to aspartic acid in a protein antibiotic at weakly acid pH has been demonstrated (Maeda & Kuromizu, 1977). Recently, Williams and John (1979) were able to generate multiple forms of aspartate transaminase from pig heart cytosol in vitro at pH 7.5 and 25°C by deamidation. The half-life was about 20 days. Moreover, the more negatively charged forms developed in vitro were almost fully active. However alternative explanations for the difference in charge between the multiple forms of this enzyme have been proposed, one of which was the presence of different amounts of sialic acid in these proteins (Denisova & Polyanovsky, 1973). The presence of carbohydrate has been shown to be partly responsible for the multiple nature of aspartate transaminase from pig heart cytosol (Denisova & Polyanovsky, 1973) or the adenosine deaminase found in human liver tissues (Swallow et al, 1977). To date, no 17 $\beta$ -HSDs of mammalian origin (Burns et al, 1971; Pons et al, 1977; Kobayashi & Kochakian, 1978) or the 3(17) $\beta$ -HSDs of bacterial origin (Schultz et al, 1977) have been shown to be glycoproteins. We have not attempted to measure the carbohydrate content (if any) of the 3(17) $\alpha$ -HSDs of rabbit kidney and liver cytosols.

The nature of the microheterogeneity of the rabbit kidney and liver 3(17) $\alpha$ -HSDs and a host of other 17-HSDs (see Introduction section 4) remains unresolved. But certainly heterogeneity is a general phenomenon among 17-HSDs and other HSDs. Aukrust et al (1976) demonstrated on polyacrylamide gel electrophoresis 2-6 forms of 3 $\alpha$ -HSD from Pseudomonas testosteroni. The rat

kidney 3 $\alpha$ -HSD has also been shown to contain three enzymes capable of catalyzing the interconversion of 5 $\alpha$ -dihydrotestosterone and 5 $\alpha$ -androstan-3 $\alpha$ ,17 $\beta$ -diol (Verhoeven et al., 1976). Indeed, the recognition of isozymes or multiple molecular forms of enzymes in general has been well documented in the literature (Markert, 1977).

Despite the universal occurrence of isozymes, the biological role of these multiple molecular forms of enzymes has not been fully evaluated. Ureta (1978) hypothesized that "metabolic pathways are unidirectional chain reactions catalyzed by specific isozymes associated as polyisozymic complexes." It was proposed that the differences in the physiochemical properties within the components of an isozyme system e.g., net charge, are responsible for such an assembly in the complex. It is tempting to speculate that the 3(17) $\alpha$ -HSDs of this study might be assembled in such a manner in the cell. The multiple specificities of these enzymes toward both 3 $\alpha$ - and 17 $\alpha$ -hydroxysteroids and their ketones, in addition to the occurrence of multiple forms of these enzymes may endow the animal with greater economy, versatility or flexibility.

#### GENERAL CONCLUDING REMARKS

A new steroid-metabolizing enzyme, the 3(17) $\alpha$ -hydroxysteroid dehydrogenase of female rabbit kidney and liver cytosols is described in this thesis. This enzyme exists in multiple forms in both tissues and catalyzes the NADP(H)-linked reversible oxidoreductions of various 3 $\alpha$ - and 17 $\alpha$ -hydroxysteroids. Conjugated estrogens particularly the 3-glucuronide derivatives of 17 $\alpha$ -estradiol or estrone are good substrates compared to their aglycones. However, in general, the C<sub>19</sub>-steroids possessing either 3 $\alpha$ - or 17 $\alpha$ -hydroxyl and their ketone functions are better substrates than the estrogens or their derivatives.

The significance of one of the multiple forms present in both the rabbit kidney and liver tissues is related to the metabolism of estrogens in this animal. This enzyme form (LIB and KIB) is highly specific for 17 $\alpha$ -estradiol 3-glucuronide or estrone 3-glucuronide which are intermediary compounds in the inactivation pathway of 17 $\alpha$ -estradiol, 17 $\beta$ -estradiol or estrone (Layne, 1970; Hasnain & Williamson, 1974; Layne et al, 1975). The 3-glucuronide of 17 $\alpha$ -estradiol is the only substrate for the transfer of N-acetylglucosamine from UDP-N-acetylglucosamine to the steroid (Jirku & Layne, 1965; Collins et al, 1968). The resulting double conjugate is then excreted in the urine (Layne, 1965). The fact that the specific activity of both N-acetylglucosaminyl transferase (Collins et al, 1968) and the 17 $\alpha$ -estradiol 3-glucuronide dehydrogenase are high in the kidney compared to those present in the liver points to the importance of the kidney in the detoxification mechanism. With reference to the metabolism of androgens in the rabbit, it is unfortunate that

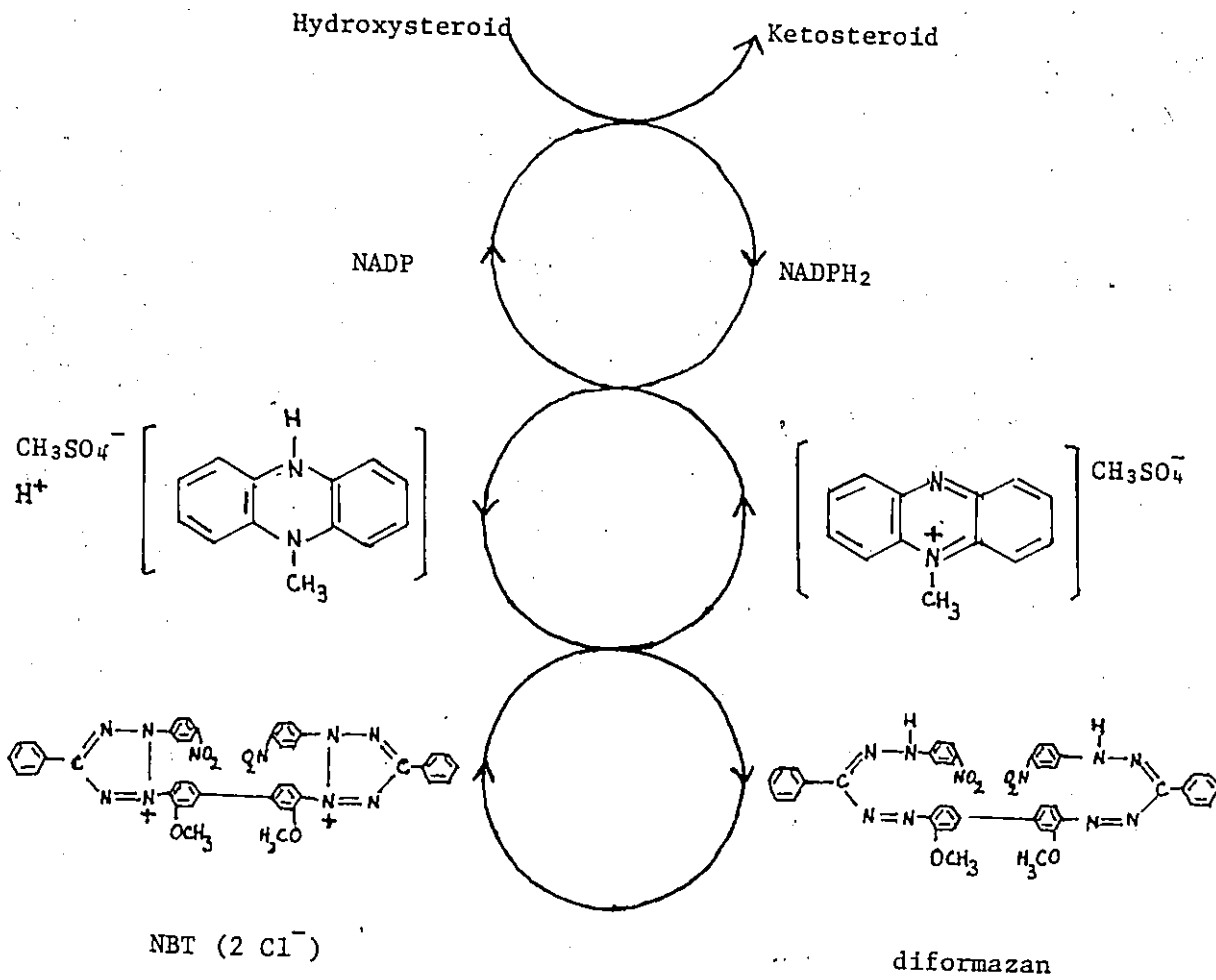
information is lacking to attach any definite significance of the existence of the various multiple forms highly specific for these steroids. However, enzymes which catalyze the interconversion of 5 $\alpha$ -dihydrotestosterone and 5 $\alpha$ -androstan-3 $\alpha$ ,17 $\beta$ -diol may possibly play a regulatory role in androgen metabolism.

Sufficient evidence has been presented that the 3 $\alpha$ - and 17 $\alpha$ -HSD activities reside in the same protein and that the multiple forms of this enzyme are not artefacts but real intracellular enzymes. The peptide maps showed that the liver IA and kidney II enzymes which exhibit similar substrate specificities may be the products of one gene. On the other hand, the liver IB enzyme which differs from the LIA and KII enzymes in substrate specificities may be derived from another gene. At present, the origin of these multiple forms remains unclarified.

The regulation of these multiple forms is another interesting area yet to be examined. Unpublished work in our laboratory has indicated that these multiple forms are age-dependent. Perhaps certain genes may not be fully activated at all times and that their activity may be modulated by hormones accompanying the development of the animal. If deamidation is an explanation for the microheterogeneity of the HSDs observed in this study the deamidation process may be age-dependent. In the latter situation, the acidic enzyme forms should accompany the older animals whereas the basic forms should prevail in the young. Indeed, age-dependent changes in the degree of deamidation of glutaminy and asparaginy residues have been observed in a large number of proteins (Robinson & Rudd, 1974). Moreover, it has also been proposed that deamidation may play a central role as a molecular timer of biological events.

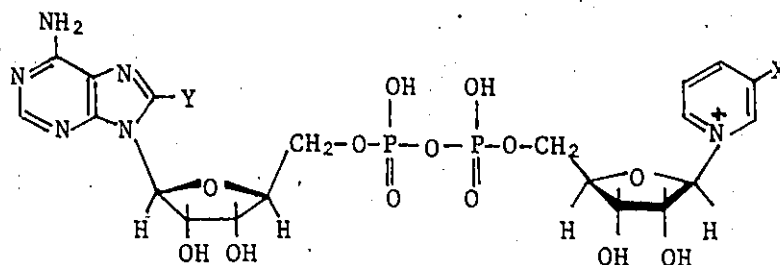
APPENDIX I

Reduction of NBT by Hydroxysteroid Dehydrogenases



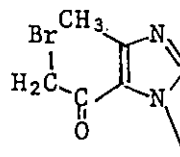
APPENDIX II

Structures of Analogues of NAD<sup>+</sup>



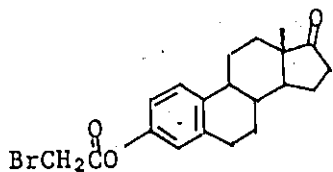
	<u>X</u>	<u>Y</u>
nicotinamide adenine dinucleotide	-CONH <sub>2</sub>	-H
pyridine adenine dinucleotide	-H	-H
3-acetylpyridine adenine dinucleotide	-COCH <sub>3</sub>	-H
3-cyanopyridine adenine dinucleotide	-CN	-H
3-iodopyridine adenine dinucleotide	-I	-H
nicotinamide 8-bromoadenine dinucleotide	-CONH <sub>2</sub>	-Br
nicotinamide 8-thioadenine dinucleotide	-CONH <sub>2</sub>	-SH
thionicotinamide adenine dinucleotide	-CSNH <sub>2</sub>	-H
3-chloroacetyl pyridine adenine dinucleotide	-COCH <sub>2</sub> Cl	-H
nicotinamide [5-bromo acetyl 4-methylimidazole] dinucleotide	-CONH <sub>2</sub>	-H

except the adenine moiety  
is modified as follows:

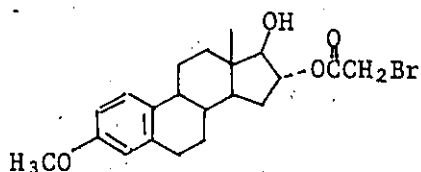


APPENDIX III

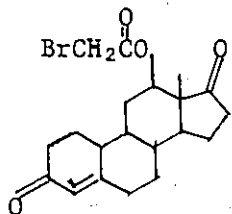
Structures of Modified Steroids used in Affinity Labeling



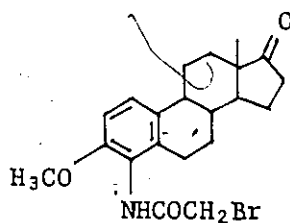
3-Bromoacetoxyestrone



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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