THE PURIFICATION AND CHARACTERIZATION OF 17α- and 17β-HYDROXYSTEROID DEHYDROGENASES OF FEMALE RABBIT LIVER CYTOSOL

BY SADIQ HASNAIN

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SUMMARY

The 17β-HSDase activity in female rabbits is concentrated mainly in three organs, liver, large intestine and kidney, while most of the 17α-HSDase activity occurs in kidney and liver. The specific activity of 17β-HSDase is higher in the liver than in the kidney but the reverse is true of the 17α-HSDase activity. The 17β-HSDase activity in rabbit liver is largely specific for the nucleotide NAD^+, while the bulk of the 17α-HSDase activity is associated with the cofactor $NADP^+$. However, the soluble 17β-HSDase activity of rabbit liver has equal specificities for both cofactors. The 17α-HSDase activity in the cytosol is highly specific for $NADP^+$.

On the basis of the intracellular distribution and specific activities of the 17-HSDases a possible pathway for estrogen metabolism can be postulated. Estrone or 17β-estradiol, upon entering the liver, can be conjugated by microsomal glucuronyl transferase to form either estrone glucuronide or 17β-estradiol 3-glucuronide. The latter can be readily oxidized by 17β-HSDase in the microsomes or in the cytosol to estrone glucuronide. In the cytosol estrone glucuronide can be reduced by 17α-HSDase to 17α-estradiol 3-glucuronide. This

order of conjugation and then reduction is probably the most likely sequence based on the relative activities of the different 17-HSDases in the microsomes and the cytosol. The monoconjugate is further conjugated at carbon 17 by N-acetylglucosaminyl transferase to form the double conjugate 17α-estradiol 3-glucuronide 17-N-acetylgucosaminide which is excreted in the urine, and is the sole urinary excretory product in the rabbit of injected estrone, 17β-estradiol or 17α-estradiol (6, 7).

The 17β-HSDase activity of rabbit liver cytosol is distinct and separable from the 17α-HSDase by the techniques of ion-exchange chromatography and isoelectricfocusing These two activities are also distinct on the basis of their nucleotide specificity, but similar in their pH optimum, adsorption properties on calcium phosphate gel and elution profiles upon gel filtration. One of the 17β-HSDases, which is pure, is also quite different from the 17α-HSDases in its primary structure as detected by peptide mapping.

The 17α-HSDase activity can be separated into three main and one minor fraction by ion-exchange chromatography. The third fraction (enzyme III) is essentially pure on the basis of polyacrylamide gel electrophoresis at pH 8.3 and

SDS-gel electrophoresis. Fractions I and II contain more than one enzyme fraction. Electrofocusing of fraction I separates the activity into 3 pure enzymes while a similar treatment of fraction II separates the activity into 4 enzymes, two of which are pure and the other two are cross contaminated. None of the purified 17α-HSDases can oxidize 17β-hydroxysteroids. All of the purified 17α-HSDases except IC have a higher affinity and a faster rate of catalysis for the substrate epitestosterone when compared with estrogen substrates. Enzyme IC has a similar Michaelis constant and Vmax for the two substrates 17α-estradiol 3-glucuronide and epitestosterone. Enzyme IC is highly specific for 17α-estradiol 3-glucuronide when compared with other estrogen substrates such as 17α-estradiol and its glucosyl and galacturonide conjugates. Enzyme IIC has a similar Vmax for both epitestosterone and 17α-estradiol 3-glucuronide, but this enzyme has almost a 10 fold lower Km for the androgen than the estrogen. The other estrogens tested with enzyme IIC gave a five fold lower specific activity than the glucuronide conjugate. This suggests that proper orientation of the glucuronide conjugate on enzymes IC and IIC involves both the stereospecific recognition of glucuronic acid and the charged carboxyl moiety. The question as to whether the higher affinity for the androgen substrates is due to an extra methyl group or

due to the lack of an aromatic A ring is not resolved by the present studies.

The activities of enzyme IIC and III are not affected by the metal ions Mg^{2+}, Mn^{2+}, Ca^{2+}, Fe^{2+} nor by EDTA and iodoacetate. However Cu^{2+} inhibits their activity by 50% at a concentration of $1x10^{-3}$M.

The isoelectric points of the multiple 17α-HSDases range from 5.72 to 6.68. The molecular weights of the purified 17α-HSDases are all very close to the mean value of 39600 daltons. Enzyme III has the lowest molecular weight (38800), while enzyme IIC, with a molecular weight of 40500, is the largest. The amino acid compositions of the purified enzymes are also very similar. Enzyme III shows distinct dissimilarities for only amino acid residues, glx (glutamine + glutamic acid) and asx (asparagine + aspartic acid), when compared to the amino acid compositions of the rest of the 17α-HSDases. However the peptide maps of these enzymes do reveal small differences in their primary structures. All of the evidence suggests that the 17α-HSDases are very similar enzymes but, nevertheless, have small differences in structure and function which mark them as distinct from each other. These differences are probably not artifactual, and are due to

either small pre-translation variations in the different messages (multigene theory) or slight post-translation gene directed modifications of one main form (one gene theory)(63).

GLOSSARY

The following compounds are referred to in this thesis by their trivial names.

Trivial Name	Compound
Estrone	Estra-1,3,5(10)-trien-3-ol-17-one
17β-Estradiol	Estra-1,3,5(10)-trien-3, 17β-diol
17α-Estradiol	Estra-1,3,5(10)-trien-3, 17α-diol
Estrone glucuronide	Estra-1,3,5(10)-trien-3-ol-17one-3-yl-β-D-glucopyranosiduronic acid
17β-Estradiol 3-glucuronide	Estra-1,3,5(10)-trien-3, 17β-diol-3-yl-β-D-glucopyranosiduronic acid
17α-Estradiol 3-glucuronide	Estra-1,3,5(10)-trien-3, 17α-diol-3-yl-β-D-glucopyranosiduronic acid

GLOSSARY (Cont'd)

Trivial Name	Compound
17α-Estradiol 3-glucoside	Estra-1,3,5(10)-trien-3, 17α-diol-3-yl-β-D-glucopyranosiduronic acid
17α-Estradiol 3-galacturonic acid	Estra-1,3,5(10)-trien-3,17α-diol-3-yl-β-D-galactopyranosiduronic acid
17α-Estradiol 3-glucuronide 17-N-Acetylglucosaminide	Estra-1,3,5(10)-trien-17α-yl-2' acetamino-2'-deoxy-β-D-glucopyranosid-3-yl-β-D-glucopyranosiduronic acid
Testosterone	17β-Hydroxy-4-androstene-3-one
Epitestosterone	17α-Hydroxy-4-androstene-3-one
Androstenedione	4-Androstene-3,17-dione
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NAD^+	Nicotinamide adenine dinucleotide

GLOSSARY (Cont'd)

Trivial Name	Compound
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NADH	Nicotinamide adenine dinucleotide (reduced)
UDPGA	Uridine-5'-diphosphoglucopyranosiduronic acid
UDPNAG	Uridine-5'-diphospho-2'-acetamido-2'-deoxy-D-glucopyranoside
Tris	Tris (hydroxymethyl) aminomethane
DEAE-cellulose	Diethylaminoethylcellulose
CM-cellulose	Carboxymethylcellulose
DTT	Dithiothreitol
EDTA	Ethylenediamine-tetraacetic acid
TEMED	N,N,N',N'-Tetramethylethylenediamine

GLOSSARY (Cont'd)

Trivial Name	Compound
PPO	2,5-Diphenyloxazole
SDS	Sodium dodecylsulphate
Ketodase	β-Glucuronidase
UDPGalA	Uridine-5'-diphosphogalactopyranosiduronic acid

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INTRODUCTION

Metabolism of Estrogens

The estrogens are eighteen carbon steroids with an aromatic A ring. They promote the appearance of female secondary sex characteristics and the growth and development of female reproductive organs. When labelled estrogen is injected into an animal, the steroid accumulates in the uterus, vagina, and the anterior pituitary. There is a concomitant increase in mitotic activity and in the content of endoplasmic reticulum, protein and RNA in the uterus and vagina (1). The importance of the estrogens in their normal physiological roles, and their palliative effects upon certain tumors of the breast and prostate gland (2) necessitated the study of the regulation of the blood levels of these steroids. In general the circulating levels of hormones are controlled by the rates of synthesis, the rates of degradation and the efficiency of the transport system.

In the rabbit, the liver is the main metabolic organ for estrogens. The steroid initially enters the liver as the circulating form, estrone (1) or 17β-estradiol but before excretion in the urine there is a complete conversion to 17α-estradiol (3, 4, 5, 6, 7), which is excreted as the double glycoside 17α-estradiol 3-glucuronide-17-N-acetylglucosaminide

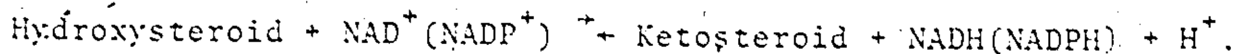
(6, 12, 13). Studies of the two transferase enzymes involved show that glucuronyl transferase, which conjugates the 3-position, can transfer glucuronic acid to the 3-hydroxyl group of estrone or either of the estradiol epimers (11). The N-acetylglucosaminyl transferase is, on the other hand, highly specific for the 17α-hydroxyl group, and requires the prior addition of glucuronic acid to the 3-position (11, 14). Thus it is of interest whether the addition of glucuronic acid may precede, or even facilitate, the conversion of estrone and 17β-estradiol in the metabolic sequence. This thesis is concerned with a study of oxido-reduction by rabbit liver enzymes of the 17 function of estrogens and their glucuronides.

Rabbit liver preparations can also form the estrogen conjugates 17α-estradiol 3-galacturonide (42), 17α-estradiol 3-galactoside (43) and 17α-estradiol 3-glucoside (46). However these conjugates are not excreted and as yet their function *in vivo* is unknown.

Hydroxysteroid Dehydrogenases

Hydroxysteroid dehydrogenases (HSDases) promote stereospecific and reversible interconversion of hydroxyl and carbonyl

groups located on the steroid skeleton or the side chain. The reactions require pyridine nucleotide cofactors and may be formulated according to the following equation (15).



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

It has been suggested that discrete enzymes are responsible for oxidations at positions 3α-, 3β-, 11β-, 17α-, 17β-, 20α-, 20β-, and 21- of the steroid molecule (16). Several studies with tissue slices and crude homogenate preparations have demonstrated the variety and distribution of these reactions, (17,18) and their presence in many organisms including bacteria, fungi, and higher animals, has been documented (19).

17-Hydroxysteroid Dehydrogenases in Animal Tissues

Purification of 17-hydroxysteroid dehydrogenases (17-HSDases) has been achieved from the cytosol of human term placenta (20, 21, 22, 23), the microsomes of porcine testes (24) and guinea pig kidney cytosol (25), while partial purification has been obtained from guinea pig liver cytosol (26), chicken liver cytosol (27), rabbit liver cytosol (9, 10), and from the membrane free haemolysate of human erythrocytes (28). The human placental 17β-estradiol dehydrogenase has been studied most extensively. It consists of two polypeptide chains with a unit molecular weight (MW) of 34,000 and an aggregate MW of 64,000 to 68,000 (29,30). Recently, Engel and Groman reported the fractionation of pure 17β-estradiol dehydrogenase by isoelec-

trofocusing in acrylamide gels. They obtained three prominent and two faint bands by either staining for enzyme activity or for protein (32). When the enzyme sample was isoelectrofocused in the presence of 8M urea three protein bands were visualized after staining for protein. On this evidence the authors (32) suggested that 3 different monomers present in unequal amounts could interact to form six dimers. Multiple forms of 17-HSDases have also been observed in preparations from female rabbit liver (9) and male guinea pig kidney (33, 34). In the latter case Kochakian et. al. (59) established that at least one of the main enzyme forms observed on acrylamide gels was hormone dependent. Upon castration of the animals, they observed a disappearance of one of the enzyme bands which re-appeared after testosterone treatment.

All of the 17β-HSDases that have been isolated or partially purified from animal tissues have a nonspecific requirement for the cofactors, NAD^+ and $NADP^+$ (9,10,25,28,35,36). However all these enzymes have an absolute stereospecificity for the 17β-hydroxyl, except for the rabbit liver 17-HSDase partially purified by Ball and Breuer (10) and by Thaler-Dao et. al. (9). Ball and Breuer suggested that, on the basis of their data, one enzyme was able to oxidize both the 17β- and 17α-hydroxyl groups. However, it should be kept in mind that their preparation was only partially purified and the possibility of the presence of two distinct enzymes was not completely excluded. Thaler-Dao

et. al. did not observe any 17α-HSDase activity in their rabbit liver preparation containing 17β-HSDase activity, but did find that one of their partially purified 17β-HSDase fractions also contained 3α-HSDase activity. They suggested that, on the basis of their evidence, the same enzyme was responsible for the oxido-reduction at positions 3α and 17β. No other steroid dehydrogenase from any source exhibits this dual stereospecificity.

The 17β-HSDases have broad substrate specificities, in that they can effect the oxido-reduction of both estrogens and androgens. One exception is the enzyme from human placenta, which has a characteristic requirement for an aromatic A or B ring or both (32). This would suggest a differentiation of function among the enzymes isolated from the placenta (a target organ) and those from the liver (a metabolic organ). The female rabbit liver 17β-HSDases (9, 10) are three to four times more active towards testosterone than estradiol, while the male guinea pig kidney 17β-HSDases are thirty five fold more active towards testosterone than estradiol. Human erythrocyte 17β-HSDase has the lowest level of specificity with activities towards androstenediol, estradiol and testosterone in the ratio of 2 to 1.5 to 1.5 (28). However, these results can not be rigidly interpreted because of the lack of homogeneity in the enzyme preparations.

The pH optima of the various 17β- and 17α-HSDases show striking similarities. The female rabbit liver 17β- and

17α-HSDases (9,10), and the chicken liver 17β- and 17α-HSDases (27) exhibit a broad pH optimum in a range from 9.0 to 9.5. The guinea pig kidney 17β-HSDases have a pH optimum in the range from 9.4 to 10.2, (34) and similarly the 17β-HSDase from human erythrocytes is optimally active within a pH range of 9.0 to 10.0 (28). Finally, the 17β-estradiol dehydrogenase from human placenta also exhibits a pH optimum at pH 10.0 (37). Langer and Engel (37) showed experimentally that this pH optimum reflects the ionization of groups on the enzyme rather than on the phenolic hydroxyl of estradiol. The porcine testicular microsomal 17β-HSDase does not show the usual pH profile and instead has a pH optimum between 6.5 and 7.5 (24).

The Michaelis constants which have been reported for the various 17-HSDases show a great variation, not only among enzymes from different sources but also for the same enzyme in the hands of different workers. For example, Km values quoted for the human placental 17β-HSDase are 2×10^{-5}M (20), 2×10^{-6}M (38) and most recently 6×10^{-7}M (36). Table I lists the rate constants for the various 17β- and 17α-HSDases that have been studied. It is interesting to note that the Vmax for human placental 17β-estradiol dehydrogenase is one hundred times higher than the Vmax for the other enzymes listed in Table I.

The study of the hydroxysteroid dehydrogenases of a group of animals including horse, mouse, sheep, chicken, turkey and rabbit gained a degree of importance when it was discovered

TABLE I

The Vmax and Michaelis Constants for 17-Hydroxysteroid Dehydrogenases from Different Sources.

Source	Substrate	Km (M)	Vmax (nmol/mg/min)	Reference
Rabbit Liver	17α-estradiol	5.8×10^{-5}	10	10
	17β-estradiol	3.5×10^{-5}	17	10
Porcine Testis	Testosterone	8.3×10^{-6}	48	24
Chicken Liver	17α-estradiol	1.2×10^{-5}	1.0	27
	17β-estradiol	1.6×10^{-5}	3.5	27
Rabbit Liver	Testosterone	3.0×10^{-6}	-	9
Human Placenta	17β-estradiol	2.0×10^{-5}	9000	20
	17β-estradiol	2.0×10^{-6}	-	38
	17β-estradiol	6.0×10^{-7}	-	36
Human Erythrocytes	Testosterone	1.7×10^{-4}	-	28
	17β-estradiol	5×10^{-5}	-	28

that these animals were able to convert the 17β-hydroxyl of estrogens or androgens to the 17α-hydroxyl and subsequently excrete the product in the urine (39). Ozon and Breuer (40) and Dollefeld and Breuer (41) reported the presence of 17α-estradiol dehydrogenase activity in chicken liver and horse placenta respectively. Renwick and Engel (27) partially purified the 17α- and 17β-HSDase activities from the chicken liver cytosol by chromatography on DEAE-cellulose and CM-cellulose. These HSDase activities co-chromatographed on either of the ion exchangers. However, a partial separation of the 17β- and 17α-HSDase was achieved on Sephadex G-200. Similar attempts to fractionate the two activities from female rabbit liver cytosol by Ball and Breuer (10) were unsuccessful. Their attempts included an ammonium sulphate fractionation, gel filtration on Sephadex G-200 and adsorption on DEAE-cellulose and CM-cellulose. On the basis of this data and the results from their kinetic experiments, they concluded that the oxido-reduction of both 17α- and 17β-estradiol in the rabbit liver is catalyzed by a single enzyme. However, recently Crastes de Paulet _et. al._ have achieved the separation of the two activities (9). By adsorption on DEAE-cellulose they fractionated the 17β-HSDase activity of female rabbit liver into three fractions, namely A, B and C. Only fraction C was tested for 17α-estradiol dehydrogenase activity and no such activity was found.

The wørk presented in this thesis, mainly the purification and characterization of the 17-hydroxysteroid dehydrogenases of rabbit liver cytosol, was undertaken for the following reasons; firstly to study the importance of the 17-hydroxysteroid dehydrogenases in the metabolic sequence of estrogens in rabbit liver; to explore the nature of the relationship between oxido-reduction of the estrogen and glucuronidation; and lastly to resolve whether the 17α- and 17β-estradiol dehydrogenase activities of rabbit liver cytosol are both due to the same enzyme (10) or are seperable enzyme activities such as the chicken liver 17α- and 17β-estradiol dehydrogenases (27).

MATERIALS AND METHODS

Materials

Chemicals and their origin are listed below.

Amersham/Searle Corporation (Arlington Heights, Illinois)

1 - ^{14}C-Acetic anhydride, 17β-[6,7-$^{3}H_2$] estradiol and [6,7-$^{3}H_2$] estrone

Calbiochem (Los Angeles, California)

Thermolysin

Canadian Laboratory Supplies (Ottawa, Ontario)

Iodoacetate, phosphoric acid and silica gel N (standard grade)

Eastman Kodak Co. (Rochester, N. Y.)

Acrylamide, bis-acrylamide and tetramethylethylenediamine (TEMED)

Fisher Scientific Co., Ltd. (Montreal, Quebec)

Ampholytes (LKB), ammonium persulfate, ethylenediaminetetraacetic acid (EDTA), and all buffer salts and solvents

Frazer Medical Supplies Ltd. (Vancouver, British Columbia)

2,5-Diphenyloxazole (PPO)

General Biochemicals (Chagrin Falls, Ohio)

Ammonium sulphate (enzyme grade)

Mandel Scientific Co., Ltd. (Montreal, Quebec)

DEAE-cellulose

New England Nuclear (Boston, Mass.)

[1,2-$^3H_2$] Androstenedione, [1,2-$^3H_2$] epitestosterone

[1,2-$^3H_2$] testosterone

Pharmacia Fine Chemicals AB (Montreal, Quebec)

Sephadex G-75, superfine

Pierce Chemical Co. (Rockford, Illinois)

Sodium dodecylsulphate

Schwarz/Mann (Orangeburg, N. Y.)

Amberlite XAD-2 resin

Sigma Chemical Co. (St. Louis, Missouri)

Almond emulsin, androstenedione, bovine serum albumin, Bromophenol Blue, Coomassie Brilliant Blue, cytochrome C, diothiothreitol, 17α-estradiol, 17β-estradiol, estrone, hyaluronidase Type IV, lysozyme, myoglobin, nicotinamide adenine dinucleotide phosphate ($NADP^+$), NADPH, nicotinamide adenine dinucleotide (NAD^+), NADH, ovalbumin and testosterone

Warner Chilcott (Toronto, Ontario)

Ketodase (beef liver β-glucuronidase)

Methods

Preparation of Substrates

The biosynthetic preparation of 17α-[6,7-$^3H_2$] estradiol and 17α-[6,7-$^3H_2$] estradiol 3-glucuronide has been reported by Collins et. al. [6], Layne et. al. [5] and Williamson et. al. (44). To synthesize the required substrates, the authors were able to take advantage of the fact that the rabbit excretes 17α-estradiol free of contamination by the β isomer (3, 12). About 5 mCi (specific activity 40 to 45 Ci/mmol) of either 17β-estradiol or estrone was injected into a virgin female New Zealand rabbit. The urine was collected for 48 hours and then passed over a column of Amberlite XAD-2 resin (44). The column was eluted first with water and then with methanol as described by Bradlow (45). The methanol eluate contained the double conjugate 17α[6,7-$^3H_2$] estradiol 3-glucuronide 17-N-Acetylglucosaminide. The methanol was evaporated and the residue dissolved in 0.1M citrate buffer (pH 4.2) and then incubated with hyaluronidase for 48 hours. The product, 17α-[6,7-$^3H_2$] estradiol 3-glucuronide, was extracted with ethyl acetate, pH 2.0, evaporated to dryness and then made up to the required concentration with methanol. When 17α-[6,7-$^3H_2$] estradiol was required for metabolic studies the glucuronide conjugate was dissolved in 0.1M acetate buffer, pH 5, and incubated with ketodase for 24 hours. The reaction mixture was then extracted with benzene, the benzene extract

evaporated, and then dissolved in methanol to give the required concentration. Substrates were tested for purity by thin layer chromatography. Final purification if required was done by thin layer chromatography in benzene-ethyl acetate (7:3).

17β-[6,7-$^3H_2$] Estradiol 3-glucuronide was prepared according to the method of Collins et. al. (11, 46). A microsomal suspension, prepared from female rabbit liver (11), was diluted with 0.15M Tris-HCl buffer, pH 8.0 and incubated with the nucleotide, UDPGA and 17β-[6,7-$^3H_2$] estradiol at 37°C for 60 minutes. The reaction mixture was extracted first with benzene and then with ethyl acetate at pH 2.0 to quantitatively remove 17β-[6,7-$^3H_2$] estradiol 3-glucuronide. Final purification for either tritiated 17α- or 17β-estradiol 3-glucuronide was done by thin layer chromatography in the solvent system methyl ethyl ketone-water-benzene-ethanol (3:1:3:3).

The synthesis of the tritiated galacturonide of 17α-estradiol was accomplished in the same manner as the preparation of tritiated 17β-estradiol 3-glucuronide, substituting UDP-galacturonic acid for UDPGA (42). The synthesis of tritiated 17β- and 17α-estradiol 3-glucoside was done according to the method of Collins <u>et</u>. <u>al</u>. (46). The methyl ester of tritiated 17α- or 17β-estradiol 3-glucuronide was synthesised by reacting the steroid in an ethanol-water solution (24:1) with diazomethane. The steroid ester was dried and the residue dissolved in methanol. The methyl ester was reacted with sodium borohydride

for 30 min at room temperature and then evaporated to dryness. The residue was taken up in a small volume of distilled water and extracted several times with ethyl acetate. Final purification, if required, was achieved by thin layer chromatography in chloroform-ethanol (4:1). All tritiated substrates were diluted with radioinert steroid to give a final specific activity of 4.5μCi/μmol.

<u>Assay for 17-Hydroxysteroid Dehydrogenase Activities</u>

The incubation mixtures contained the following in a total volume of 3 ml; steroid substrate (specific activity 4.5 μCi/μmol), 0.003 μmol in 30 μl of methanol; $NADP^+$ or NADPH, 0.5 μmol; glycine buffer, pH 9.5, 200 μmol; enzyme fraction, 5-400 μl. Samples were incubated at 37°C for 30 min. The amount of enzyme added to an incubation mixture was adjusted so that the extent of substrate oxidation (reduction) was less than 40%.

Incubations with 17β- or 17α-estradiol or estrone were terminated by extraction of the incubation medium with 5 ml of benzene. The organic layer was evaporated to dryness under nitrogen. Radioinert 17β-estradiol, 17α-estradiol and estrone were added to each benzene extract residue and the mixture was then separated by thin-layer chromatography on silica gel N in the solvent system benzene-ethyl acetate (7:3). The estrogens were visualised by spraying with 2% sulfuric acid in ethanol. The coloured zones were scraped off the plates and assayed for radioactivity in a Nuclear Chicago

Isocap 300 scintillation counter operating at a trituim efficiency of 40%. Enzyme activity was determined by calculating the percentage of radioactivity present in the estrone zone (for the oxidation reaction) or in the 17α- or 17β-estradiol zone (for the reduction reaction). When estrogen glucuronides or galacturonides were incubated, the steroids were extracted with 5 ml of ethyl acetate after adjusting the incubation mixture to pH 2.0 with 1.0 N HCl. The conjugates were hydrolysed for 14 hrs with ketodase (1,000 units) in 3 ml of 0.1M soduim acetate buffer, pH 5.0. The free steroids were extracted with 5 ml of benzene and analyzed by thin-layer chromatography as described above for 17α- and 17β-estradiol. In incubations with estrogen glucosides, the steroids were extracted with 5 ml of ethyl acetate and the ethyl acetate layer was evaporated to dryness. The conjugates in each incubation were hydrolysed with almond emulsin (0.2 mg) in 3 ml of sodium acetate buffer, pH 5.0 for 14 hrs. The aglycone was then extracted with 5 ml of benzene and the substrate and product analyzed as described above for the free estrogens. When androgens were incubated, the steroids were extracted with 5 ml of ethyl acetate and the organic layer removed and evaporated to dryness. Radioinert testosterone, epitestosterone and androstenedione in methanol were added to the residue and the mixture chromatographed on silica gel N thin-layer plates in the solvent system chloroform-acetone (95:5). The androgens were visualized by spraying with a

saturated solution of phosphomolybdic acid in ethanol. The zones of colour were then treated as described for the estrogen substrates.

The recovery of incubated radioactivity after extraction or extraction and hydrolysis was 85-95%. Control incubations were performed either without enzyme fraction or without nucleotide cofactors. Neither the extraction procedure nor the enzymatic hydrolysis to form the aglycone caused the oxidation of the 17β- or 17α-hydroxyl group. The accuracy of the assay method was ± 3% as determined by incubations carried out in triplicate.

Protein concentration was determined either by the method of Lowry _et. al._ (47) or by measurement of the absorbance at 280 and 260 nm (48).

Crude Enzyme Preparation

All procedures for the purification of 17-HSDases were carried out at 0-4°C. A virgin female New Zealand white rabbit was killed by cervical dislocation and the liver was removed and immersed in ice cold 0.15M potassium chloride. The liver was homogenized for one min. in a Sorvall omnimixer with four volumes of 0.25M sucrose, containing 0.5mM dithiothreitol (DTT). The homogenate was centrifuged for 30 min

at 10,000 g and the supernatant recentrifuged for 90 min at 105,000 g. The resulting supernatant was used in the purification studies.

Calcium Phosphate Gel Fractionation

Calcium phosphate gel was prepared according to the method of Swingle and Tiselius (49). A solution of 0.5M sodium phosphate buffer, pH 7.0, was added to the rabbit liver supernatant (20 mg protein/ml) to a final concentration of 1mM. The supernatant was mixed with a calcium phosphate gel suspension (30 mg solids/ml) in a proportion of 1 ml of gel to 3-5 mg of protein. The mixture was stirred in an ice bath for 45 min and then centrifuged at 2,500 g for 10 min. The supernatant was decanted and the pellet resuspended in a volume of 70mM phosphate buffer (pH 7.0, 0.5mM DTT) that was 1.3 times the volume of the original calcium phosphate gel slurry. The mixture was stirred for 45 min and centrifuged at 20,000 g. The supernatant was decanted and concentrated to about 15 ml by ultrafiltration in a Diaflo apparatus (Amicon Corp) with a PM-10 membrane. The concentrate was centrifuged for 15 min at 48,000 g to remove insoluble material.

Sephadex G-75 Gel Filtration

The concentrated enzyme fraction (1300-1700 mg protein) obtained by calcium phosphate gel fractionation was applied to a 5.0 x 90 cm column of Sephadex G-75 (superfine) that had been previously equilibrated in 10mM Tris-HCl, pH 8.0, 0.5mM DTT. The column was eluted with the same Tris-HCl buffer at a flow rate of 20 ml/hr. Ten ml fractions were collected and assayed for 17-hydroxysteroid dehydrogenase activity.

DEAE-Cellulose Chromatography (Type I)

The gel filtration fractions having 17-HSDase activity were pooled and concentrated to approximately 15 ml by ultrafiltration. The concentrate (300 mg protein) was applied to a 2.5 x 40 cm column of DEAE-cellulose, previously equilibrated with 10mM Tris-HCl, 0.5mM DTT, pH 8.0 (Tris-buffer). Protein was eluted with a 2.4 l linear gradient of potassium chloride from 0 to 0.25M in Tris-buffer at a flow rate of 40 ml/hr. Ten ml fractions were collected and assayed for 17-HSDase activity.

DEAE-Cellulose Chromatography (Type II)

In this procedure a protein sample (300 mg) purified by gel filtration was applied to a DEAE-cellulose column

(2.5 x 40 cm) previously equilibrated with Tris-buffer containing 25mM sodium chloride. Protein was eluted first with a 2.0 l linear gradient of sodium chloride from 25mM to 70mM in the equilibrating buffer at a flow rate of 40 ml/hr. Then a second 1.5 l linear gradient from 70mM to 160mM was passed through the column to elute the remaining 17-hydroxysteroid dehydrogenase activity. Ten ml fractions were collected and assayed for 17-HSDase activity.

Electrophoresis in Polyacrylamide Gels

Polyacrylamide disc gel electrophoresis was carried out in the pH 8.3 Tris-glycine buffer system as described by Davis (50) except that the acrylamide monomer concentration was 10%. When gels were pre-run, a stacking gel was omitted from the procedure. After electrophoresis of the sample, the gels were fixed with trichloroacetic acid (12.5%), stained with Coomassie Brilliant Blue (0.2%) and destained with a solution of ethanol-acetic acid (25%-10%) and then acetic acid (10%) (51). By this method of staining, a fraction of a μgm of protein can be visualized. Protein samples were dialysed against Tris-HCl buffer prior to electrophoresis.

Determination of Enzyme Activity in Polyacrylamide Gels

Polyacrylamide gels were prepared as described above except that 10 times the amount of enzyme was required to visualize

bands of enzyme activity. The 17-HSDase activity staining procedure of Liu and Kochakian (34) was followed with slight modification. The reaction mixture contained; 9 mmol of sodium carbonate buffer, pH 9.5; 63 μmol of either epitestosterone or testosterone in 3 ml of propylene glycol; 2 mmol of $NADP^+$; 23 mg of Nitro Blue tetrazolium; 0.54 mg of phenazine methosulphate; in a final volume of 90 ml. The gels were incubated in the dark at room temperature until bands of enzymatic activity could be seen.

Isoelectric Focusing

Isoelectric focusing was performed according to the procedure described in the manufacturer's instruction manual. In a preliminary experiment ampholytes with a pH range of 3-9 were used to establish the conditions required for the successful isoelectric focusing of 17-HSDases over a narrow pH range. A sample of 17α- and 17β-HSDase (40 mg), partially purified by calcium phosphate gel fractionation and gel filtration on Sephadex G-75, was dialysed to reduce the ion content to below 0.5 mmol. A linear gradient of glycerol from 65% to 4%, including the enzyme and ampholytes (2%; pH 3-9), was formed manually in an LKB 4102 electrofocusing column of 110 ml capacity. The gradient was layered over the anode solution (70% glycerol, 1% phosphoric acid) and finally the cathode solution (1% sodium hydroxide) was layered over

the gradient. One watt of power output was maintained for the duration (5 days) of the electrofocusing procedure. After electrofocusing was completed, the column contents were drained and collected in one ml fractions. The pH of the fractions was determined immediately and the fractions were assayed for 17α- and 17β-estradiol dehydrogenase activity.

In the ensuing electrofocusing experiments ampholytes in a pH range of 5-7 or 5-8 were used to obtain a fine resolution of enzyme activities. Also, the LKB 8102 electrofocusing column with a capacity of 440 ml was substituted for the smaller columns mentioned above. In most experiments a linear gradient of sucrose (47% to 5%, w/v) replaced the glycerol gradient and in all experiments DTT was added to the gradient to give a final concentration of 0.5mM. The salt concentration in the sample to be focused was reduced to lower than 1.5 mmol. When a narrower pH range was required, it was prepared by one of two methods; a preliminary electrofocusing of ampholytes (pH 5-7) without enzyme to obtain the desired range; or electrofocusing ampholytes and enzyme in the preliminary run to obtain the desired narrow pH range. The fractions containing the required range of ampholytes were pooled and used to form the second gradient as described before. When a narrow pH range of ampholytes was required the ampholyte concentration was 5% in the preliminary run. The usual ampholyte concentration in the second electrofocusing experiment was about 1.5%. Electrofocusing was carried out for 5 days and a total power

load of 2 watts was maintained for that duration. After electrofocusing the column contents were drained and 2 ml fractions collected. The pH of the fractions was determined and the fractions assayed for enzyme activity. Fractions containing enzyme activity were pooled, concentrated and then dialyzed against 2 l of 10mM glycine-HCl, 0.5mM DTT, pH 9.5 and concentrated again. Dialysis and concentration of the sample was done in an Amicon ultrafiltration cell attached to a 2.5 l reservoir. The concentrated fractions were used for further characterization studies.

Molecular Weight Determination

The procedure of Weber and Osborn (52) was followed to calculate the molecular weights of the enzyme fractions. Samples and standard proteins were incubated at 60°C for 10 min in sodium phosphate buffer, pH 7.0, containing 1% sodium dodecyl sulphate (SDS). The gels were stained as described for Davis gels. The following protein standards were used; bovine serum albumin, ovalbumin, myoglobin, lysozyme and cytochrome C.

Amino Acid Analysis

Samples were dialysed extensively against distilled water and lyophilized. Each sample (about 50µg) was dissolved in 0.5 ml of a 5.7 N HCl solution and transferred to a heavy wall

pyrex glass tube. The solution was frozen, the tube constricted, evacuated for 15 min, sealed and then placed in an oven at 110°C for 24 hr. The hydrolysate was dried under nitrogen, resuspended in lithium-citrate buffer, pH 2.0, and applied to a cartridge. The analyser used was a Durrum D-500 single column multisample amino acid analyser equipped with an automatic integrator. The analysis was done in Dr. M. Yaguchi's laboratory at National Research Council by Mr. C. Roy.

Peptide Mapping with Micro Quantities of Enzyme

Because of the small amount of protein available after the purification procedure, the conventional peptide mapping, which employs staining with ninhydrin, could not be used. Therefore a new procedure was devised which involved the derivatization of lysyl residues with the radioactive reagent, acetic anhydride. This technique, which is similar to that used by Kaplan _et_. _al_. (53) to isolate lysyl peptides in competitive labeling studies, permits the comparison of the primary structures of extremely small amounts of protein in the following manner.

About 100 µg of each enzyme fraction was extensively dialysed against 8M urea at room temperature. Each sample was adjusted to a final volume of 2.5 ml to which 0.5 mg of sodium borate was added. The pH Stat was adjusted to an end

point of pH 9.0 and the syringe was filled with 6N sodium hydroxide. The sample was placed in a cell equipped with a stirrer. To the sample 125 μCi of acetic anhydride (specific activity 30 mCi/mmol) in 0.4 ml of aceto nitrile was added and the mixture allowed to react for 5 min with constant agitation. An excess (50 μl) of inert acetic anhydride was added to the sample in 12.5 μl volumes over a period of 5 min. The mixture was stirred for another 5 min and then the next sample was treated in the same manner. After acetylation, all the samples were dialysed extensively against 0.5% ammonium bicarbonate and the dialysate monitored by liquid scintillation counting to determine when all the unbound acetic acid had been removed. The contents of the dialysis sacs were emptied into test-tubes and to each sample 2 μg of Thermolysin was added. The samples were incubated for 4 hr at 37°C and then lyophilized. All samples were subjected to high voltage electrophoresis in the following manner. The samples were dissolved in 0.2 ml of pH 6.5 electrophoresis buffer (acetic acid-pyridine-water, 0.3:10:90) and spotted on a large sheet of Whatman No. 3 paper. Dansyl arginine and dansyl sulfonic acid were also applied to the sheet as fluorescent markers. The sheet was then buffered and electrophoresed in the pH 6.5 buffer system described above. After 40 min of electrophoresis at 3 kilovolts the sheet was removed from the tank, dried, and then placed under photographic film for 5 days. With the aid of the developed film,

a strip containing the radioactive peptides was cut out for each of the samples. This strip was sewn onto Whatman No. 3 paper and the dansylated markers were applied to the length of the strip. The sheet was buffered and re-electrophoresed in the pH 2.1 buffer system (acetic acid-formic acid-water, 8:2:90) for 40 min at 2.8 kV. Each sheet was dried and placed under photographic film for sufficient time to fully expose and develop the areas of radioactivity. Before placing under film, sheets were marked with radioactive ink to allow proper alignment after developing.

RESULTS

Part I: Preliminary Investigations on 17-Hydroxysteroid Dehydrogenases from Rabbit

1. Tissue Distribution of 17-Hydroxysteroid Dehydrogenases

The liver, spleen, kidney, small intestine, large intestine and uterus, were excised from a virgin female rabbit and homogenized as described earlier. Assays were conducted to determine whether the various tissue homogenates contained either NAD^+ or $NADP^+$ dependent 17-hydroxysteroid dehydrogenase activity towards the substrates 17β-estradiol, 17α-estradiol, 17β-estradiol 3-glucuronide and 17α-estradiol 3-glucuronide. Table 2 lists the results from this experiment. The liver homogenate contained the highest levels of 17β-estradiol and 17β-estradiol 3-glucuronide dehydrogenase activities. NAD^+ was the preferred cofactor for the liver 17β-HSDases while the kidney enzymes had little or no preference for either nucleotide. The large intestine 17β-HSDases were 2 to 3 fold more active with NAD^+ than $NADP^+$. The small intestine, spleen and uterus had relatively low levels of 17β-HSDase activity with both cofactors.

The kidney homogenate appeared to contain the highest level of 17α-HSDase activity for the substrates 17α-estradiol

TABLE 2

The homogenates of all tissues were prepared as described for rabbit liver in the section on methods. The small and large intestines were first flushed extensively with water to eliminate all contents. The crude homogenate fractions were incubated with the different substrates as described.

TABLE 2

Tissue Distribution of NAD^+ and $NADP^+$ Dependent

17-hydroxysteroid dehydrogenases

Tissue	Enzyme Activity μunits/mg							
	17β-Estradiol		17α-Estradiol		17β-Estradiol 3-glucuronide		17α-Estradiol 3-glucuronide	
	NAD	NADP	NAD	NADP	NAD	NADP	NAD	NADP
Liver	350	60	0.5	9.6	250	27	0.3	54
Spleen	1.5	1.8	0.03	0.03	0.17	0	0.3	0
Kidney	24	39	8.4	17	13	13	20	116
Small Intestine	4.0	8.5	0	0.04	1.9	3.4	0.6	0.04
Large Intestine	41	16	0	0.07	24	7.5	0.34	1.8
Uterus	0.12	0.3	0.01	0.02	0.07	0.07	0.2	0.2

and 17α-estradiol 3-glucuronide. In the assay containing 17α-estradiol, the kidney 17α-HSDase had a 2 fold greater activity with $NADP^+$ than NAD^+, whereas in the assay containing 17α-estradiol 3-glucuronide the activity was 5 fold greater with $NADP^+$. Although the level of 17α-HSDase activity was lower in the liver homogenate when compared with the kidney, the activity with $NADP^+$ was 20 and 180 fold greater than with NAD^+ in the assays with substrates 17α-estradiol and 17α-estradiol 3-glucuronide, respectively. The small intestine, spleen, large intestine and uterus contained relatively lower levels of 17α-HSDase activity when compared with kidney and liver.

In the liver homogenate, the NAD^+ specific 17β-HSDase activity was almost 1000 fold higher than the NAD^+ specific 17α-HSDase activity. However the $NADP^+$ specific 17α-estradiol 3-glucuronide dehydrogenase activity was equivalent to the 17β-estradiol dehydrogenase $NADP^+$ specific activity. The $NADP^+$ specific 17α-estradiol dehydrogenase in the liver homogenate had the lowest level of activity of any of the 4 enzyme activities examined.

2. Substrate Specificity of 17-HSDase in Rabbit Liver Cytosol

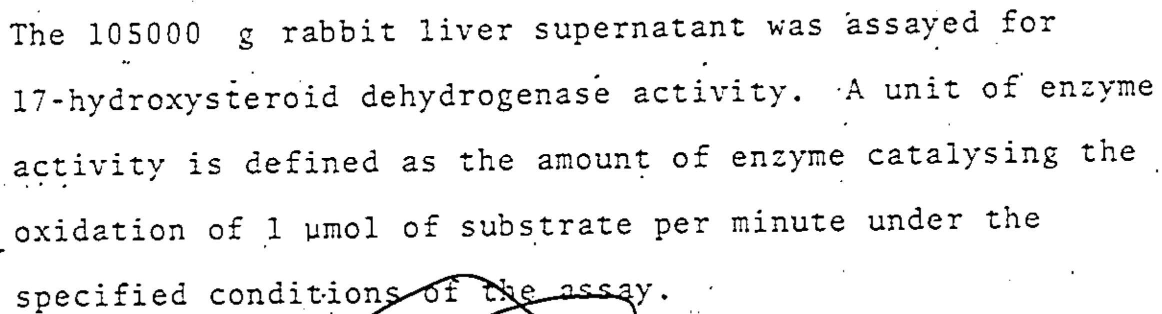
The supernatant, prepared from the 105000 g centrifugation of rabbit liver homogenate, was assayed for $NADP^+$ specific 17-HSDase activity with the substrates 17β- and 17α-estradiol

TABLE 3

17-Hydroxysteroid Dehydrogenase Activity in Rabbit Liver Supernatant

Substrate	Enzyme activity (μunits/mg protein)	
	$NADP^+$	NAD^+
17β-estradiol	88	91
17α-estradiol	61	1.5
17β-estradiol 3-glucuronide	40	-
17α-estradiol 3-glucuronide	275	-

The 105000 g rabbit liver supernatant was assayed for 17-hydroxysteroid dehydrogenase activity. A unit of enzyme activity is defined as the amount of enzyme catalysing the oxidation of 1 μmol of substrate per minute under the specified conditions of the assay.



and their glucuronide conjugates (Table 3). Enzyme activity toward 17β-estradiol was 1.5 times that observed with 17α-estradiol. The activity was, however reversed in the case of the glucuronide substrates, since 17α-estradiol 3-glucuronide was oxidized at 7 times the rate of the 17β-epimer. The 17α-estradiol 3-glucuronide dehydrogenase activity was the highest observed for any of the substrates. NAD^+ was not an effective cofactor for 17α-estradiol dehydrogenase since the activity with $NADP^+$ was about 40 fold higher. However 17β-estradiol dehydrogenase was equally active with either $NADP^+$ or NAD^+.

3. Dehydrogenase Activity as a Function of Time

The 17-HSDase activity in the rabbit liver cytosol was partially purified by 35-75% ammonium sulphate fractionation. This preparation was used to establish the time course of the 17-HSDase reaction under the assay conditions described earlier. Assays with the substrates, 17α-estradiol 3-glucuronide and 17β-estradiol, were terminated after 10, 20, 30, 40 and 50 minutes and the time course of the reactions plotted (Fig. 1). The curves for both substrates were linear up to at least 30 min. The rate of the oxidation of 17α-estradiol 3-glucuronide was slightly higher than that of 17β-estradiol.

FIGURE 1

The 17-Hydroxysteroid Dehydrogenase Activities as a Function of Time

The 35-75% ammonium sulphate fraction of rabbit liver cytosol, containing 17-HSDase activity, was incubated with 17α-estradiol 3-glucuronide and 17β-estradiol. The reactions were stopped at 10, 20, 30, 40 and 50 min intervals.

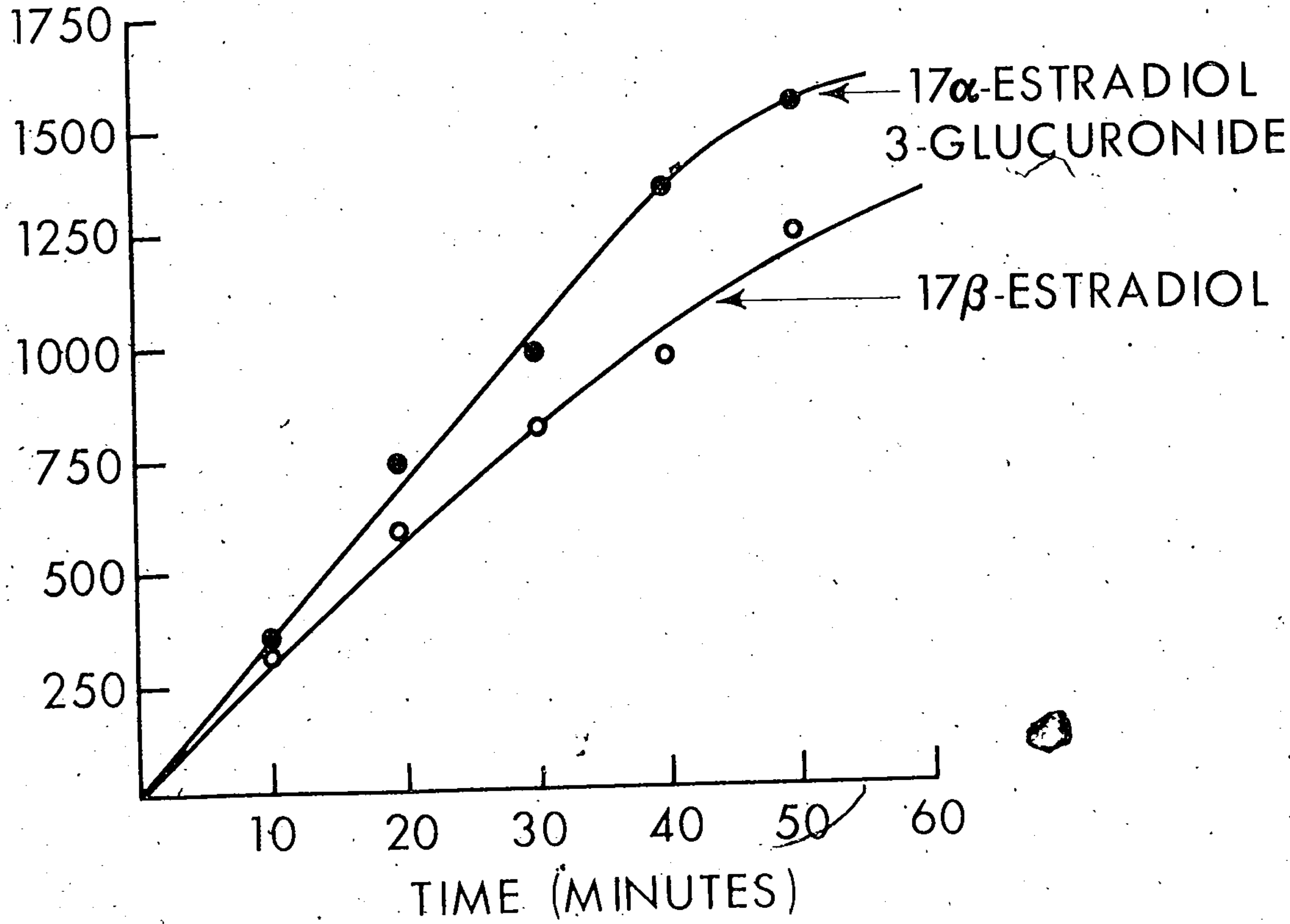
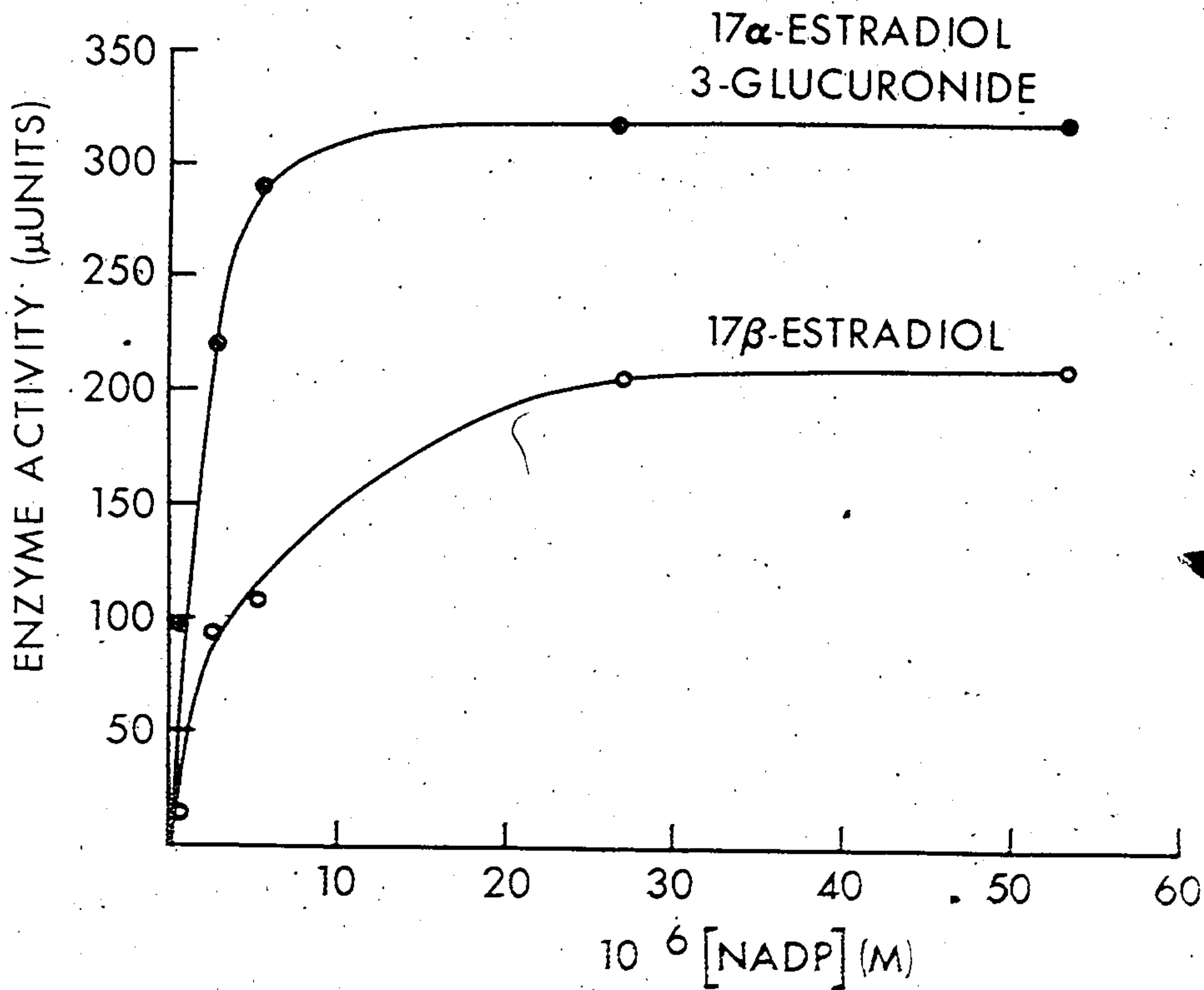

P MOL PRODUCT FORMED
1750
1500
1250
1000
750
500
250
10
20
30
40
50
60
TIME (MINUTES)
17α-ESTRADIOL
3-GLUCURONIDE
17β-ESTRADIOL

FIGURE 2

The Dependence of 17-Hydroxysteroid Dehydrogenase Activity on $NADP^+$ Concentration

The 35-75% ammonium sulphate fraction of rabbit liver cytosol, containing the 17-HSDase activity, was incubated with 17α-estradiol 3-glucuronide or 17β-estradiol and increasing concentrations of $NADP^+$.


17α-ESTRADIOL
3-GLUCURONIDE
17β-ESTRADIOL
ENZYME ACTIVITY (μUNITS)
350
300
250
200
150
100
50
10
20
30
40
50
60
10 6 [NADP] (M)

4. Dehydrogenase Activity as a Function of $NADP^+$ Concentration

The 17-HSDase activity in the 35-75% ammonium sulphate fraction was incubated with the substrates 17α-estradiol 3-glucuronide or 17β-estradiol and increasing concentrations of $NADP^+$ to determine its saturation level. The curves plotted in Fig. 2 indicate that 17α-estradiol 3-glucuronide dehydrogenase was saturated at a lower concentration (33 μmol/l) than 17β-estradiol dehydrogenase (167 μmol/l). The curves also suggest a higher Vmax and a lower Km for the reduction of $NADP^+$ in the presence of 17α-estradiol 3-glucuronide than 17β-estradiol.

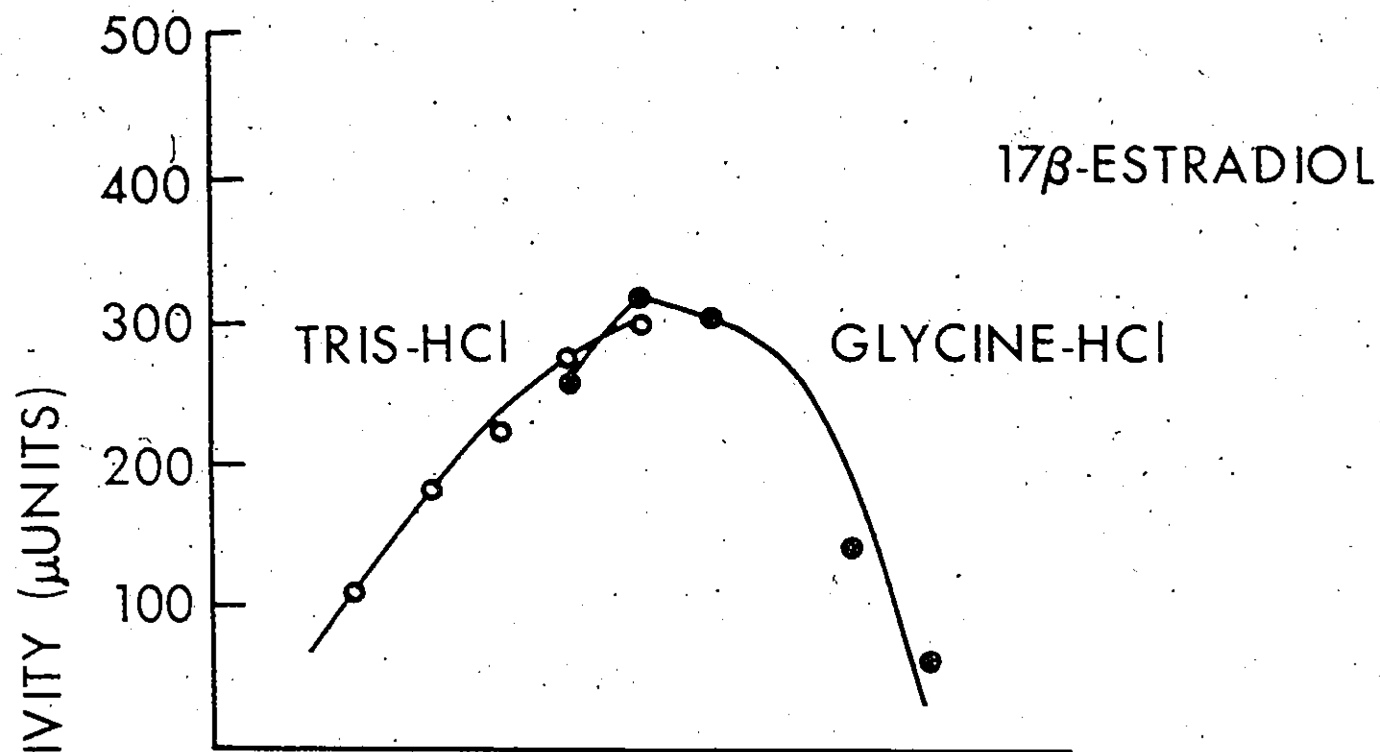
5. Dehydrogenase Activity as a Function of pH

The pH dependance of the 17-HSDase, fractionated by ammonium sulphate precipitation, was determined by incubating the substrates 17α-estradiol 3-glucuronide and 17β-estradiol at several different pH in the range of pH 7.0 to 11.0. Tris-HCl (0.1M) was used to buffer the assays in the lower pH range while glycine-HCl (0.1M) was used to buffer the assays at higher pH values. The curves plotted for the oxidation reaction for the two substrates in Fig. 3 show that both enzyme activities have broad pH optima. The 17β-estradiol dehydrogenase activity was optimal from pH 9 to 9.5 while the 17α-estradiol 3-glucuronide dehydrogenase activity

FIGURE 3

The Dependence of 17-Hydroxysteroid Dehydrogenase Activity on pH

The 35-75% ammonium sulphate fraction of rabbit liver cytosol, containing 17-HSDase activity, was assayed with 17α-estradiol 3-glucuronide and 17β-estradiol from pH 7.0 to 11.0. Tris-HCl (0.1M) was used in the assay with 17α-estradiol 3-glucuronide from pH 7.0 to 10.0 and in the assay with 17β-estradiol from pH 7.0 to 9.0. Glycine-HCl (0.1M) was used in the assays in the pH range from 8.5 to 11.0.


500
400
300
200
100
17β-ESTRADIOL
TRIS-HCl
GLYCINE-HCl
ENZYME ACTIVITY (μUNITS)
500
400
300
200
100
17α-ESTRADIOL
3-GLUCURONIDE
7.0
8.0
9.0
10.0
11.0
12.0
pH

was optimal from pH 9.5 to 10.0. The 17α-estradiol 3-glucuronide dehydrogenase activity declined more steeply at lower pH values than did the 17β-estradiol dehydrogenase activity.

DISCUSSION

The results on the tissue distribution of 17-HSDase in the rabbit, as listed in Table 2, reaffirm previous evidence that the liver is the main site of estrogen metabolism (1). The 17β-HSDase activity in the liver is significantly higher than that in any other organ that was examined. This activity is associated mainly with the NAD^+ specific 17β-estradiol dehydrogenase. The relatively high level of 17α-estradiol and 17α-estradiol 3-glucuronide dehydrogenase activities in the kidney is, at first, difficult to reconcile with the results of Layne & Quamme (54). When these workers perfused rabbit liver in situ with blood containing tritiated estrone, in order to examine its metabolites, they discovered that complete conversion to the double conjugate 17α-estradiol 3-glucuronide 17-N-acetylglucosaminide occurred within the liver. One possible explanation for the high levels of 17α-HSDase activity found in the kidney may be that it serves as a safety mechanism for the detoxification of estrogens which could possibly be de-conjugated after leaving the liver. This explanation is supported by fact that the rabbit kidney also contains N-acetylglucosaminyl transferase.

From a comparison of the levels of 17α-estradiol and 17α-estradiol 3-glucuronide dehydrogenase activities in the

liver homogenate it would appear that the pathway for estrogen metabolism probably involves first, the conjugation with glucuronide at position 3 and then a conversion from either 17β-estradiol 3-glucuronide or estrone 3-glucuronide to 17α-estradiol 3-glucuronide. The conversion to the 17α-epimer prior to conjugation at C 3 on the steroid ring is probably not a major step in the pathway for estrogen metabolism.

It is interesting to note in Table 3 that the levels of activities of the 17-HSDase in the soluble fraction of rabbit liver homogenate is quite different when compared to the whole homogenate. It becomes apparent that, while the majority of the 17α-HSDase activity is localized in the soluble fraction, a considerable amount of the 17β-HSDase activity of rabbit liver resides in the microsomal fraction. The NAD^+ to $NADP^+$ ratio for 17β-estradiol dehydrogenase activity decreases from 5 in the crude homogenate to 1 in the soluble fraction while the similar ratio for 17α-estradiol dehydrogenase activity decreases 25 fold. The lack of nucleotide specificity exhibited by 17β-estradiol dehydrogenase is similar to the results reported for the human placental 17β-estradiol dehydrogenase (35). On the basis of this result and kinetic data a transhydrogenase function was proposed for the placental 17β-estradiol dehydrogenase (55). This function may

also be applicable to the 17β-estradiol dehydrogenase from rabbit liver cytosol.

The data presented in Figs. 1, 2 and 3 indicated that under the conditions of the assay there is a difference in the behaviour of the 17α- and 17β-HSDase activities. This data and the evidence presented in Tables 2 and 3 clearly suggested the presence of two distinct HSDase activities, one for the oxido-reduction of the 17α-hydroxyl and the other active towards the 17β-hydroxyl, in the rabbit liver cytosol. This possibility was supported by earlier work on the separation of 17α- and 17β-HSDase activities from chicken liver (27) but was called into question by Breuer's inability to separate the same activities from rabbit liver (10). We therefore attempted to determine whether 17α- and 17β-HSDases are in fact two distinct and separable enzyme activities in rabbit liver cytosol as was indicated in the preliminary results. During the course of separation of these two activities in our laboratory, Thaler-Dao _et. al_. (9) published results on the partial purification of three 17β-HSDase fractions from rabbit liver cytosol. They showed that one of these fractions did not contain 17α-HSDase activity. They did not, however, monitor the 17α-HSDase activity during their purification procedure.

RESULTS

Part II: Purification of 17-Hydroxysteroid Dehydrogenases

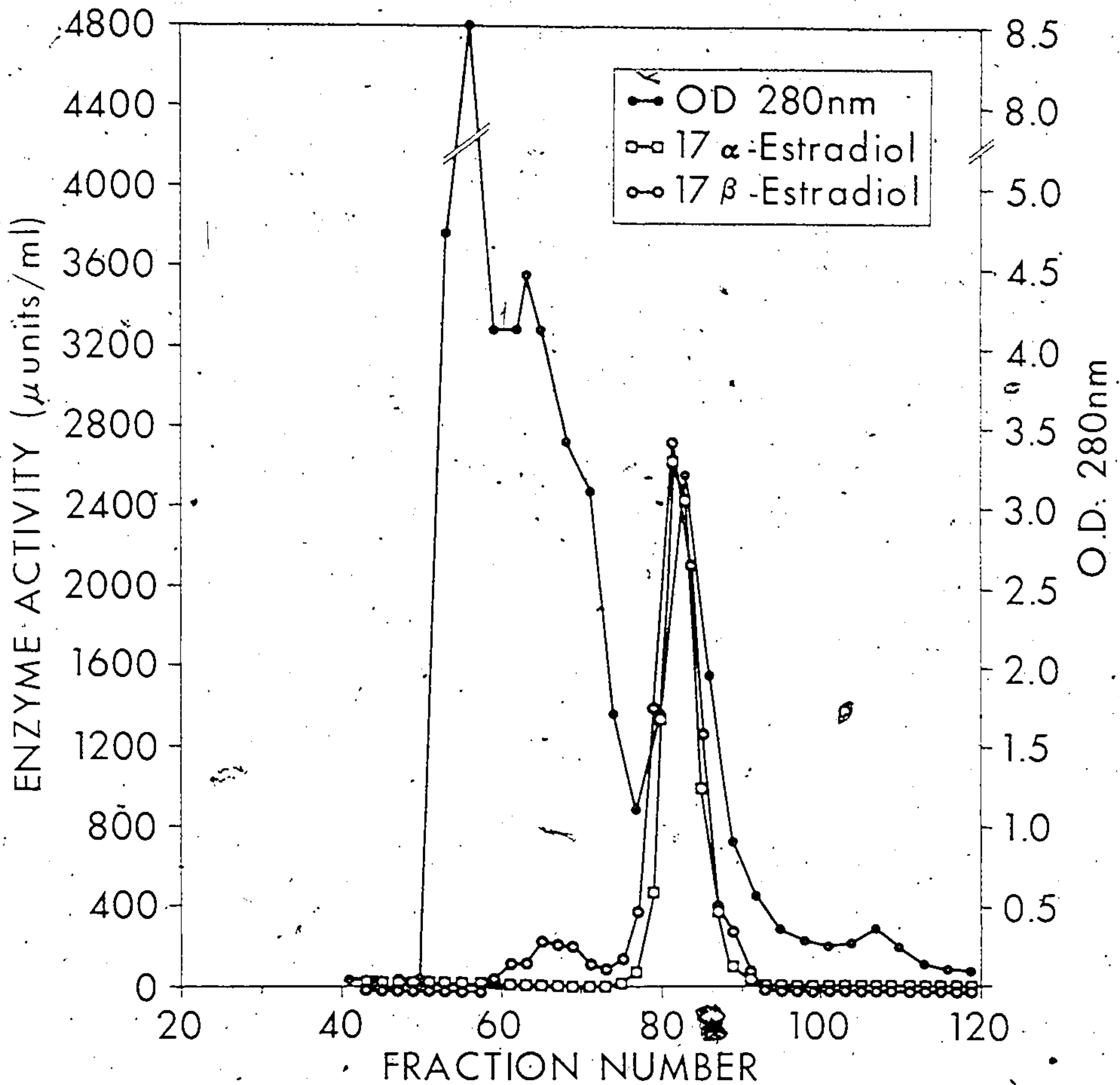
1. Calcium Phosphate Gel Fractionation and Gel Filtration

The calcium phosphate gel fractionation of the 105000 g supernatant of rabbit liver resulted in a 2 fold purification of the 17-HSDase activities (Table 4) but produced no separation of the 17α- and 17β-HSDase activities. Almost total recovery of the enzyme activities was obtained with this procedure. This fraction was further purified by gel filtration on Sephadex G-75 superfine as shown in Fig. 4. The column fractions were assayed for activity toward both 17α- and 17β-estradiol and their glucuronide conjugates. All four enzyme activities were retained on the gel and co-chromatographed, but only the curves for 17α- and 17β-estradiol dehydrogenase activities were plotted in Fig. 4. A small proportion of the total 17β-HSDase activity migrated faster than the bulk of the activity and was eluted between fractions 65 to 70, free of 17α-HSDase activity. When it was necessary to further reduce the level of the contaminating protein, the 17-HSDase fraction was re-chromatographed on the same gel filtration column. This procedure usually resulted in another 1.5 to 2 fold increase in specific activity (Table 4). The

FIGURE 4

Gel Filtration of 17-Hydroxysteroid Dehydrogenase Activity

The 17-HSDase activity fractionated by calcium phosphate gel was further purified by gel filtration on a Sephadex G-75 column (5 x 90 cm), equilibrated in 10mM Tris-HCl, pH 8.0 and 0.5mM DTT (Tris-HCl buffer). The void volume of the column was 500 ml. The column fractions (10 ml) were assayed for 17-HSDase activity with the substrates 17α-estradiol and 17β-estradiol. Usually 10 to 20 μl aliquots of each column fraction were used in the assay.



4800
4400
4000
3600
3200
2800
2400
2000
1600
1200
800
400
0
ENZYME ACTIVITY (μunits/ml)
8.5
8.0
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
O.D. 280nm
OD 280nm
17 α-Estradiol
17 β-Estradiol
20
40
60
80
100
120
FRACTION NUMBER

fold purification of enzyme activity and the percent recovery at each step of purification are listed in Table 4.

2. DEAE-Cellulose Chromatography (Type I)

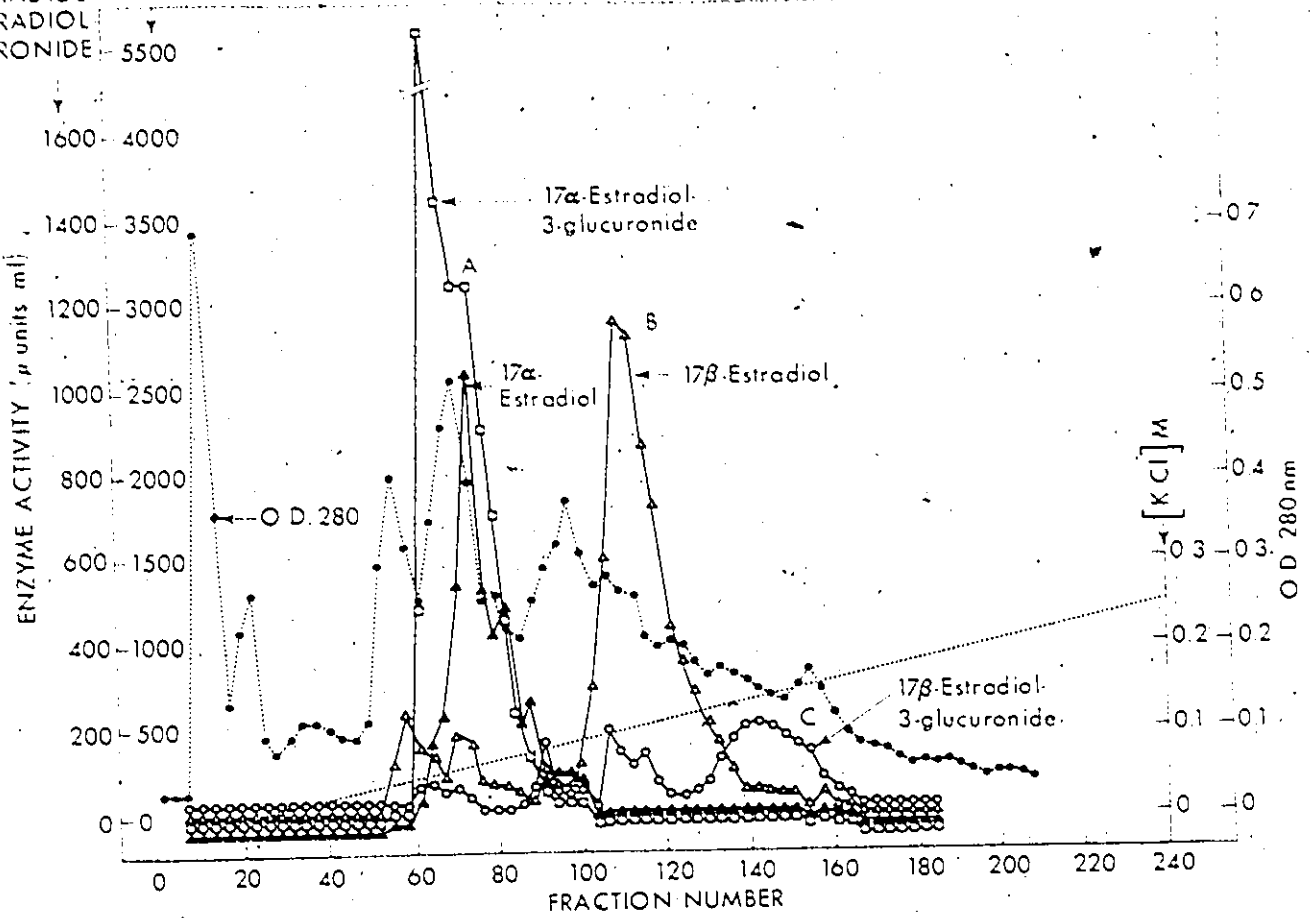
The combined 17-HSDase fractions from the gel filtration column were concentrated and subjected to DEAE-cellulose chromatography. The elution pattern for this procedure is shown in Fig. 5. The column fractions were assayed for activity towards 17α- and 17β-estradiol and their glucuronide conjugates. The dehydrogenase activities for 17α-estradiol and 17α-estradiol 3-glucuronide (Fig. 5, peak A) were eluted together with 0.08M NaCl. Although no separation of 17α-estradiol dehydrogenase from the 17α-estradiol 3-glucuronide dehydrogenase was achieved, the enzyme activities were resolved into 2 peaks which occurred in tubes 64 and 73 respectively.

The majority of the 17β-HSDase activity was eluted with 0.1M NaCl (Fig. 5, peak B). No 17α-HSDase activity was associated with this peak. Minor peaks of 17β-HSDase activity were eluted at lower NaCl concentration, and one of these minor peaks was associated with the 17α-HSDase activity in peak A. Some 17β-estradiol 3-glucuronide dehydrogenase activity was also present in peak B. A larger proportion of

FIGURE 5

DEAE-Cellulose Chromatography (Type I) of the 17-Hydroxysteroid Dehydrogenase Activity

The 17-HSDase activity fractionated by gel filtration was chromatographed on a DEAE-cellulose column (2.5 x 40 cm). The enzyme activities were eluted with a 2.4 l linear gradient of 0 to 250mM KCl in the usual Tris-HCl buffer. The column fractions (10 ml) were assayed for HSDase activity with the substrates 17α-estradiol, 17β-estradiol and their glucuronide conjugates. Enzyme aliquots of 20, 50 and 100 µl were usually used in the assays for 17α-estradiol 3-glucuronide, 17α- and 17β-estradiol, and 17β-estradiol 3-glucuronide dehydrogenase activities respectively.



17α ESTRADIOL
17β ESTRADIOL
17β ESTRADIOL 3 GLUCURONIDE
17α ESTRADIOL 3 GLUCURONIDE
ENZYME ACTIVITY (μ units ml)
O D. 280
17α-Estradiol-3-glucuronide
17α-Estradiol
17β-Estradiol
17β-Estradiol-3-glucuronide
A
B
C
[KCl] M
O D 280 nm
FRACTION NUMBER

17β-estradiol 3-glucuronide dehydrogenase activity was eluted with a NaCl concentration of 0.14 M (Fig. 5, peak C). Since major contaminants were present in each fraction, as indicated by disc gel electrophoresis, an attempt was made to achieve a better purification of the desired fractions by decreasing the slope of the linear salt gradient.

3. DEAE-Cellulose Chromatography (Type II)

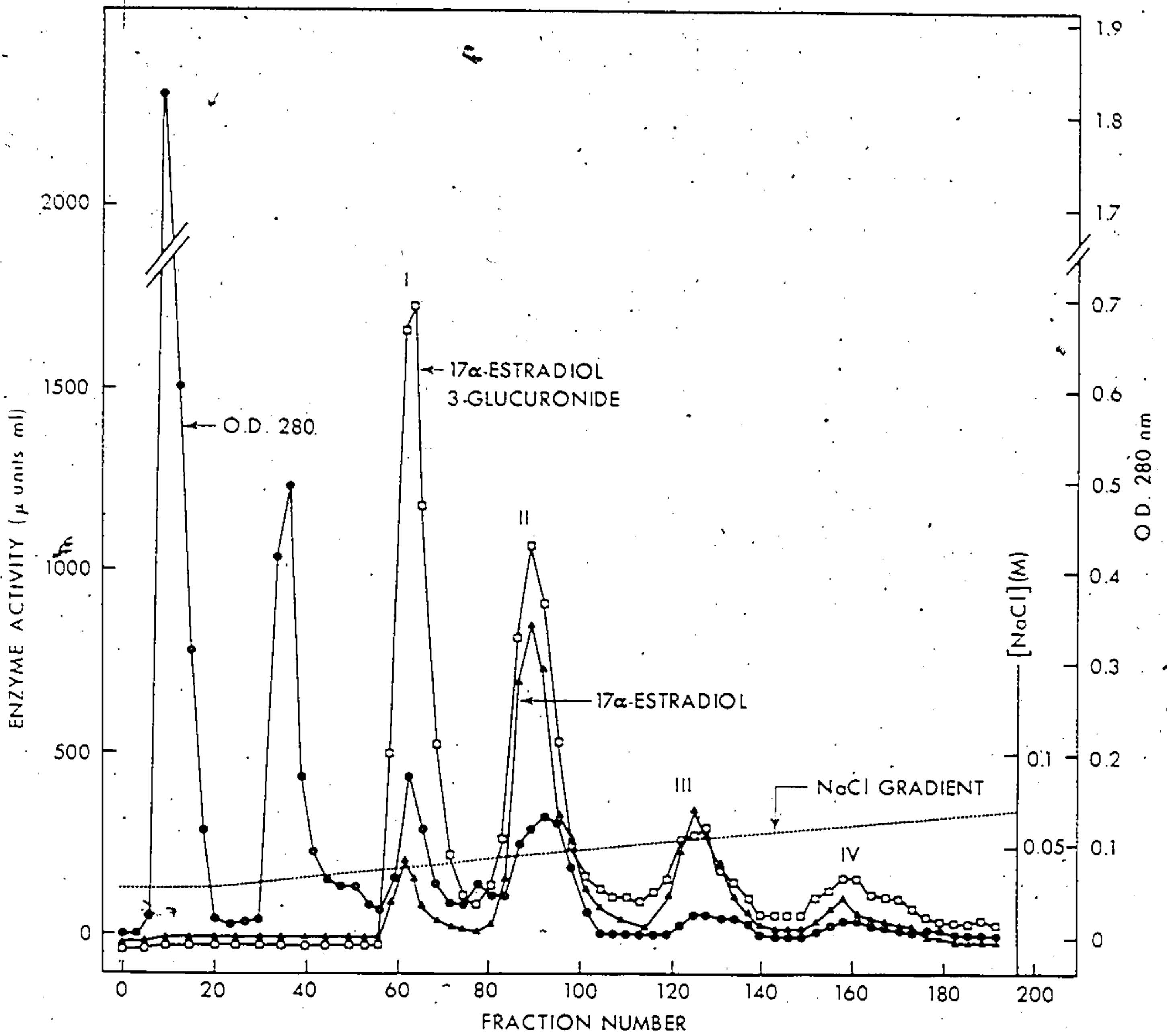
Rabbit liver 17α- and 17β-HSDase, partially purified by calcium phosphate gel fractionation and Sephadex gel filtration, were further purified by DEAE-cellulose chromatography. First, the 17α-estradiol and 17α-estradiol 3-glucuronide dehydrogenase activities were eluted from the column with a linear salt gradient from 0.025 to 0.07 M (Fig. 6) and then the 17β-estradiol and 17β-estradiol 3-glucuronide activities were eluted with a linear NaCl gradient from 0.07 to 0.16 M (Fig. 7).

The first NaCl gradient eluted two major and 2 minor 17α-HSDase activities (Fig. 6, peaks I, II, III and IV). Peak I contained a much higher proportion of 17α-estradiol 3-glucuronide dehydrogenase activity than 17α-estradiol dehydrogenase activity. Peaks II, III and IV contained approximately equal amounts of activity for both substrates. Total recovery of

FIGURE 6

DEAE-Cellulose Chromatography (Type II) of 17α-Hydroxysteroid Dehydrogenase Activity

The 17-HSDase activity fractionated by gel filtration was chromatographed on a DEAE-cellulose column (2.5 x 40 cm), equilibrated in the usual Tris-HCl buffer containing 25mM NaCl. The 17α-HSdase activities were eluted with a 2 l linear gradient of 25 to 70 mM NaCl in Tris-HCl buffer. The column fractions (10 ml) were assayed for 17α-HSDase activity with 17α-estradiol and its glucuronide conjugate. The enzyme aliquot size used in the assay was as described in Fig. 5.



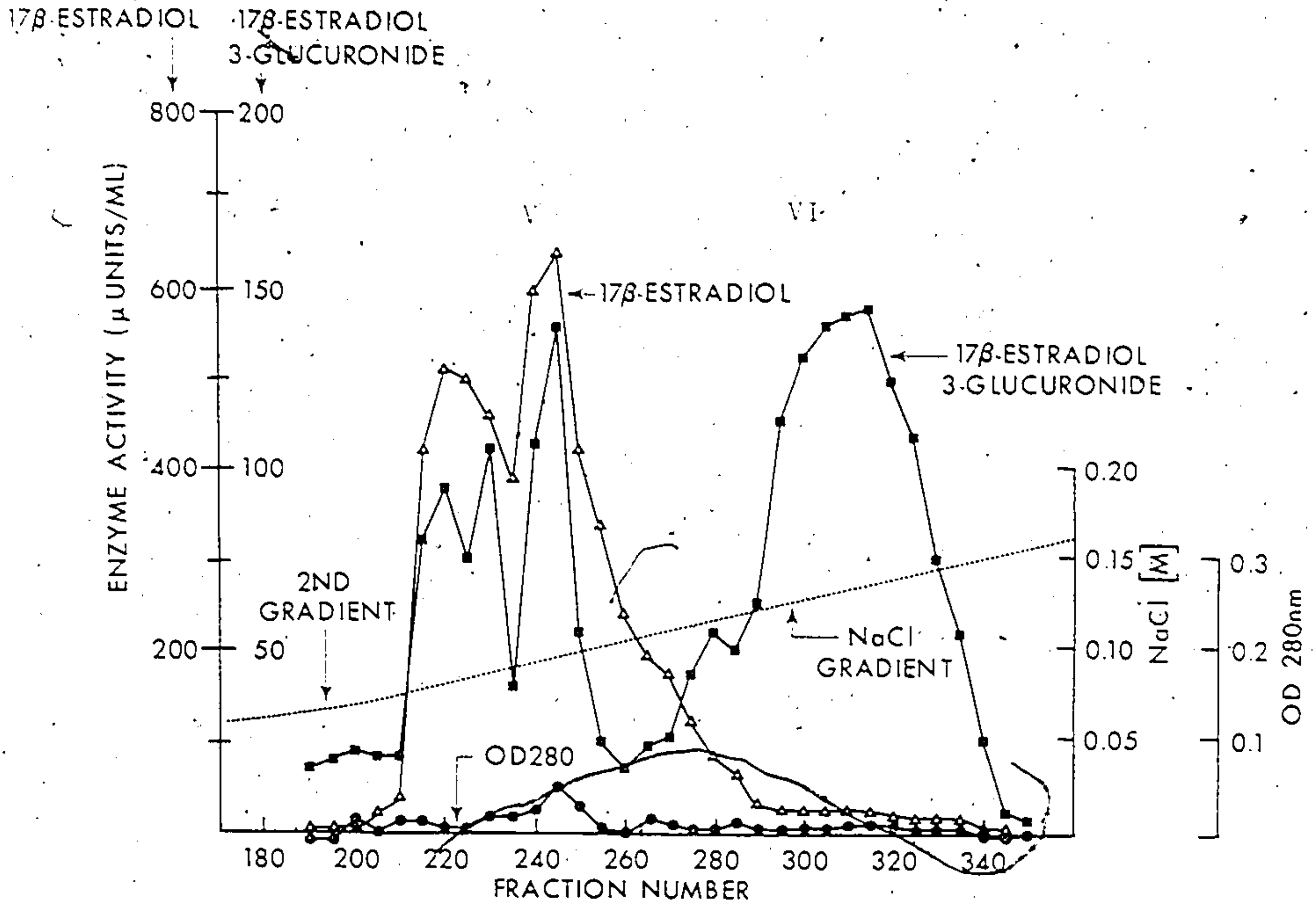
ENZYME ACTIVITY (μ units ml)
O.D. 280 nm
[NaCl](M)
FRACTION NUMBER
O.D. 280
17α-ESTRADIOL 3-GLUCURONIDE
17α-ESTRADIOL
NaCl GRADIENT
I
II
III
IV
0
500
1000
1500
2000
0
20
40
60
80
100
120
140
160
180
200
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
1.7
1.8
1.9
0.05
0.1

FIGURE 7

DEAE-Cellulose Chromatography (Type II) of

17β-Hydroxysteroid Dehydrogenases

After the elution of the 17α-HSDases from the DEAE-cellulose column (as described in Fig. 6), the 17β-HSDase activities were eluted with a 1.5 l linear gradient of 70 to 160mM NaCl in the usual Tris-HCl buffer. The column fractions (10 ml) were assayed for 17β-HSDase activity toward the substrates 17β-estradiol and 17β-estradiol 3-glucuronide using 50 and 10 μl enzyme aliquots respectively.


17β-ESTRADIOL
17β-ESTRADIOL 3-GLUCURONIDE
800
200
600
150
400
100
200
50
ENZYME ACTIVITY (μ UNITS/ML)
V
VI
17β-ESTRADIOL
17β-ESTRADIOL 3-GLUCURONIDE
2ND GRADIENT
NaCl GRADIENT
OD280
0.20
0.15
0.10
0.05
NaCl [M]
0.3
0.2
0.1
OD 280nm
180
200
220
240
260
280
300
320
340
FRACTION NUMBER

17α-estradiol dehydrogenase and 17α-estradiol 3-glucuronide dehydrogenase activity was 40% and 24% respectively (Table 4). Disc gel electrophoresis (Fig. 8) and enzyme activity staining on acrylamide gels showed that only peak III contained one protein band and one enzyme band. Peaks I, II and IV contained at least 2 protein bands and at least 2 enzyme bands.

The second NaCl gradient eluted the 17β-estradiol dehydrogenase activity and the 17β-estradiol 3-glucuronide dehydrogenase activity. The 17β-estradiol dehydrogenase activity in peak V was 8 times higher than the dehydrogenase activity for the glucuronide conjugate (Table 4), whereas, in peak VI the 17β-estradiol 3-glucuronide dehydrogenase activity was 4 times higher than the 17β-estradiol dehydrogenase activity. Disc gel electrophoresis and activity staining in acrylamide gels revealed multiple bands of protein corresponding to multiple bands of enzyme activity for both peaks V and VI (Fig. 7, peak V and VI). Total recovery of 17β-estradiol and 17β-estradiol 3-glucuronide dehydrogenase activities was 56% and 35% respectively (Table 4).

4. Preliminary Electrofocusing of 17-Hydroxysteroid Dehydrogenase Activity

Since activity staining and acrylamide gel electrophoresis of 17-HSDase fractions revealed a lack of purity or homogeneity

TABLE 4.

Purification of 17-Hydroxysteroid Dehydrogenases

The 17-HSDase activities were assayed at different stages of purification to determine the relative degree of purification and the recovery of enzyme activity. The fold purification is based on the increase in specific activity of the enzyme when compared to the enzyme activity in the 105000 xg supernatant. Total recovery * represents the sum of the 17α- and 17β-HSDase activities after DEAE-cellulose chromatography.

TABLE 4

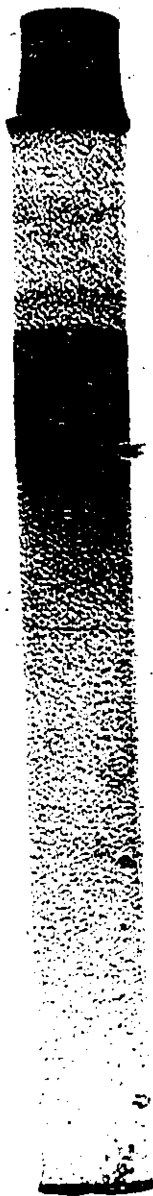
Purification of 17-Hydroxysteroid Dehydrogenases

Purification Step	17α-estradiol		17β-estradiol		17α-estradiol 3-glucuronide		17β-estradiol 3-glucuronide	
	Fold Purification	% Recovery	Fold Purification	% Recovery	Fold Purification	% Recovery	Fold Purification	% Recovery
Calcium Phosphate Gel Fractionation	2	83	2	82	3	88	2	92
Gel Filtration 1	12	97	17	129	8	70	10	76
Gel Filtration 2	18	61	32	100	15	36	13	43
DEAE								
Pk I	19	5	-	-	57	11	-	-
Pk II	63	22	-	-	31	9	-	-
Pk III	75	10	-	-	27	3	-	-
Pk IV	28	3	-	-	6	1	-	-
Pk V	-	-	90	53	-	-	-	13
Pk VI	-	-	-	3	-	-	50	22
Total % Recovery *		40		56		24		35

FIGURE 8

Polyacrylamide Gel Electrophoresis of the 17α-Hydroxysteroid Dehydrogenase Activities Fractionated by DEAE-Cellulose Chromatography

Aliquots of the pooled 17α-HSDase fractions I, II, III and IV were dialysed against Tris-HCl buffer and then electrophoresed in 10% Davis gels (50) with protein loads of 5-10 μg per gel. A current of 3mAmp per gel was maintained during electroporesis. The gels were stained for protein with Coomassie Brilliant Blue. All gels were pre-run prior to electrophoresis of samples except for the gel with sample I.



I II III IV

of the enzyme activities, it was decided to attempt a further fractionation of the activities by isoelectrofocusing. A rabbit liver 17-HSDase sample, partially purified by calcium phosphate gel fractionation and gel filtration on Sephadex G-75 was electrofocused over a pH range from 3 to 9 to establish the conditions required for the separation of 17-HSDases activities over a narrower pH range. The results of the preliminary electrofocusing experiment are detailed in Fig. 9. The column fractions were assayed for dehydrogenase activity using as substrates 17α-estradiol and 17β-estradiol. Both activities focused together in the pH range from 5 to 6.

5. Electrofocusing of 17α-Hydroxysteroid Dehydrogenase Activity over a pH Range of 5-7

A 17α-HSDase sample, partially purified by calcium phosphate gel fractionation, gel filtration on Sephadex G-75 and by DEAE-cellulose chromatography (Type I) (Fig. 5, peak A) was electrofocused over a pH range from 5 to 7. The column fractions were assayed for both 17α-estradiol and 17α-estradiol 3-glucuronide dehydrogenase activities and the activity profile is shown in Fig. 10. The single peak of 17α-HSDase activity, purified by DEAE-cellulose chromatography, fractionated into at least 6 peaks of activity after electrofocusing. Peaks 1, 3 and 5 contained equal levels of activity for both substrates while peaks 2, 4 and, in particular, 6 contained

FIGURE 9

Isoelectric Focusing of 17-Hydroxysteroid Dehydrogenase Activity

A partially purified 17-HSDase sample (40 mg) was focused in a 2% ampholine gradient from pH 3 to 9 in an LKB 4102 column with a 110 ml capacity. A linear glycerol gradient (65%-4%) was used to stabilize the pH gradient. Column fractions (1 ml) were assayed for 17α- and 17β-estradiol dehydrogenase activity.

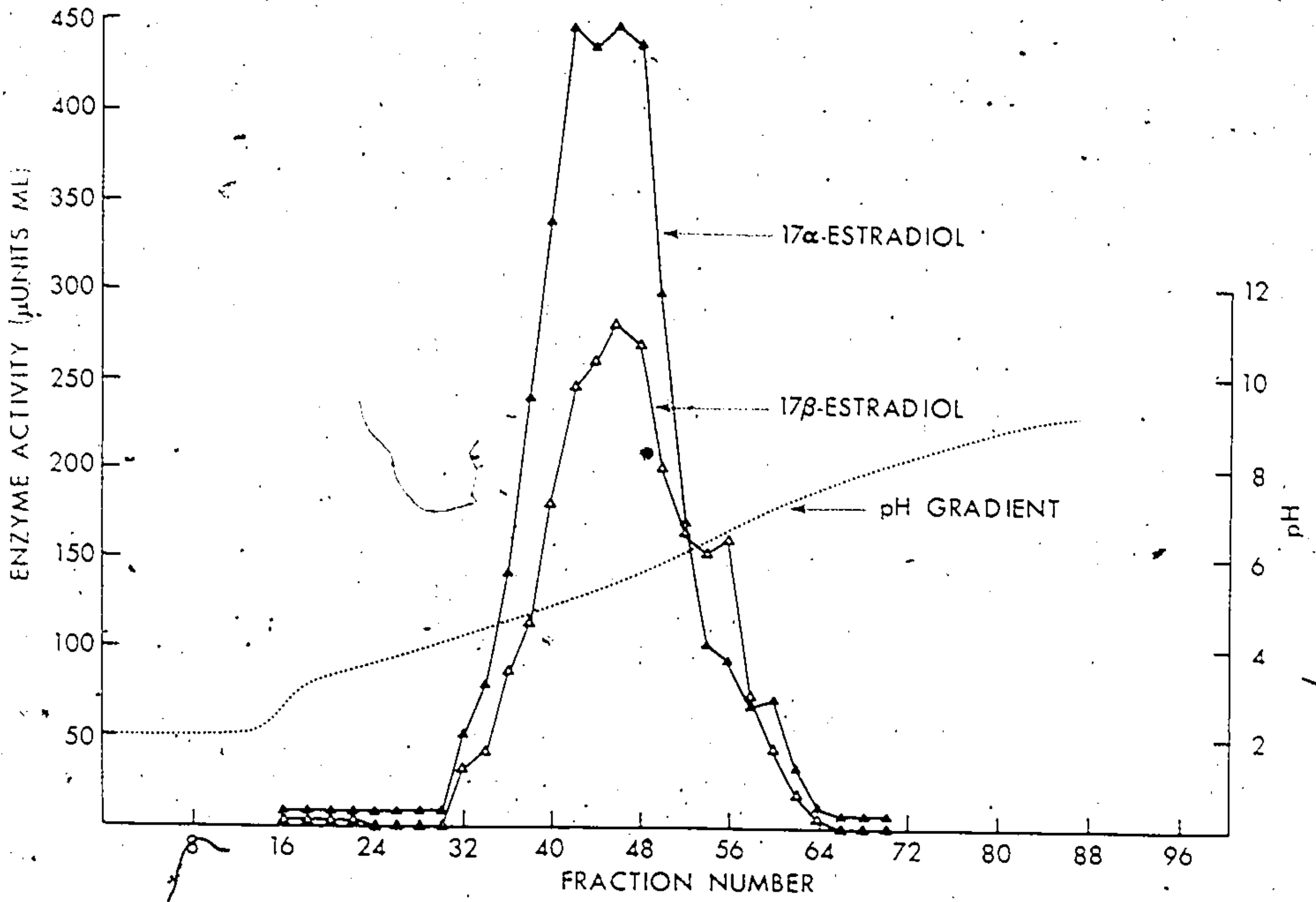
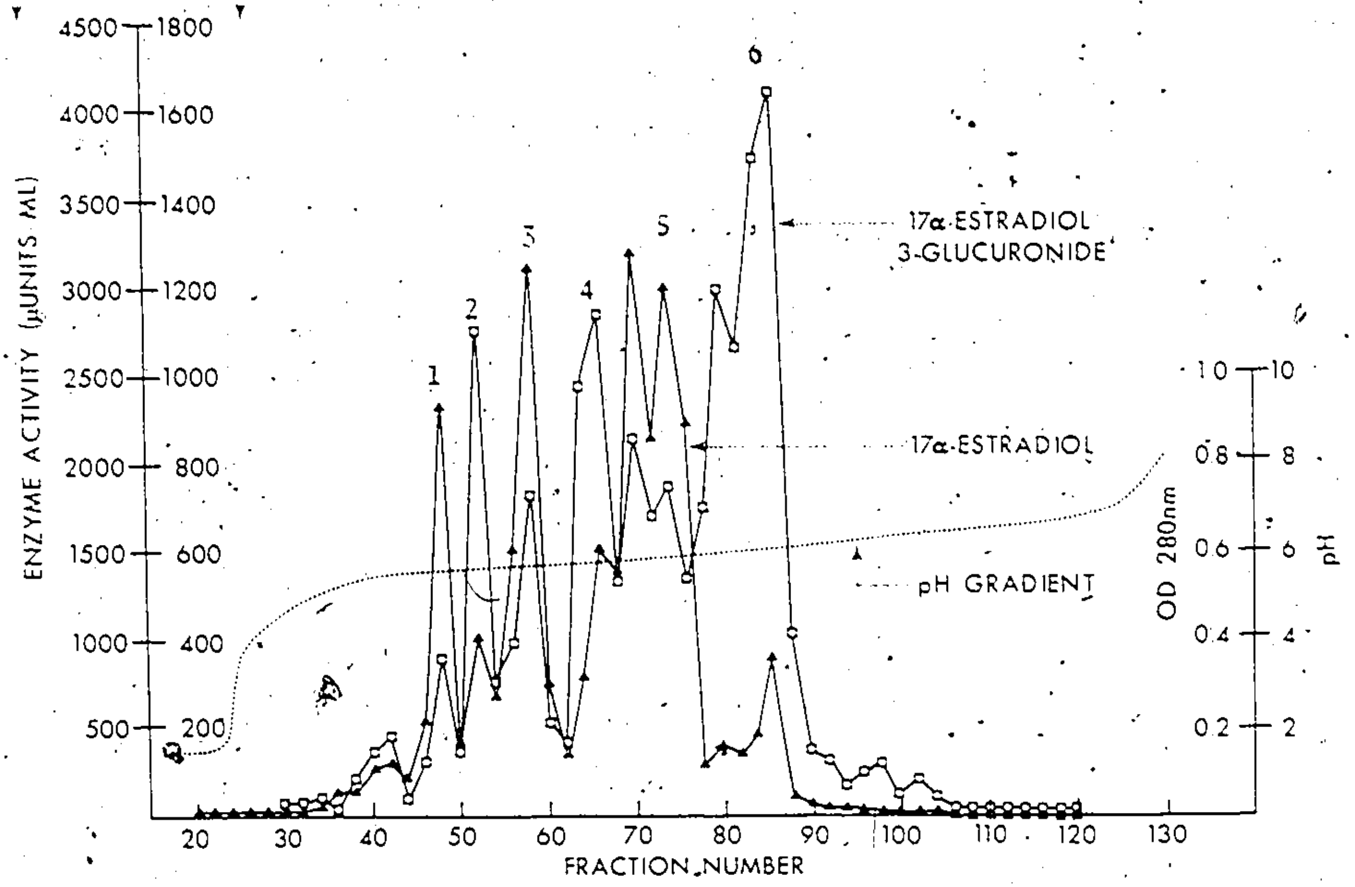

ENZYME ACTIVITY (µUNITS ML)
450
400
350
300
250
200
150
100
50
17α-ESTRADIOL
17β-ESTRADIOL
pH GRADIENT
pH
12
10
8
6
4
2
8
16
24
32
40
48
56
64
72
80
88
96
FRACTION NUMBER

FIGURE 10

Isoelectrofocusing of 17α-Hydroxysteroid Dehydrogenase Activity

A partially purified 17α-HSDase fraction (Fig. 5, peak A) was electrofocused in a 2% ampholine gradient from pH 5 to 7 in an LKB 8102 column with a 440 ml capacity. A sucrose gradient of 47% to 5% (w/v) with 0.5mM DTT was used to stabilize the pH gradient. The column fractions (2 ml) were assayed for 17α-estradiol and 17α-estradiol 3-glucuronide dehydrogenase activities using 50 and 20 μl enzyme aliquots respectively.



17α ESTRADIOL 3 GLUCURONIDE
17α-ESTRADIOL
ENZYME ACTIVITY (μUNITS/ML)
4500
4000
3500
3000
2500
2000
1500
1000
500
1800
1600
1400
1200
1000
800
600
400
200
1
2
3
4
5
6
17α-ESTRADIOL 3-GLUCURONIDE
17α-ESTRADIOL
pH GRADIENT
OD 280nm
pH
1.0
0.8
0.6
0.4
0.2
10
8
6
4
2
20
30
40
50
60
70
80
90
100
110
120
130
FRACTION NUMBER

much higher activity towards 17α-estradiol 3-glucuronide than towards the free steroid. Most of the enzyme activity focused in the pH range from 5.4 to 6.1. Acrylamide gel electrophoresis revealed a lack of homogeneity in all of these fractions.

6. Electrofocusing of 17α-Hydroxysteroid Dehydrogenase Fraction I Purified by DEAE-Cellulose Chromatography

The 17α-HSDase fraction I purified by DEAE-cellulose chromatography (Type II) (Fig. 6, peak I) was electrofocused in a pH gradient from 5 to 7. After focusing, the column fractions were assayed for dehydrogenase activity towards the substrates 17α-estradiol and 17α-estradiol 3-glucuronide. Three peaks of 17α-HSDase activity were separated as shown in Fig 11. All three peaks had activity towards both, 17α-estradiol and 17α-estradiol 3-glucuronide, but peak IC exhibited greater activity toward the glucuronide conjugate. These enzyme fractions were pooled and re-focused over a narrower pH range from 5.6 to 6.6. The column fractions were assayed for 17α-estradiol dehydrogenase activity. The same enzyme profile was obtained over the narrower pH range (Fig. 12) but a better separation of enzyme activities was achieved.

FIGURE 11

Isoelectrofocusing of 17α-Hydroxysteroid Dehydrogenase Fraction I

A 17α-HSDase fraction (Fig. 6, peak I) was electrofocused in a 5% ampholine gradient from pH 5 to 7 in an LKB 8102 column with a 440 ml capacity. A sucrose gradient of 47% to 5% (w/v) with 0.5 mM DTT was used to stabilize the pH gradient. The column fractions (2 ml) were assayed for 17α-estradiol and 17α-estradiol 3-glucuronide dehydrogenase activities using 100 and 10 µl enzyme aliquots respectively.

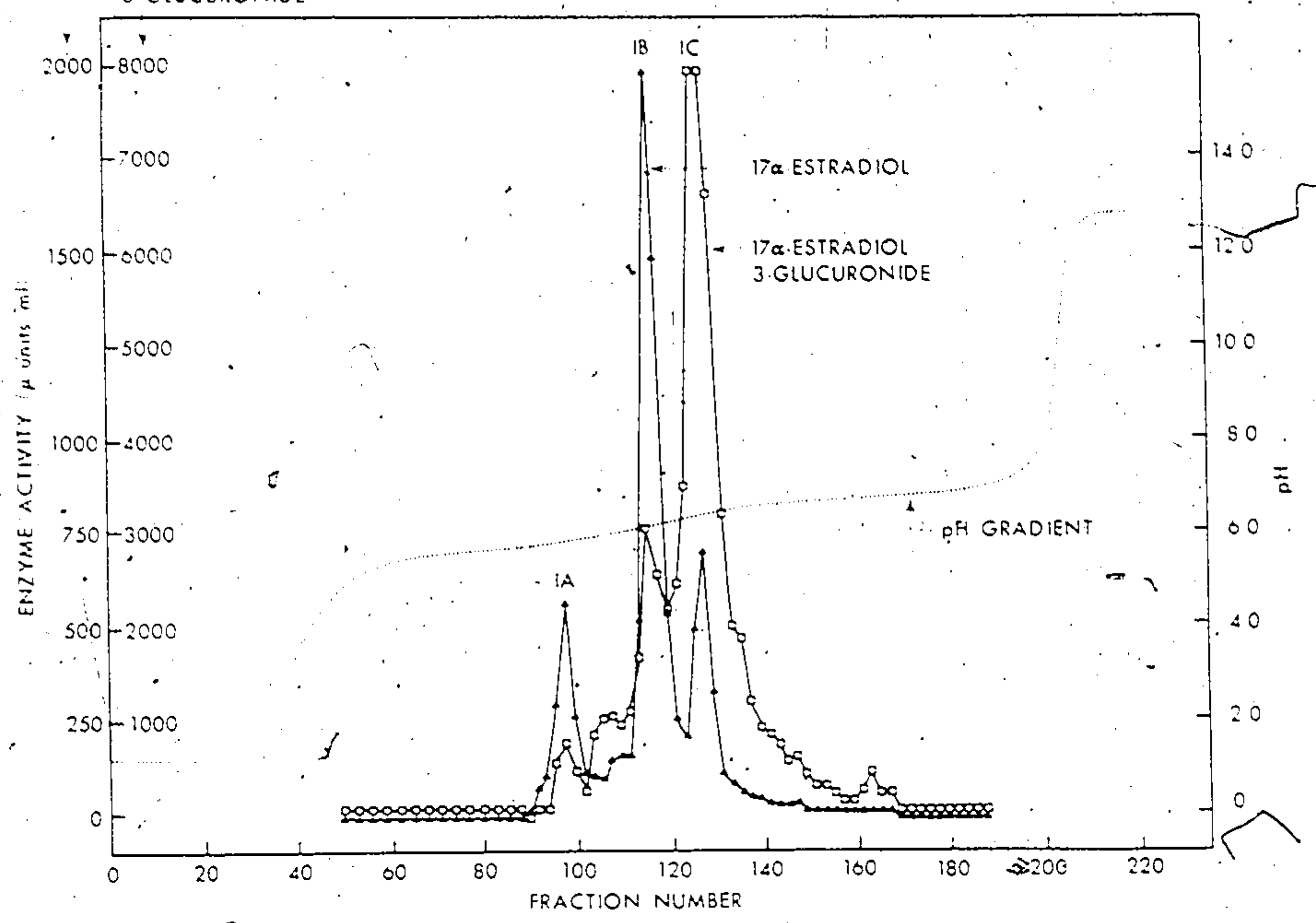
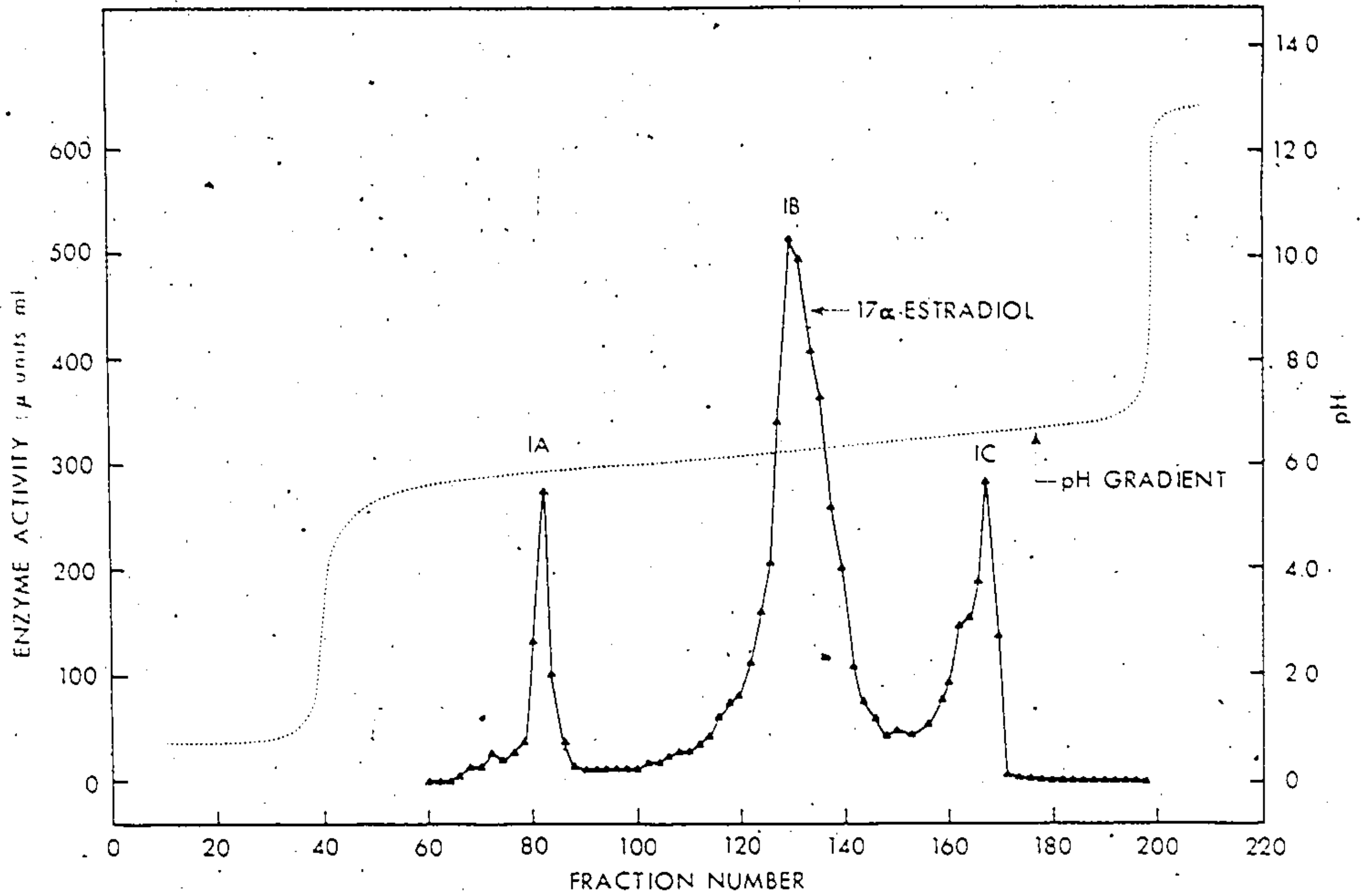

17α ESTRADIOL
17α-ESTRADIOL 3-GLUCURONIDE
IB
IC
17α-ESTRADIOL
17α-ESTRADIOL 3-GLUCURONIDE
IA
pH GRADIENT
ENZYME ACTIVITY (μ units/ml)
pH
FRACTION NUMBER

FIGURE 12

Re-focusing of 17α-Hydroxysteroid Dehydrogenase Fraction I

The pooled 17α-HSDase activities (Fig. 11 peaks IA, IB and IC) were re-focused in a linear sucrose gradient (47% to 5% (w/v) without additional ampholine. Column fractions (2 ml) were assayed for 17α-estradiol dehydrogenase activity using 100 μl enzyme aliquots.


ENZYME ACTIVITY (μ units/ml)
pH
FRACTION NUMBER
IA
IB
IC
17α-ESTRADIOL
pH GRADIENT
0
100
200
300
400
500
600
0
20
40
60
80
100
120
140
160
180
200
220
2.0
4.0
6.0
8.0
10.0
12.0
14.0

7. Electrofocusing of 17α-Hydroxysteroid Dehydrogenase Fraction II Purified by DEAE-Cellulose Chromatography

The 17α-HSDase fraction II purified by DEAE-cellulose chromatography (Fig. 6, peak II) was fractionated into two peaks of activity by electrofocusing in a 5% ampholine pH gradient from 5 to 8 (Fig. 13). When a similar fraction was electrofocused over a much narrower pH range (5.4-6.4) four enzyme activities were observed (Fig. 14). Peak IIA and IIC contained much higher activity toward 17α-estradiol 3-glucuronide than towards the free steroid and peak IID had approximately the same level of activity for both substrates. The enzyme peaks IIA and IIB were not clearly separated. Re-focusing of the pooled fractions IIA and IIB did not resolve the two activities.

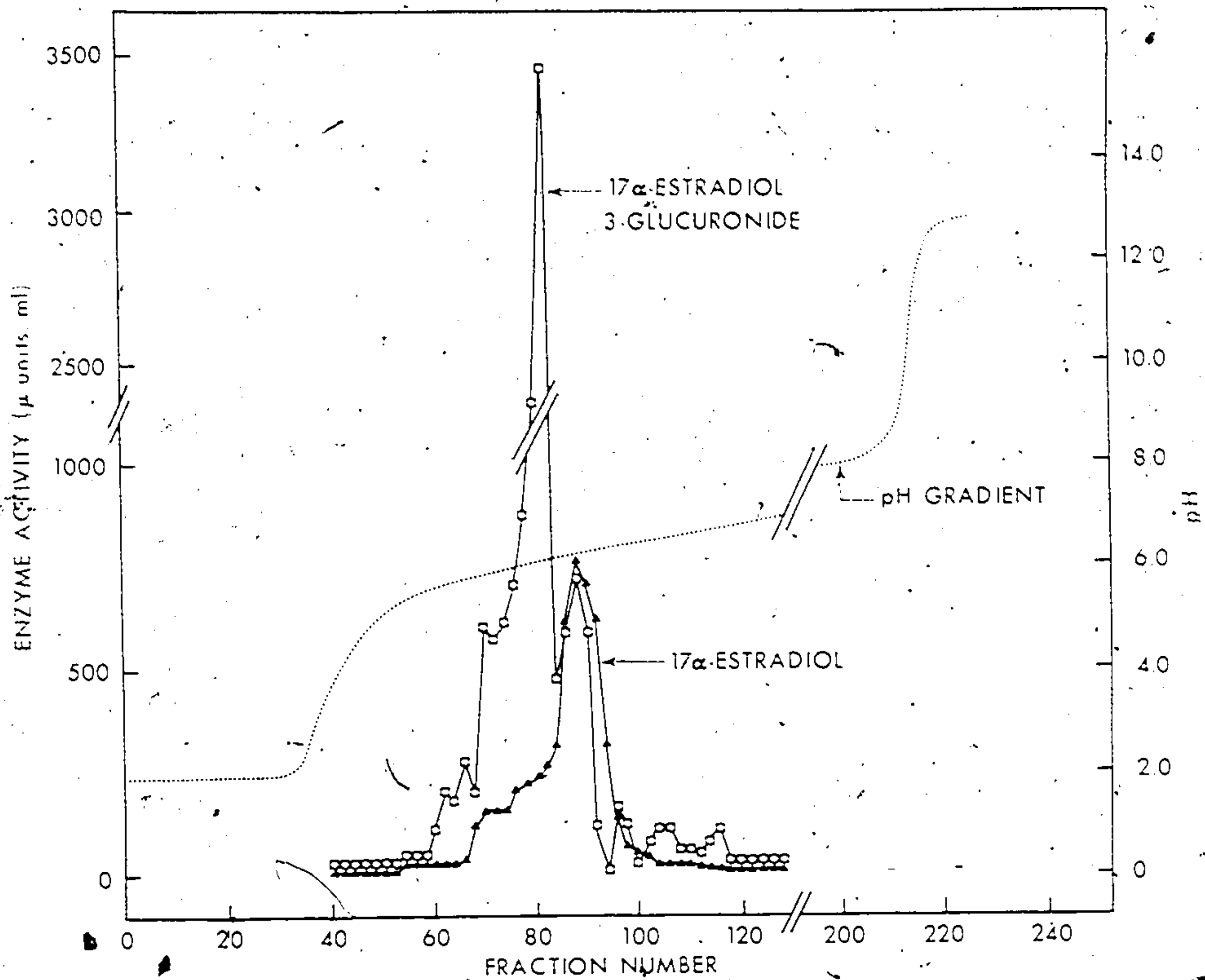
The purity of the 17α-HSDase was established by electrophoresis in polyacrylamide gels by the method of Davis (50) and Weber and Osborn (52). The electrophoretic profile of isolated enzymes IA, IB, IC, IIC, IID and III are presented in Fig. 15. All enzyme samples, except IA, migrated as a single band on the basic acrylamide gels. Two protein bands were visible after electrophoresis of fraction I A. However, after electrophoresis on SDS gels (Fig. 16) the protein in fraction IA appeared as only one protein band.

FIGURE 13

Isoelectrofocusing of 17α-Hydroxysteroid

Dehydrogenase Fraction II

A 17α-HSDase fraction (Fig. 6, peak II) was electrofocused in a 5% ampholine gradient from pH 5 to 8 in an LKB 8102 column with 440 ml capacity. A sucrose gradient of 47% to 5% (w/v) with 0.5mM DTT was used to stabilize the pH gradient. The column fractions (2 ml) were assayed for 17α-estradiol and 17α-estradiol 3-glucuronide dehydrogenase activities using 100 and 20 µl enzyme aliquots respectively.



3500
3000
2500
1000
500
0
ENZYME ACTIVITY (μ units/ml)
17α-ESTRADIOL
3-GLUCURONIDE
17α-ESTRADIOL
pH GRADIENT
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0
pH
0
20
40
60
80
100
120
200
220
240
FRACTION NUMBER

FIGURE 14

Isoelectrofocusing of 17α-Hydroxysteroid

Dehydrogenase Fraction II

A 17α-HSDase (Fig, 6, peak II) was focused in a 1.5% ampholine narrow range pH gradient (pH 5.4 to 6.4) stabilized by a sucrose gradient from 47% to 5% (w/v). The narrow pH range ampholytes were prepared by electrofocusing a 5% ampholine solution without the protein sample. The column fractions (2 ml) were assayed for 17α-estradiol and 17α-estradiol 3-glucuronide dehydrogenase activities using 50 and 20 μl enzyme aliquots respectively.

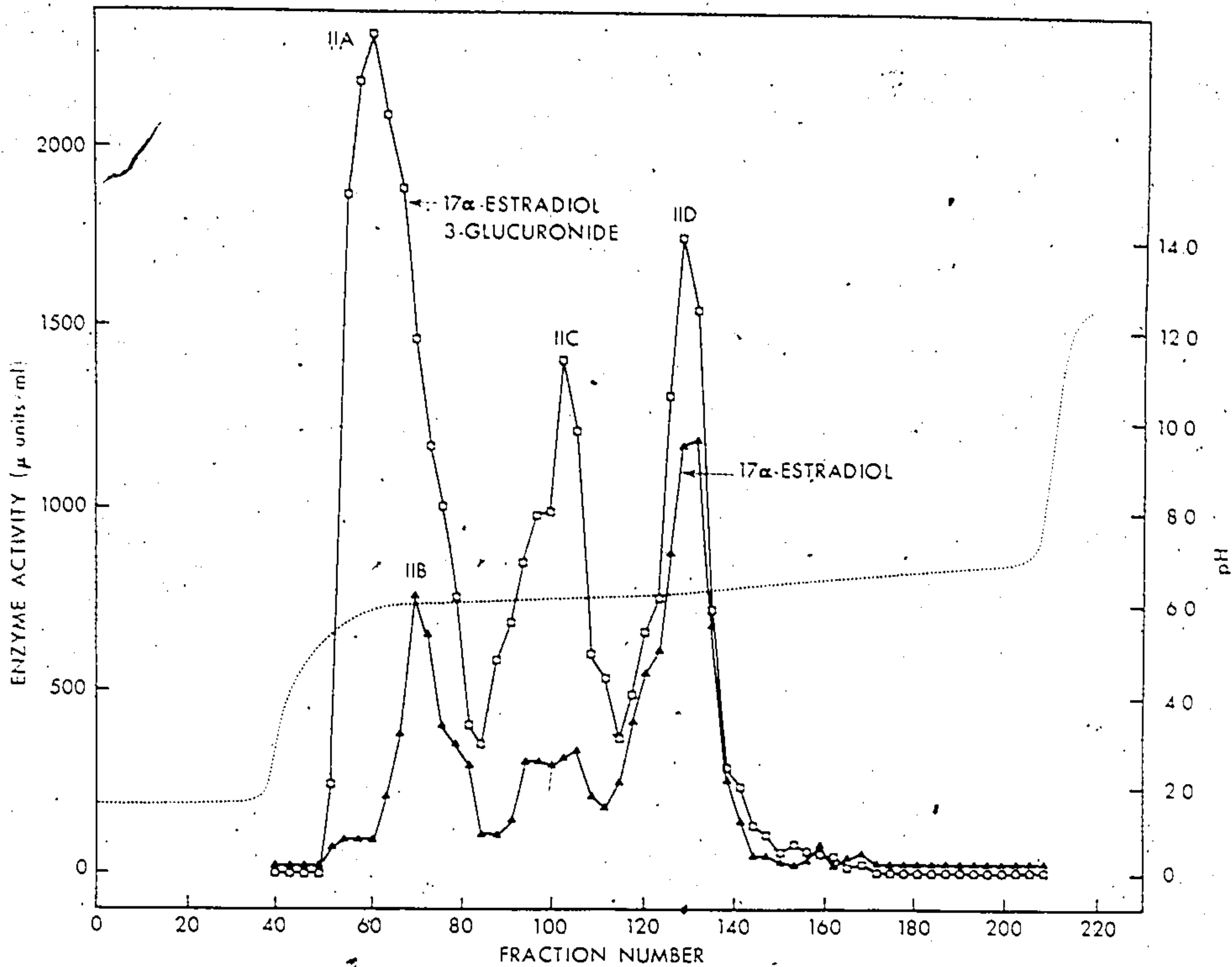

IIA
IIB
IIC
IID
17α-ESTRADIOL
3-GLUCURONIDE
17α-ESTRADIOL
ENZYME ACTIVITY (μ units·ml)
pH
FRACTION NUMBER
0
500
1000
1500
2000
2.0
4.0
6.0
8.0
10.0
12.0
14.0
0
20
40
60
80
100
120
140
160
180
200
220

FIGURE 15

Polyacrylamide Gel Electrophoresis of the

17α-Hydroxysteroid Dehydrogenases

The 17α-HSDases fractionated by DEAE-cellulose chromatography and isoelectrofocusing were electrophoresed in 10% Davis' gels (50). The concentrations of the proteins applied to the gels were in the range of 1 to 5 μg. The gels were stained for protein with Coomassie Brilliant Blue. All gels were pre-run except for IC.

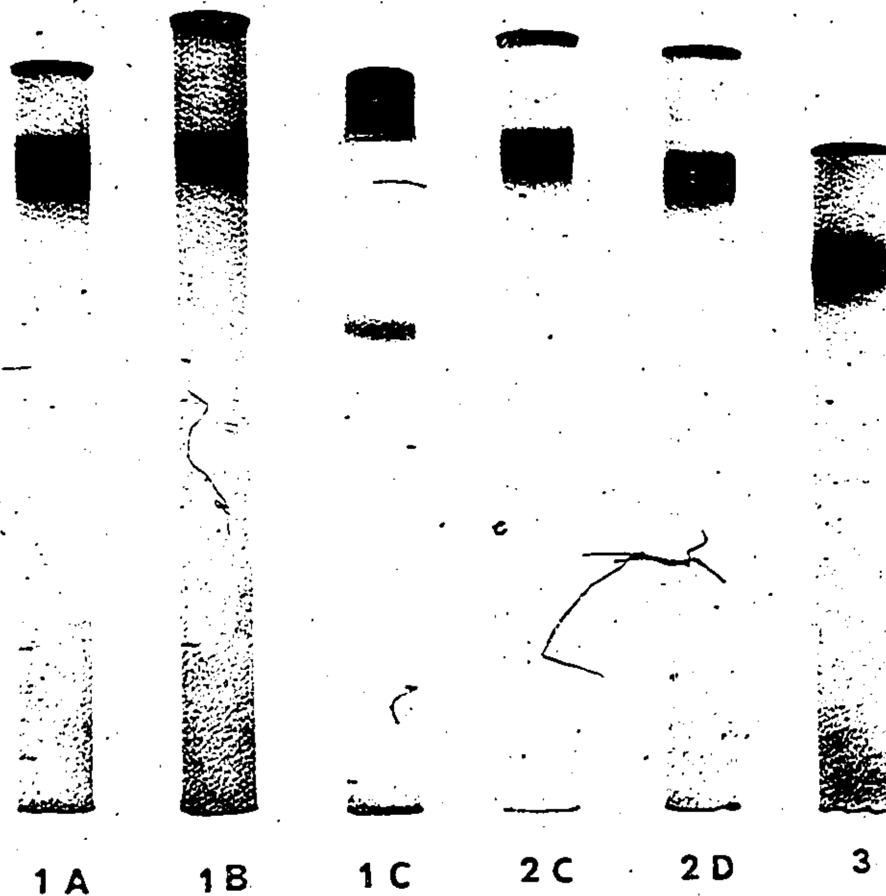
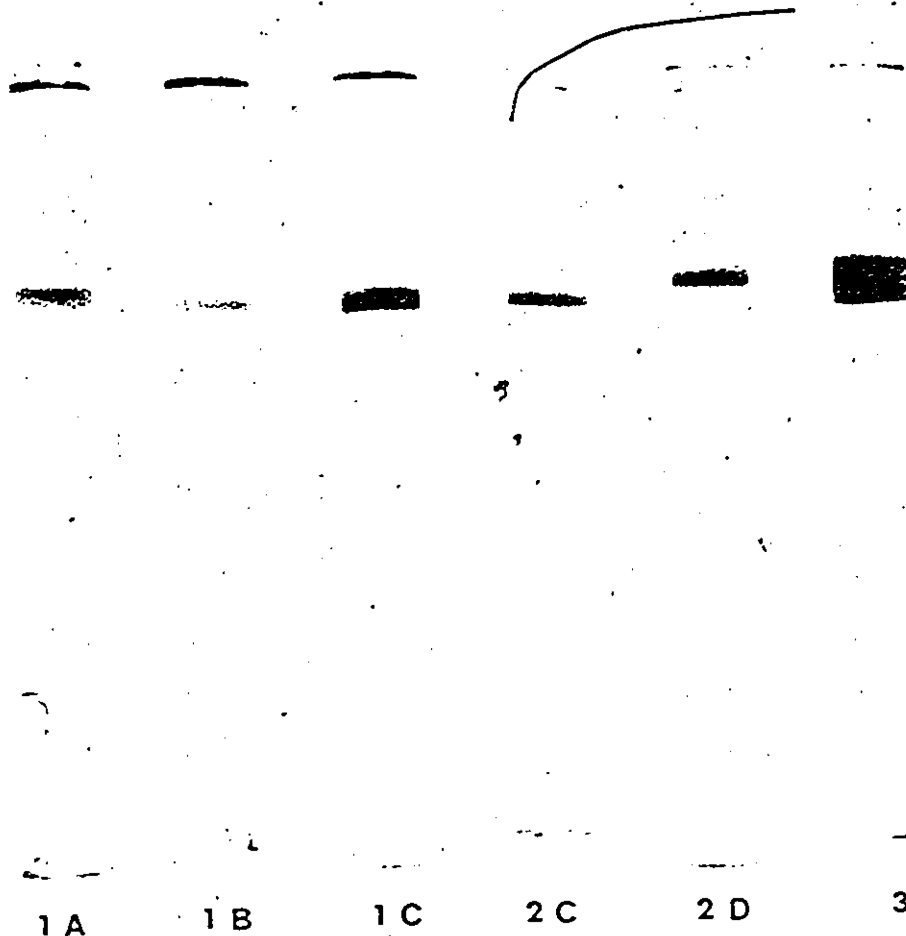


FIGURE 16

SDS-Polyacrylamide Gel Electrophoresis of the 17α-Hydroxysteroid Dehydrogenases

The 17α-HSDases purified by DEAE-cellulose chromatography and electrofocusing were electrophoresed in SDS-acrylamide gels (52). All samples were incubated at 65°C for 10 min in SDS sample buffer containing mercaptoethanol. A protein load of 1-2 μg was used for proteins 1A to 2D. For protein III a 5 μg sample was applied to the gel. The gels were stained for protein with Coomassie Brilliant Blue.



Each of the remaining isolated fractions also was visualized as one protein band on SDS acrylamide gels as shown in Fig. 16.

Table 5 lists the specific activities of each of the purified enzymes. Fractions IA, IB, IID and III were equivalent in their activities for 17α-estradiol and its glucuronide conjugate. Fraction IIC was approximately 4 times more active toward 17α-estradiol 3-glucuronide, while fraction IC was 30 fold more active toward the glucuronide conjugate than the free steroid. Total recovery of enzyme activity after pufification was 16% for 17α-estradiol dehydrogenase and 12% for 17α-estradiol 3-glucuronide dehydrogenase. The highest purification achieved was 190 fold for the 17α-estradiol 3-glucuronide dehydrogenase activity of enzyme IC when compared to the specific activity in the 105000 g supernatant. None of the purified 17α-HSDases was active on the 17β-epimer.

8. Electrofocusing of 17β-Hydroxysteroid Dehydrogenase

Electrofocusing of 17β-HSDase, partially purified by DEAE-cellulose chromatography (Fig. 7, peak V), did not result in a clear resolution of the multiple enzyme activities. However electrofocusing of fraction VI (Fig. 7, peak VI) resolved this 17β-HSDase activity into two peaks of enzyme activity when assayed with 17β-estradiol and its glucuronide

TABLE 5

Specific Activities of the Purified

17α-Hydroxysteroid Dehydrogenases

Enzyme	Specific Activity (nmol of prod/min/mg prot)	
	Substrate	
	17α-estradiol	17α-estradiol 3-glucuronide
IA	800	1100
IB	3500	4400
IC	1100	38,000
IIC	2900	11,000
IID	3700	3300
III	2300	2300

The six purified 17α-HSDase fractions were assayed for 17α-estradiol and 17α-estradiol 3-glucuronide dehydrogenase activities.

conjugate (Fig. 17). When the pooled enzyme fractions were refocused over a narrower pH range, and the column fractions assayed with 17β-estradiol 3-glucuronide, the enzyme activity clearly resolved into two peaks of activity (Fig. 18). Both fractions exhibited greater activity toward 17β-estradiol 3-glucuronide than toward 17β-estradiol (Fig. 17). Acrylamide electrophoresis of the two fractions revealed the less acidic fraction (VIB) to be homogeneous (Fig. 19). However, the more acidic fraction (VIA) contained one major protein band and also some lighter staining contaminants.

FIGURE 17

Isoelectrofocusing of 17β-Hydroxysteroid Dehydrogenase Fraction VI

A 17β-HSDase fraction (Fig. 7, peak VI) was electrofocused in a 5% ampholine gradient from pH 5 to 7 in an LKB 8102 column with 440 ml capacity. A sucrose gradient of 47% to 5% (w/v) with 0.5mM DTT was used to stabilize the pH gradient. The column fractions were assayed for 17β-estradiol and 17β-estradiol 3-glucuronide dehydrogenase activities.

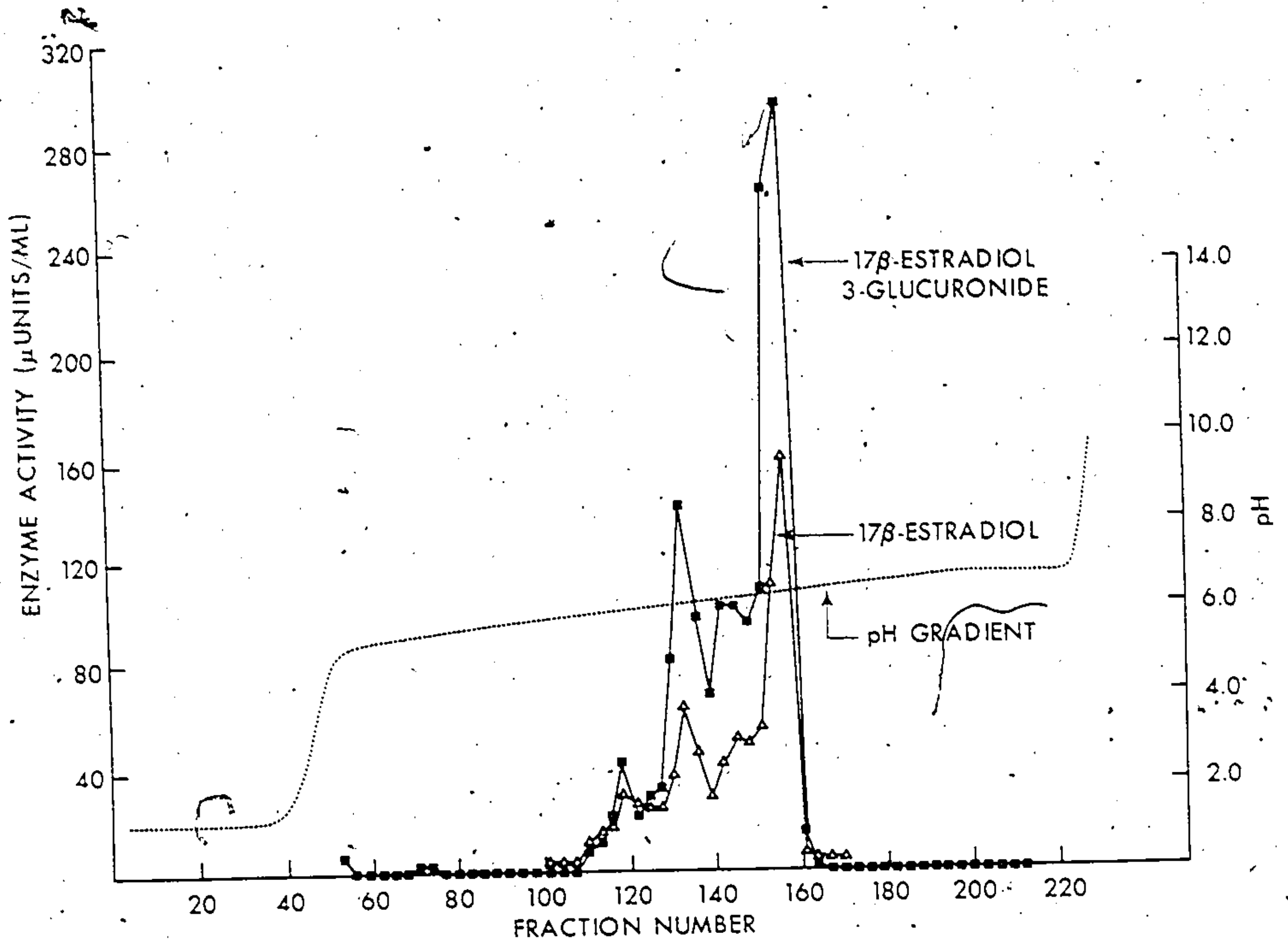

320
280
240
200
160
120
80
40
ENZYME ACTIVITY (μUNITS/ML)
14.0
12.0
10.0
8.0
6.0
4.0
2.0
pH
17β-ESTRADIOL 3-GLUCURONIDE
17β-ESTRADIOL
pH GRADIENT
20
40
60
80
100
120
140
160
180
200
220
FRACTION NUMBER

FIGURE 18

Re-focusing of 17β-Hydroxysteroid Dehydrogenase Fraction VI

The pooled 17β-HSDase activities (Fig. 17) were re-focused in a linear sucrose gradient (47% to 5% w/v) without additional ampholine. The column fractions (2 ml) were assayed for 17β-estradiol 3-glucuronide dehydrogenase activity.

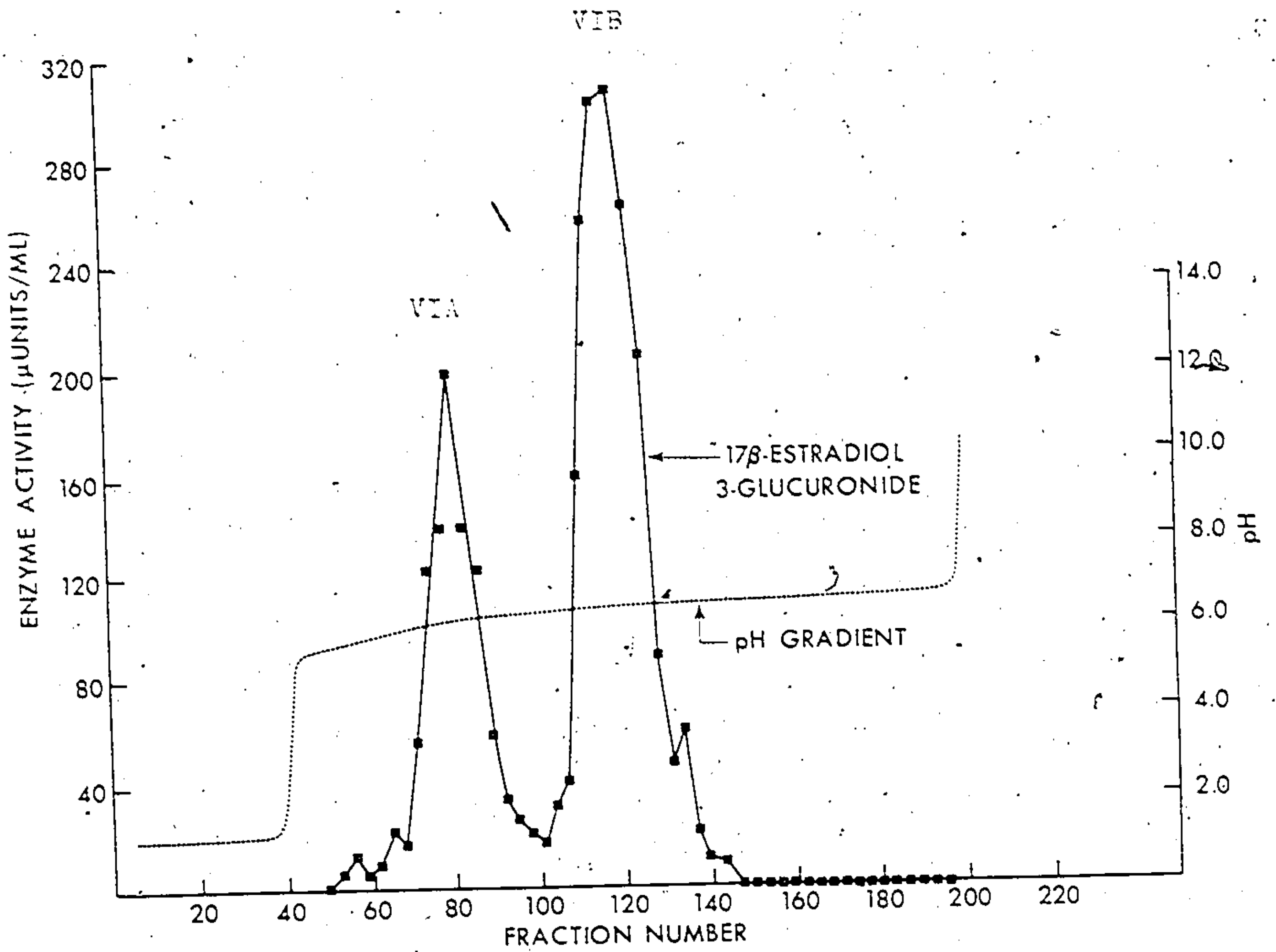

VIB
VIA
ENZYME ACTIVITY (μUNITS/ML)
320
280
240
200
160
120
80
40
14.0
12.0
10.0
8.0
6.0
4.0
2.0
pH
17β-ESTRADIOL
3-GLUCURONIDE
pH GRADIENT
20
40
60
80
100
120
140
160
180
200
220
FRACTION NUMBER

FIGURE 19

<u>Polyacrylamide Gel Electrophoresis of</u>
<u>17β-Hydroxysteroid Dehydrogenases</u>

The 17β-HSDases fractionated by electrofocusing (Fig. 18) were electrophoresed in 10% Davis gels (50). The concentrations of the proteins applied to the gels were in the range of 1-2 μg. The gels were stained for protein with Coomassie Brilliant Blue. The gels were pre-run before applying the protein sample.

VIB VIA

DISCUSSION

These results support the earlier assumption that 17α-HSDase and 17β-HSDase activities are distinct and separable. Also, 17α-HSDase cannot oxidize 17β-estradiol nor can 17β-HSDase oxidize 17α-estradiol.

The heterogeneity of both 17α- and 17β-HSDase activities is an interesting problem. According to recent evidence, it appears that heterogeneity may be a common feature of 17-HSDases of different species. Thaler-Dao et. al. (9) recently reported the heterogeneity of rabbit liver 17β-HSDase (while this work was in progress). Multiple forms of 17β-HSDase of guinea pig kidney have been observed (33, and 34) and recently one of these forms has been purified (25). Multiple forms have also been observed for the human placental 17β-estradiol dehydrogenase after isoelectric focusing (32). These reports clearly eliminated the possibility that the multiple forms could be explained as artifacts produced by the purification procedure. The results detailed in this thesis show that at least eight forms of the 17α-HSDase and three forms of 17β-HSDase can be isolated from rabbit liver cytosol by DEAE-cellulose chromatography and isoelectric focusing. Six of these enzymes were pure on the basis of

polyacrylamide gel electrophoresis. Of these, five have only 17α-HSDase activity and one has 17β-HSDase activity.

The molecular heterogeneity of these 17-HSDases could result from multiple genes, a single gene, chemical modification or binding to small molecules (56 and 57). It is unlikely that the multiple 17-HSDases were artifacts produced during the purification procedure, since the same relative amounts of the different enzymes were obtained from different rabbit liver preparations. Storage of the supernatant for different periods of time did not alter the enzyme pattern, either in number or elution profile. This excludes the possibility of protease action, methylation or deamidation.

There have been reports of artifacts formed during electrofocusing due to Ampholine protein interaction (58). However, this may possibly arise, instead, by oxidation of the proteins at the electrode solutions. This problem is eliminated by including a strong reducing agent, such as dithiothreitol (DTT), in the solution to be electrofocused. An altered enzyme profile upon refocusing of an enzyme at a different Ampholine concentration indicates artifact formation during electrofocusing. In this study re-focusing of the isolated enzymes did not alter the observed enzyme profile.

Furthermore, heterogeneity of 17-HSDases was evident after DEAE-cellulose chromatography, prior to electrofocusing.

As stated above, five 17α-HSDases have been purified in this work. Enzyme IA, isolated by electrofocusing, resolved into 2 bands of protein after electrophoresis on Davis acrylamide gels. Since it is unlikely that two distinct proteins would have the same mobility on SDS-acrylamide gels and on Sephadex G-75, the same surface charge, and the same isoelectric point, it is possible that the two protein bands result from a chemical or conformational change of the enzyme prior to or during polyacrylamide electrophoresis (56 and 57). The nature of these multiple forms will be dicussed more fully in the next section.

RESULTS

Part III Characterization of 17α-Hydroxysteroid Dehydrogenases

1. Substrate Specificities

Table 6A lists the substrate specificities of the purified 17α-HSDases. The specific activity of each of the enzymes, except IC, is much higher with epitestosterone than with any of the estrogens. Enzyme IC has a slightly higher specific activity for 17α-estradiol 3-glucuronide than for epitestosterone. Enzyme IC and IIC have a significantly higher specific activity for 17α-estradiol 3-glucuronide than for the free steroid. All the enzymes exhibit similar specific activities with 17α-estradiol and 17α-estradiol 3-glucoside. However, the specific activities of all the enzymes (except IIC) for the galacturonide conjugate of 17α-estradiol are significantly lower than the specific activities with any other substrate. The specific activity of enzyme IIC is the same for 17α-estradiol and for its glucoside and galacturonide conjugates. The highest specific activity observed was with enzyme IB and epitestosterone as substrate. In all cases the specific activities for the reduction of estrone, estrone 3-glucuronide and androstenedione were about 10 to

TABLE 6

Substrate Specificities of the 17α-Hydroxysteroid Dehydrogenases

A. Specific Activities

The purified 17α-HSdases were assayed for activity towards several estrogens and androgens.

The following abbreviations are used:

$E_2\alpha GA$ 17α-estradiol 3-glucuronide
E_1GA .... estrone glucuronide
$E_2\alpha$ 17α-estradiol
E_1 .... estrone
$E_2\alpha G$ 17α-estradiol 3-glucoside
$E_2\alpha GalA$ .... 17α-estradiol 3-galacturonic acid
EpiT .... epitestosterone
And .... androstenedione

B. Rates of Oxidation of Substrates Compared to the Oxidation of 17α-estradiol 3-glucuronide.

TABLE 6A

Specific Activities (ηmoles/min/mg)

Enzyme	Substrates							
	$E_2\alpha GA$	E_1GA	$E_2\alpha$	E_1	$E_2\alpha G$	$E_2\alpha GalA$	EpiT	And
IA	1.1	-	0.8	-	1.1	0.4	33.0	4.3
IB	4.4	-	3.5	-	4.4	0.1	70.0	6.6
IC	37.9	1.5	1.1	-	1.1	0.2	32.6	2.7
IIC	10.7	-	2.9	-	3.9	2.8	29.6	3.0
IID	3.3	-	3.7	0.1	3.9	0.8	35.4	1.8
III	4.3	-	2.3	-	2.8	1.3	29.7	4.1

TABLE 6B

Rate of Oxidation and Reduction of Substrates as Compared to Oxidation of 17α-Estradiol 3-Glucuronide

Enzyme	Substrates							
	$E_2\alpha GA$	E_1GA	$E_2\alpha$	E_1	$E_2\alpha G$	$E_2\alpha GalA$	EpiT	And
IA	100	-	71	-	97	32	3000	390
IB	100	-	79	-	99	2	1600	150
IC	100	4	3	-	3	<1	86	7
IIC	100	-	27	-	36	27	280	28
IID	100	-	110	4	120	25	1100	56
III	100	-	53	-	66	30	690	95

40 fold lower than for the oxidation reaction.

The rates of the oxidation (reduction) of the various substrates by the purified enzymes were compared to the rates of oxidation of 17α-estradiol 3-glucuronide (Table 6B). For each enzyme the rate of the oxidation of 17α-estradiol 3-glucuronide was considered as 100 and the rate of oxidation (reduction) for the remaining substrates was calculated as a percent of the 17α-estradiol 3-glucuronide dehydrogenase activity. This method gives a substrate specificity profile for each enzyme and therefore allows a simple comparison of differences or similarities between the purified 17-HSDases. No two enzymes exhibit the same substrate specificity profile in Table 6B. Enzymes IA and IID are the most similar in their substrate specificities. The two enzymes exhibit approximately equivalent rates for each substrate except for epitestosterone and androstenedione. Enzyme IA oxidizes epitestosterone 30 times faster than it does 17α-estradiol 3-glucuronide while, enzyme IB shows an 11 fold preference for epitestosterone. Enzyme IA also reduces androstenedione about 4 times faster than it oxidizes the estrogen conjugate. However enzyme IID reduces androstenedione at about half the rate of oxidation of 17α-estradiol 3-glucuronide. Enzyme IC has the most distinct substrate specificity profile, exhibiting a marked preference, firstly, for 17α-estradiol 3-glucuronide and, secondly, for epitestosterone.

2. Rate Determination of 17α-Hydroxysteroid Dehydrogenases

Table 7 lists the Vmax and Michaelis constant for each of the purified enzymes. All of the enzymes except IC had a significantly lower Km value with epitestosterone as substrate than with 17α-estradiol or its glucuronide conjugate. The Km values of enzyme IC were similar whether epitestosterone or 17α-estradiol 3-glucuronide were used as substrate. Further, only a slight difference in the Vmax was observed for the two substrates. Enzyme IIC also exhibited a similar Vmax for epitestosterone and 17α-estradiol 3-glucuronide, although the apparent Km was 8 fold lower with the androgen as substrate. For the remaining enzymes (IA, IB, IID and III), when epitestosterone was used as substrate, the Vmax was 5 to 9 fold higher and the Km 4 to 11 fold lower than when either 17α-estradiol or 17α-estradiol 3-glucuronide were used. When 17α-estradiol and its glucuronide conjugate were used as substrates the Km values for enzymes IA, IB, and III were about 2 to 3 fold lower for the conjugate. The Vmax values for enzymes IA, IID and III were approximately equal for the two estrogens, while the Vmax for enzyme IB was 5 fold higher for 17α-estradiol. Figures 20, 21, 22 show the Michaelis-Menten plots for enzymes IB, IC and IID with epitestosterone, 17α-estradiol 3-glucuronide and 17α-estradiol respectively. The Michaelis Menten plot for enzyme IB, when incubated with epitestosterone, showed a characteristic substrate inhibition at steroid concen-

TABLE 7

Vmax and Michaelis Constants of
17α-Hydroxysteroid Dehydrogenases

Enzyme	Vmax (nmol/min/mg)			Km (M)		
	Substrates					
	$E_2\alpha$	EpiT	$E_2\alpha GA$	$E_2\alpha$	EpiT	$E_2\alpha GA$
IA	8	74	5	$8.8x10^{-6}$	$7.4x10^{-7}$	$4.7x10^{-6}$
IB	15	110	3	$2.7x10^{-6}$	$6.3x10^{-7}$	$1x10^{-6}$
IC	13	200	180	$8.6x10^{-6}$	$5.2x10^{-6}$	$5.1x10^{-6}$
IIC	9	41	47	$2.4x10^{-6}$	$4.3x10^{-7}$	$3.6x10^{-6}$
IID	11	72	9	$3.1x10^{-6}$	2.0x10-7	$2.7x10^{-6}$
III	7	48	6	$2.8x10^{-6}$	$4.4x10^{-7}$	$1.0x10^{-6}$

The Vmax and Michaelis constants of the purified 17α-hydroxysteroid dehydrogenases were determined for the following substrates; 17α-estradiol ($E_2\alpha$); epitestosterone (EpiT); 17 -estradiol 3-glucuronide ($E_2\alpha GA$). All values were determined by the least squares method.

FIGURE 20

Lineweaver-Burk Plot for Enzyme IB and Epitestosterone

The curve for the Lineweaver-Burk plot was drawn by the least squares method. The insert shows the curve for the rate of oxidation of epitestosterone by enzyme IB as a function of substrate concentration. Velocity (v) has the units nmol of product formed per mg of protein per min (nmol prod/mg/min).

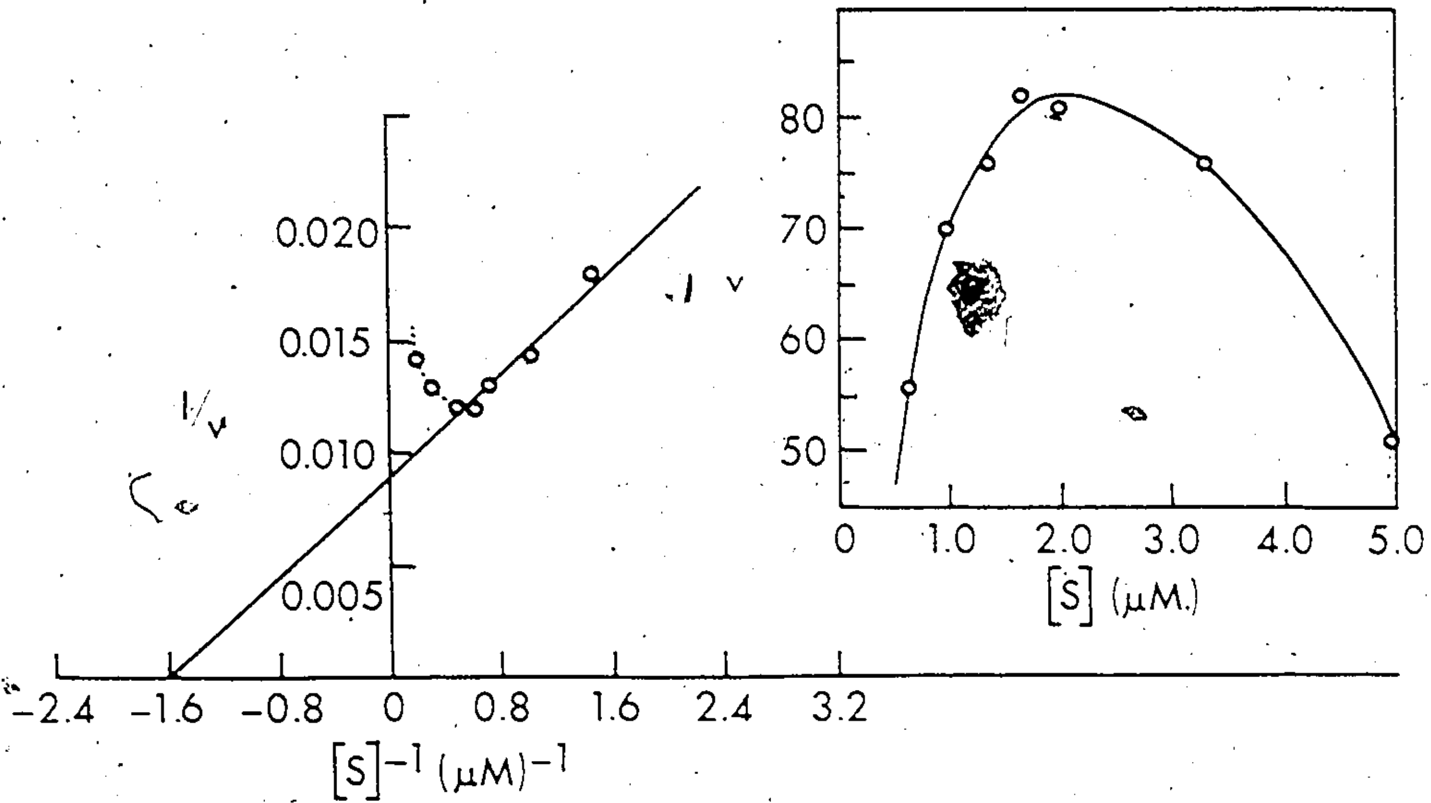
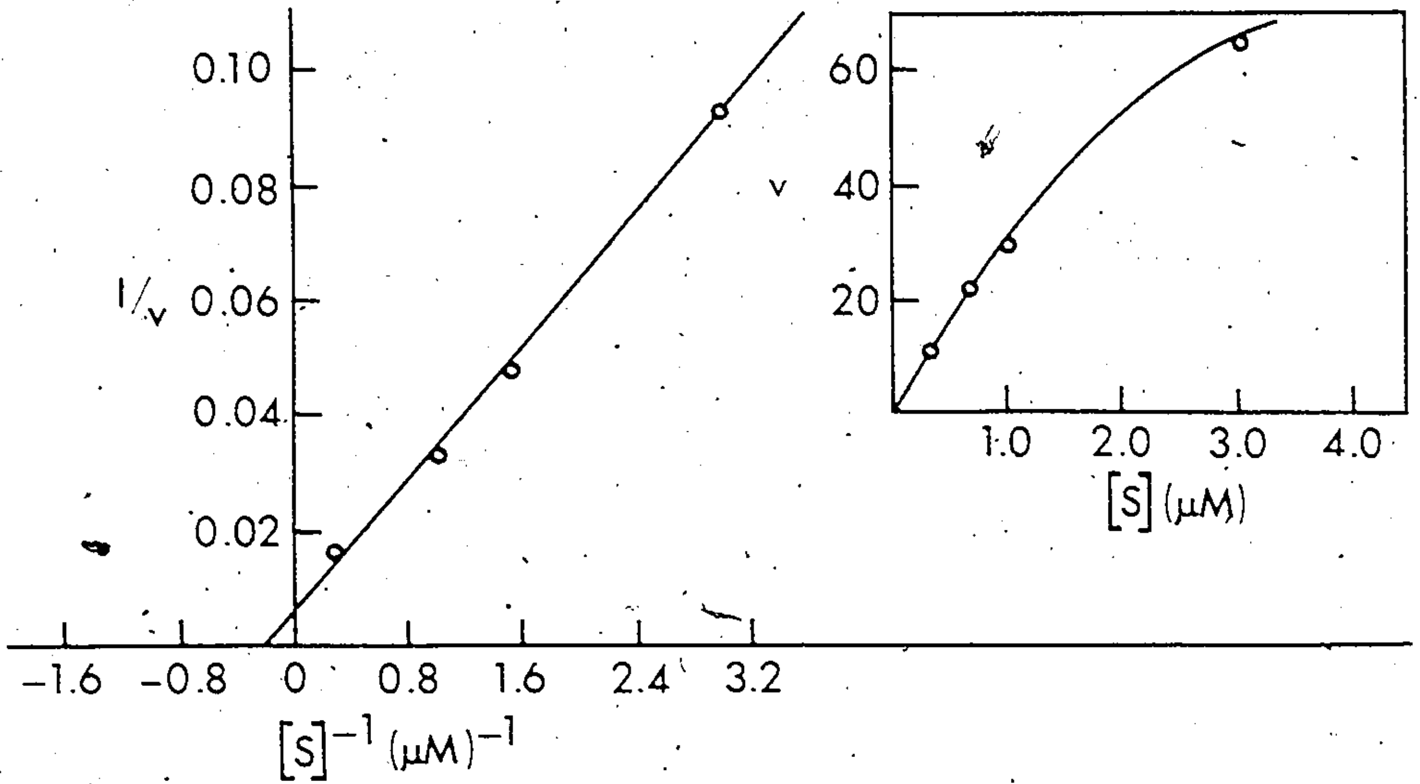

0.020
0.015
0.010
0.005
1/v
-2.4
-1.6
-0.8
0
0.8
1.6
2.4
3.2
[S]-1 (μM)-1
v
80
70
60
50
0
1.0
2.0
3.0
4.0
5.0
[S] (μM.)

FIGURE 21

Lineweaver-Burk Plot for Enzyme IC and 17α-Estradiol 3-Glucuronide

See Fig. 20 for conditions.

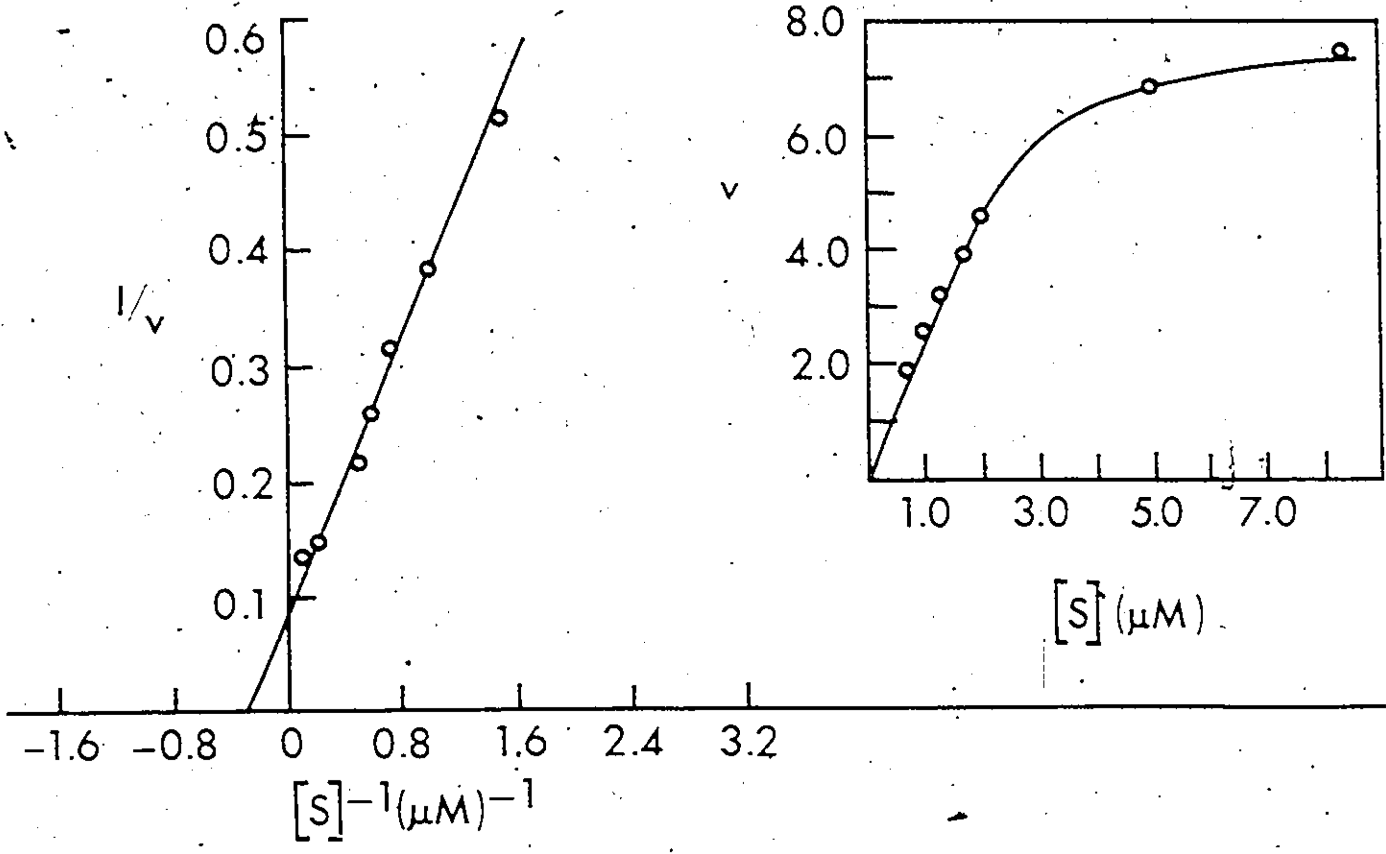


0.10
0.08
0.06
0.04
0.02
1/v
−1.6
−0.8
0
0.8
1.6
2.4
3.2
[S]−1 (μM)−1
60
40
20
v
1.0
2.0
3.0
4.0
[S] (μM)

FIGURE 22

Lineweaver-Burk Plot for Enzyme IID and 17α-Estradiol

See Fig. 20 for conditions.


0.6
0.5
0.4
0.3
0.2
0.1
1/v
-1.6
-0.8
0
0.8
1.6
2.4
3.2
[S]-1(μM)-1
8.0
6.0
4.0
2.0
v
1.0
3.0
5.0
7.0
[S] (μM)

trations higher than 3 μM. A similar inhibition was observed for all of the enzymes when incubated with epitestosterone. Inhibition of 17α-HSDase was not observed when 17α-estradiol or its glucuronide conjugate were incubated with the enzymes at concentrations as high as 8 μM.

3. Effects of Metal Ions and Sulfhydryl Reagents

Enzymes IIC and III were tested for their sensitivity to several metal ions as well as iodoacetate. Table 8 lists the results as a percent of the activity of the enzyme in a control incubation. Magnesium, calcium, manganese or iron had no significant effect on the activity of either enzyme IIC or III. However, both enzymes lost approximately half of their activity when incubated in the presence of 10^{-3} M copper ion. The presence of EDTA in the incubation mixture did not affect either enzyme, nor did magnesium ions in the presence of EDTA. Finally, no effect was observed on either enzyme from the addition of iodoacetate to the incubation mixture.

TABLE 8

Effect of Metal Ions and Iodoacetate on
17-Hydroxysteroid Dehydrogenase Activity

Enzymes IIC and III were incubated in the presence of metal ions and iodoacetate to determine their effect on enzyme activity. The results are presented as a percent of the enzyme activity in a control incubation.

TABLE 8

Reagent or Ion	Concentration (M)	% Activity of Control	
		Enzyme IIC	Enzyme III
Mg^{2+}	10^{-5}	96	107
	10^{-4}	100	103
	10^{-3}	96	103
Ca^{2+}	10^{-5}	100	100
	10^{-4}	102	-
	10^{-3}	102	93
Mn^{2+}	10^{-5}	101	101
	10^{-4}	100	104
	10^{-3}	100	93
Cu^{2+}	10^{-5}	100	97
	10^{-4}	88	95
	10^{-3}	48	55
Fe^{2+}	10^{-5}	88	97
	10^{-4}	88	100
	10^{-3}	95	99
EDTA	10^{-5}	-	96
	10^{-4}	93	99
	10^{-3}	99	95
EDTA + Mg^{2+}	10^{-5} 10^{-4}	93	99
Iodoacetate	10^{-5}	89	-
	10^{-4}	91	93
	10^{-3}	96	95

4. Isoelectric Points of 17α-Hydroxysteroid Dehydrogenases

The isoelectric ponts of the 17α-HSDases, fractionated by electrofocusing, ranged from 5.72, for enzyme IIA, to 6.68, for enzyme IC (Table 9). Enzymes IA and IIB had the same isoelectric point (5.80) and enzymes IB and IID had almost identical isoelectric points (6.18 and 6.10 respectively).

5. Molecular Weights of 17α-Hydroxysteroid Dehydrogenases

The molecular weights calculated for the purified 17α-HSDases were all within a 3% variation of their average molecular weight of 39,600 daltons (Table 10). Since this variation is strictly within the experimental error of the technique of MW determination on SDS polyacrylamide gels, all the MW's can be considered as being essentially the same. The average molecular weight of 39,600 also compares favourably with the MW estimation of 35,000 calculated on the basis of the elution volume of the HSDases after gel filtration on a column of Sephadex G-75.

6. Amino Acid Composition of 17α-HYdroxysteroid Dehydrogenases

Since the majority of the data accumulated on the properties of the purified 17α-HSDases indicated that the enzymes

TABLE 9

Isoelectric Points of 17α-Hydroxysteroid Dehydrogenases

Enzyme	Isoelectric Point
IA	5.82
IB	6.18
IC	6.68
IIA	5.72
IIB	5.80
IIC	5.95
IID	6.10

The isoelectric points of the 17α-HSDases were determined by isoelectrofocusing.

TABLE 10

Molecular Weights of 17α-Hydroxysteroid Dehydrogenases

Enzyme	Molecular Weight (Daltons)
IA	40,000
IB	39,500
IC	39,300
IIC	40,500
IID	39,500
III	38,800
avg	39,600

The molecular weights of the purified 17α-HSDases were calculated by the SDS-acrylamide gel electrophoresis method (52).

were distinct entities, it became necessary to investigate whether there were differences in the chemical constitution of the multiple forms isolated. All six purified 17α-HSdases were therefore subjected to amino acid analysis. The data presented in Table 11 are listed as ηmol % of the total ηmol of amino acid mixture analysed for each protein sample. Proteins IA, IB, IC, IIC, and IID were all remarkably similar in their total amino acid composition. However, two clear differences were apparent in the composition of protein III as compared to the rest. Proteins IA to IID all had an Asx (aspartic acid + asparagine) content from 9.8 to 10.6 ηmol % while protein III had an Asx content of 6.9 ηmol %. Similarly, the Glx (glutamic acid + glutamine) content of proteins IA to IID was between 10.7 to 11.8 ηmol % while the Glx content of protein III was 7.9 ηmol %. In both cases the difference is in the range of 20 - 30%. The other differences shown in Table 11 are minor, and the variation is less than 10%, so that they cannot be considered significant.

The amino acid composition data in Table 11 was used to calculate the number of residues of each amino acid as they occurred in each of the purified 17α-HSDases (Table 12). The accuracy of the data was checked by calculating the MW of each protein on the basis of its amino acid residue content.

TABLE 11

Amino Acid Compositions of the

17α-Hydroxysteroid Dehydrogenases

Each enzyme (100μg) was hydrolysed for 24 hrs at 110°C in an evacuated tube. Nor-leucine (12.5 nmole) was added to each sample as a standard. The data has not been corrected for destructive hydrolysis of serine, methionine, threonine, and tyrosine. The tryptophan and amide content of the proteins was not measured. Cysteine was determined as cysteic acid. The data is presented as ηmol percent.

Asx = aspartic acid + asparagine
Glx = glutamic acid + glutamine

TABLE 11

Amino acid (nmoles %)	Enzyme Number					
	IA	IB	IC	IIC	IID	III
Cys	0.5	0.6	0.3	0.2	0.6	0.6
Asx	9.8	9.8	10.3	10.6	10.2	6.9
Thr	3.0	3.2	3.4	3.6	3.7	4.1
Ser	4.5	5.1	5.8	5.5	5.1	5.4
Glx	11.0	10.7	11.5	11.8	11.1	7.9
Pro	5.2	5.3	6.0	5.7	5.2	6.2
Gly	6.7	7.9	7.1	6.8	7.1	7.9
Ala	6.5	7.0	7.4	8.0	7.9	5.9
Val	6.5	7.4	7.2	7.5	7.2	5.9
Met	1.3	1.9	1.6	1.7	1.7	1.4
Ile	5.1	4.9	5.2	5.0	5.7	5.3
Leu	10.2	11.7	11.0	11.0	10.7	10.6
Tyr	3.0	4.2	3.8	4.0	4.1	3.4
Phe	3.9	3.7	3.8	3.6	4.3	3.6
His	2.7	3.7	3.2	2.8	2.6	2.7
Lys	6.8	8.2	8.4	7.5	9.1	7.6
Arg	4.8	5.2	4.6	5.0	4.3	5.4

TABLE 12

Amino Acid Composition of the
17α-Hydroxysteroid Dehydrogenases

The table presents the number of residues for each amino acid. The residue number was calculated by multiplying the nmol percent of each amino acid by a number until the molecular weight of the sum of the amino acid residues approximated the calculated molecular weight. The molecular weights listed in the table are the sums of the MW of the amino acid residues of each protein.

TABLE 12

Amino acid (Residue No.)	Enzyme Number					
	IA	IB	IC	IIC	IID	III
Cys	2	2	1	1	2	2
Asx	33	29	31	32	33	23
Thr	10	10	10	11	11	14
Ser	15	15	17	17	16	18
Glx	37	32	35	35	34	26
Pro	18	16	18	17	16	20
Gly	22	24	21	20	22	26
Ala	22	21	22	24	24	19
Val	21	22	22	23	22	19
Met	4	6	5	5	5	5
Ile	17	15	16	15	17	17
Leu	35	35	33	33	32	35
Tyr	10	13	11	12	12	11
Phe	13	11	11	11	13	12
His	9	11	10	8	8	9
Lys	23	25	25	23	27	25
Arg	16	16	14	15	13	18
Molecular Weight	39,900	40,200	39,800	39,700	39,700	38,500

The ratio of the basic amino acid residues and the acidic amino acid residues for each protein was less than one. This ratio and the isoelectric points both indicate that the proteins are acidic.

7. A Method For Fingerprinting Lysine Containing Peptides

Since amino acid analysis of the purified 17α-HSDases showed the multiple forms to be very similar, the question arose whether the primary sequence of these proteins was also similar. Fingerprinting of the enzyme peptides after proteolytic digestion would have been the obvious answer if large enough quantities of the enzymes had been available. A test run was attempted by the usual fingerprint method (73) using 300 μg of protein III. However, this proved unsuccessful since the low intensity of peptide staining would not have permitted an adequate comparison of the multiple forms. It was clear therefore that the proteins would have to be modified by some radioactive reagent which would allow the generation of radioactive peptides. The peptide pattern after electrophoresis could then be recorded by radioautography. Lysine acetylation was selected as the method of choice for the following reasons: a) Lysine residues usually occur in sufficient abundance in proteins to ensure that an

adequate number of radioactive spots would arise to facilitate the detection of differences betweeen the proteins;
b) Lysine residues usually occur on the surface of proteins. Since it is known that changes which occur in the primary sequence of closely related enzymes are usually associated with the outer core of the protein, then lysine residues could be a sensitive tool to detect any alteration in its environment. This method of lysine peptide detection would then best illustrate any differences occurring between multiple forms of the enzymes.

As described in the section on methods, the thermolysin digest of each acetylated protein was first electrophoresed at pH 6.5. A comparison of the mobilities of the peptides after radioautography showed no discernible differences when proteins IA, IB and IC were compared and similarly no differences could be discerned between proteins IIC and IID. However, the profile of the group of proteins IA, IB and IC was slightly different from the group profile of IIC and IID. The profile of protein III was slightly different from that of both groups I and II. The peptide profile of VIB (17β-HSdase) was, however, distinctly different from all the other proteins. A better separation of the radioactive peptides was achieved after each strip of radioactive material was electrophoresed at pH 2.1 (Figs. 23, 24, 25, 26, 27, 28).

FIGURE 23

The ε-Amino-^{14}C-acetyl Lysine Peptide Map of Enzyme IA

The thermolysin digest of the ^{14}C-acetylated enzyme IA was electrophoresed first at pH 6.5 and then at pH 2.1. The chromatogram was radioautographed to detect the ε-amino-^{14}C-acetyl lysine containing peptides. The relative mobilities of the peptides were calculated relative to the migration of dansyl arginine.

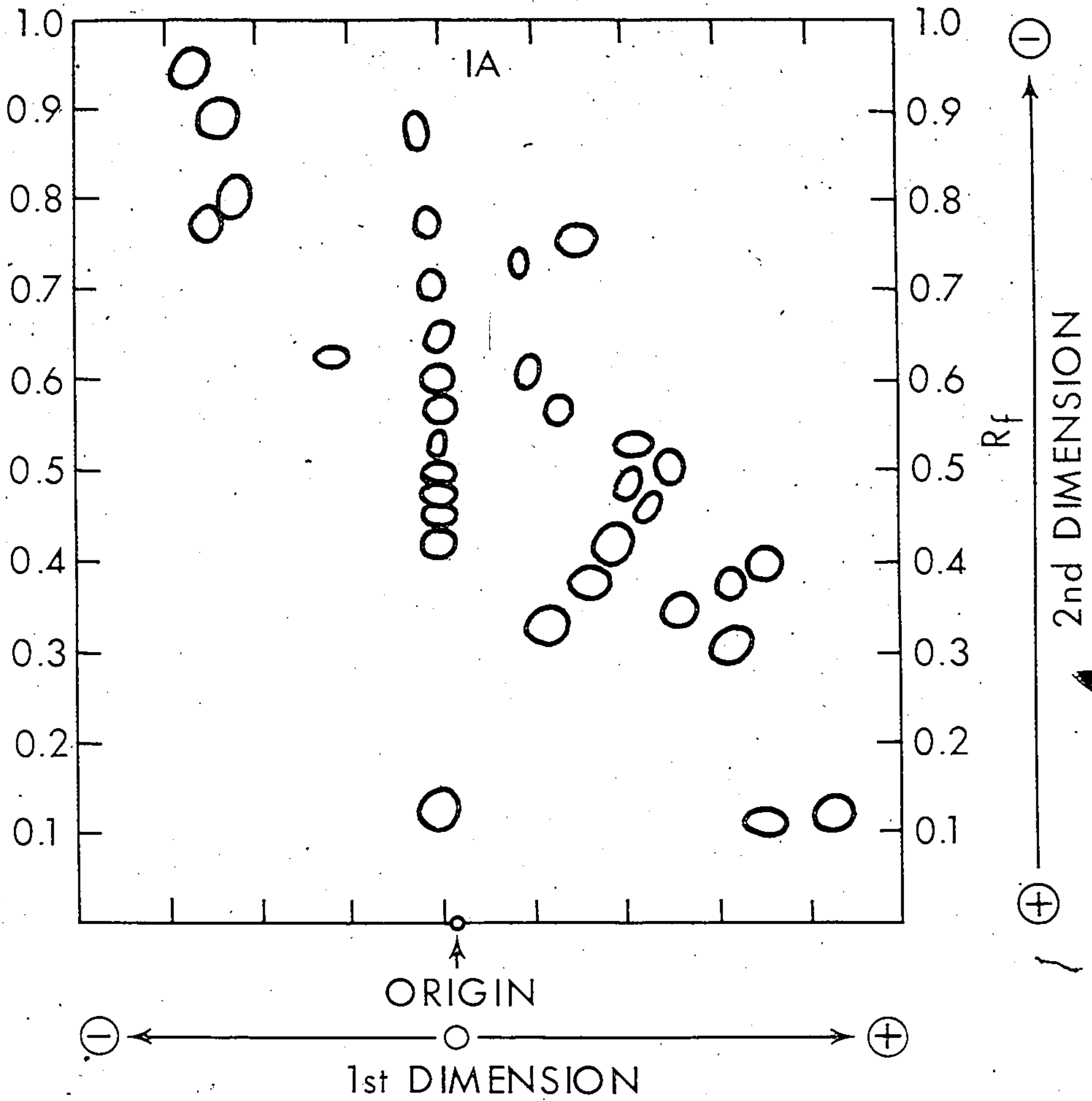
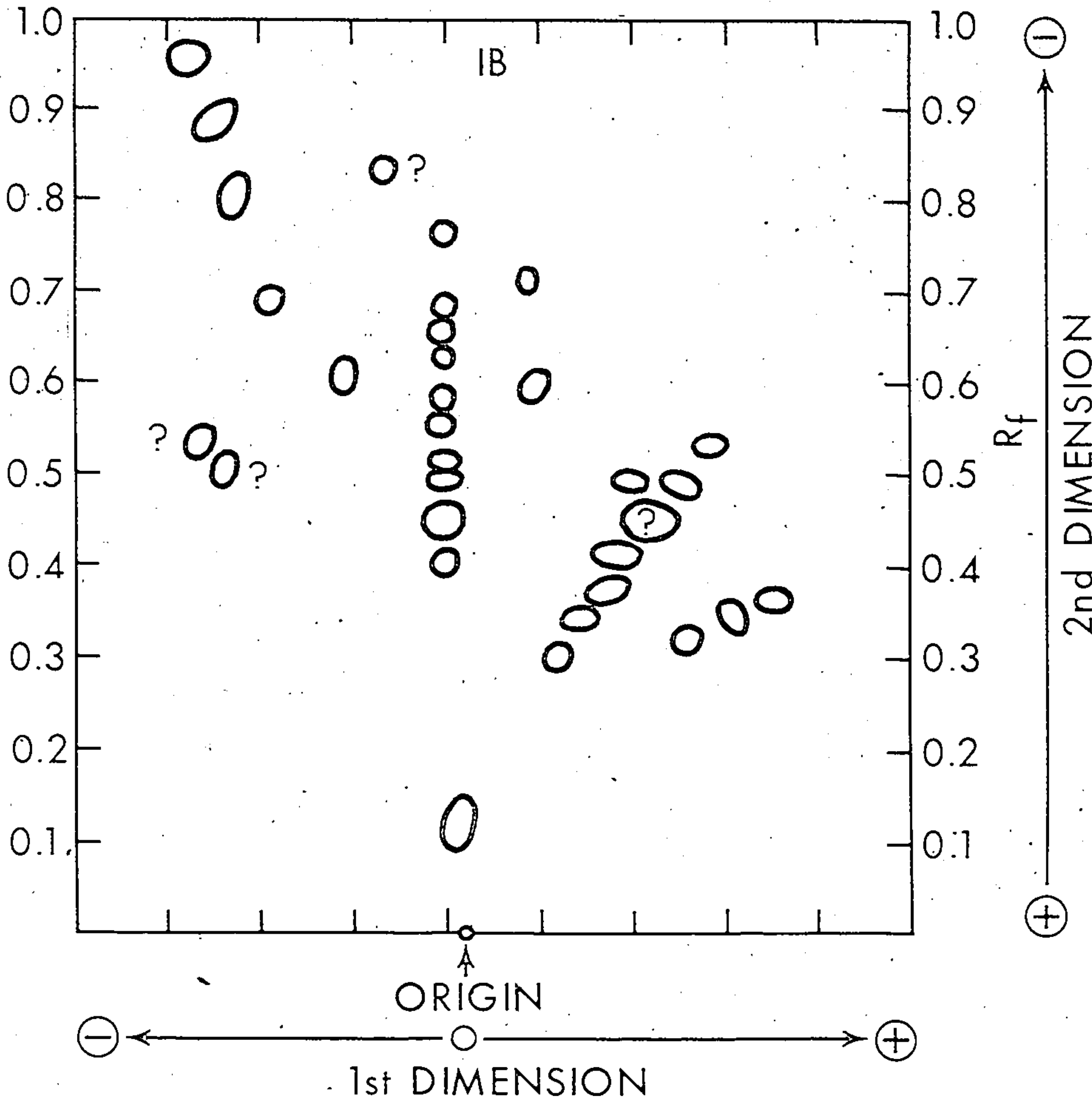

IA
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
R_f
2nd DIMENSION
ORIGIN
1st DIMENSION

FIGURE 24.

The ε-amino-^{14}C-acetyl Lysine Peptide Map of Enzyme IB

See Fig. 23 for details. Spots with dotted lines are questionable due to their low intensity as detected on the exposed film.



1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
IB
?
?
?
?
Rf
2nd DIMENSION
ORIGIN
1st DIMENSION

FIGURE 25

The ε-amino-^{14}C-acetyl Lysine Peptide Map of Enzyme IC

See Fig. 23 for details.

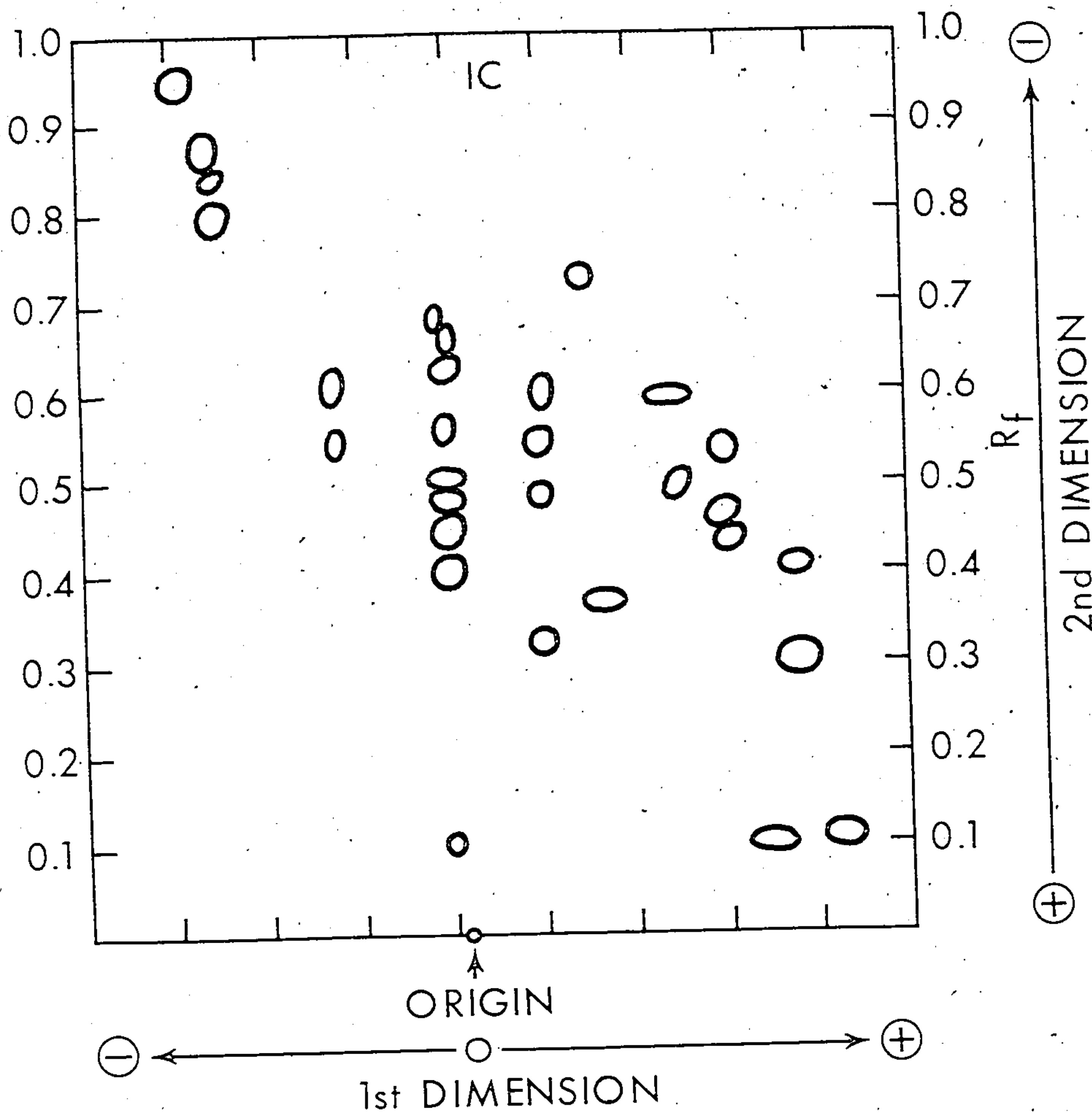

IC
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
R_f
2nd DIMENSION
ORIGIN
1st DIMENSION

FIGURE 26

The ε-amino-^{14}C-acetyl Lysine Peptide Map of Enzyme IIC

See Fig. 23 for details.

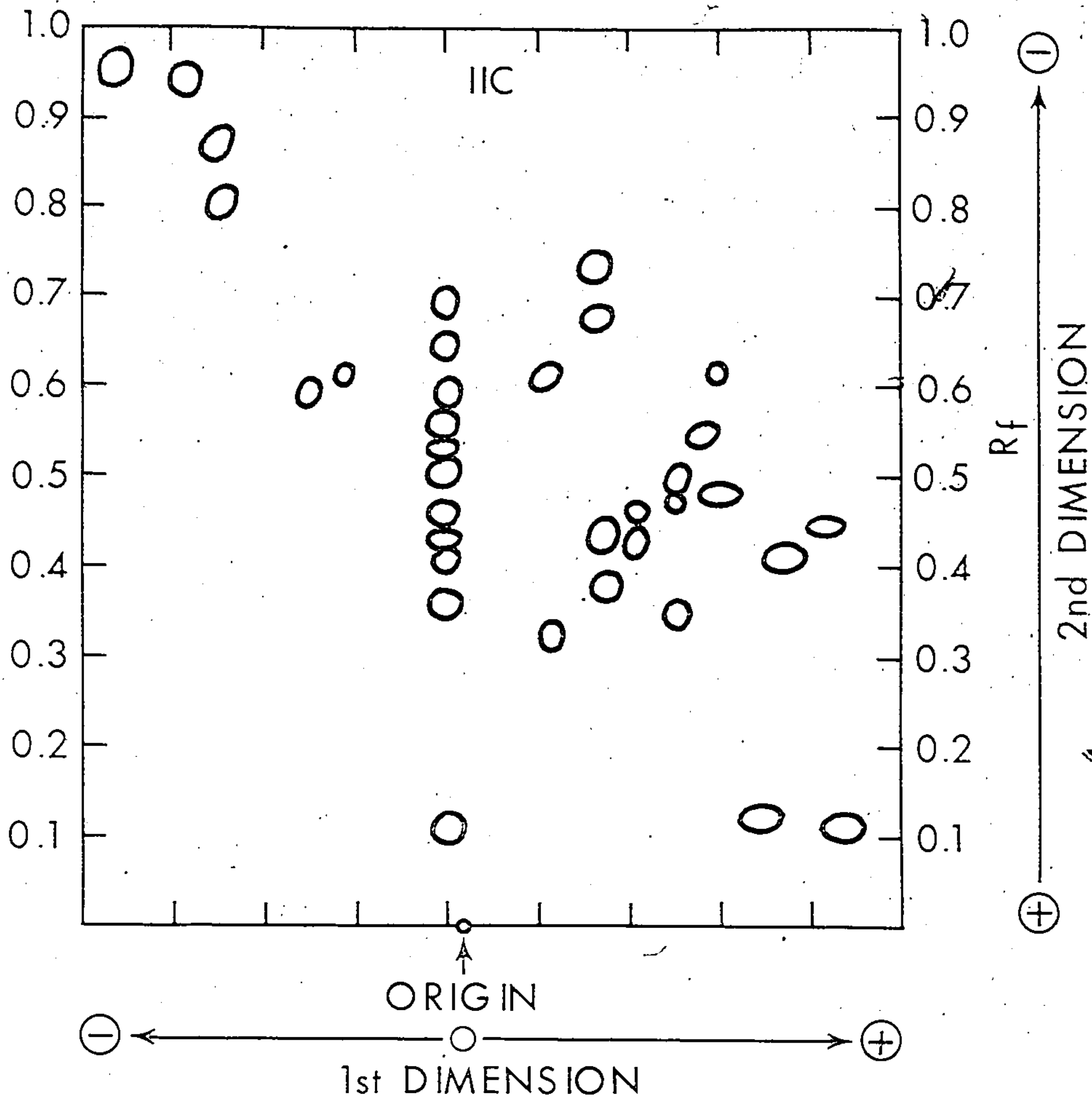

IIC
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
R_f
2nd DIMENSION
ORIGIN
1st DIMENSION

FIGURE 27

The ε-amino-^{14}C-acetyl Lysine Peptide Map of Enzyme IID

See Fig. 23 for details.

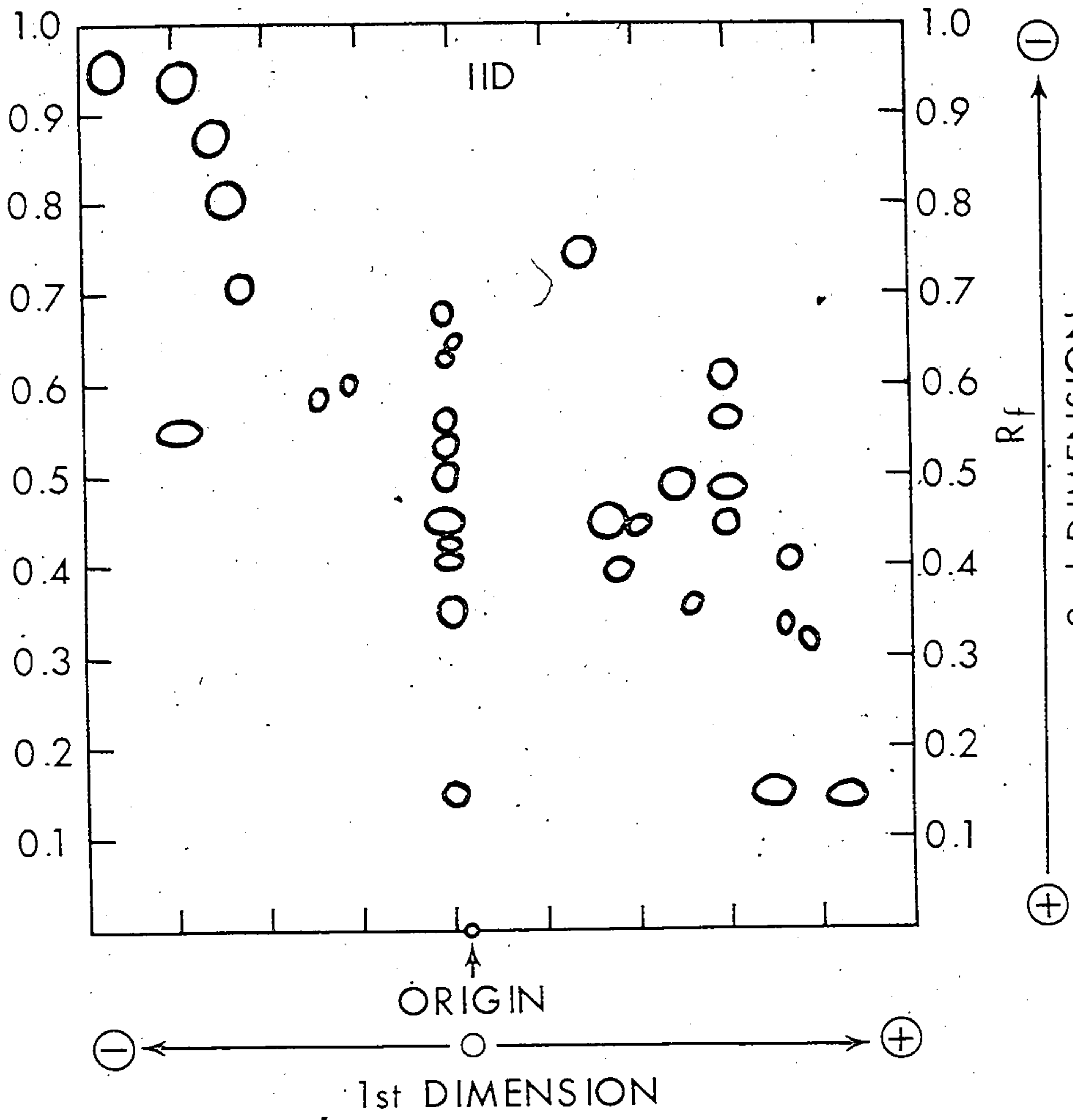
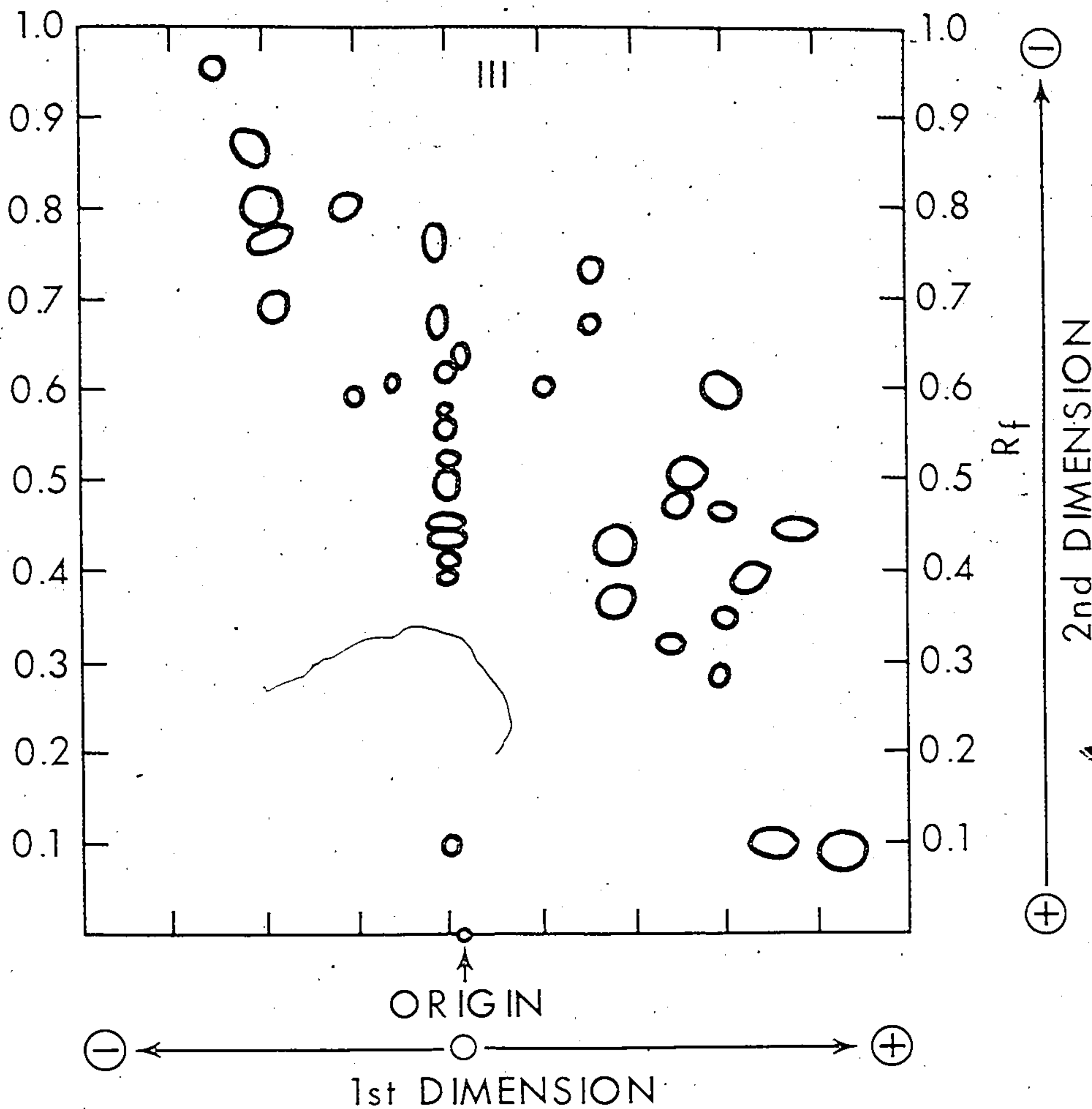

IID
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
R_f
2nd DIMENSION
ORIGIN
1st DIMENSION

FIGURE 28

The ε-amino-^{14}C-acetyl Lysine Peptide Map of Enzyme III

See Fig. 25 for details.


III
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
R_f
2nd DIMENSION
ORIGIN
1st DIMENSION

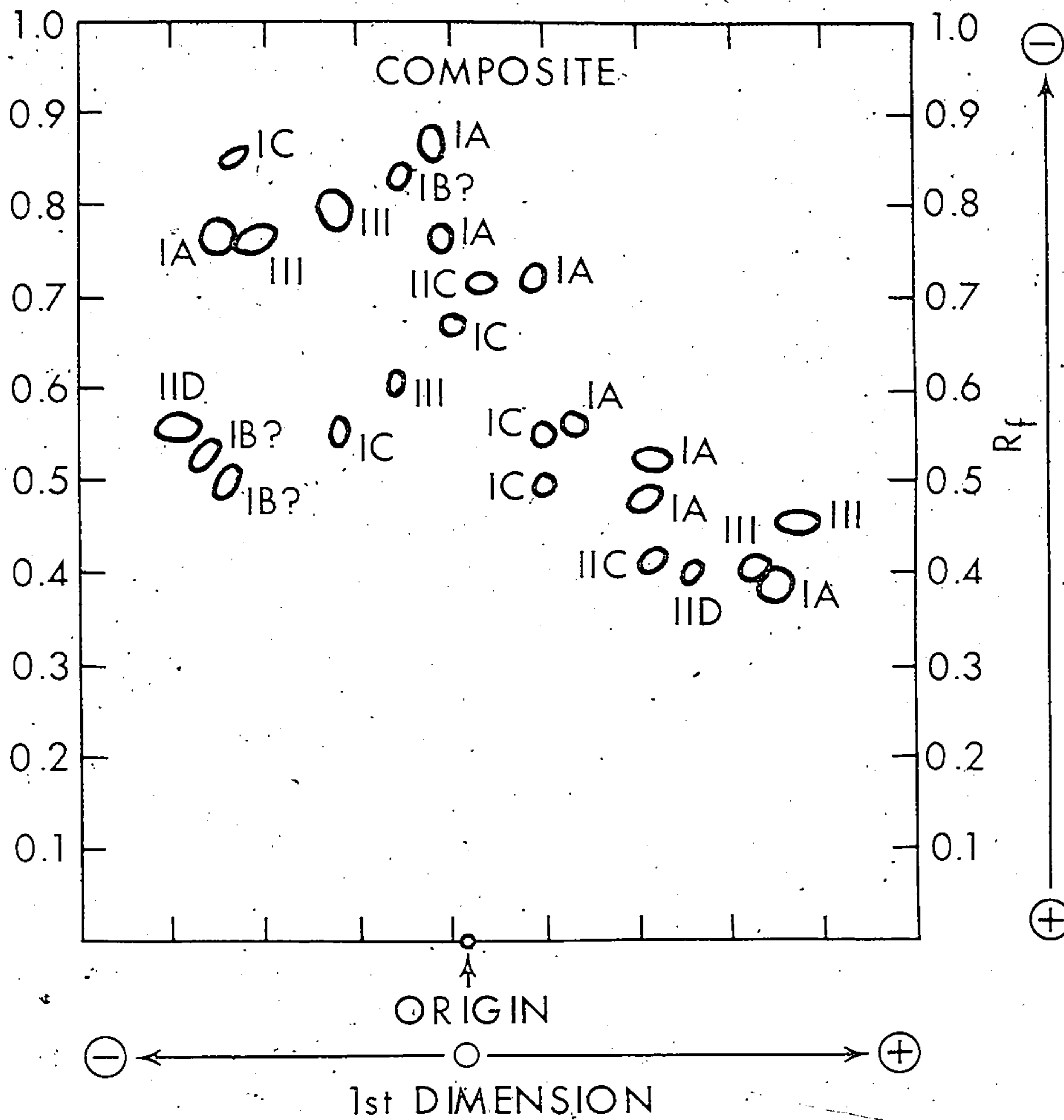
Since all acidic groups were protonated at this pH, all the peptides migrated toward the cathode. Each peptide had at least one positive charge contributed by a free terminal amino group. Some peptides also had positive charges contributed by either arginine or histidine residues or both at pH 2.1.

Since the peptide profiles in the first dimension of the group of proteins IA, IB and IC were quite similar, as were the profiles of IIC and IID, it is important to first compare the profiles of the enzymes within these two groups. To simplify a discussion of the peptide maps, those peptides that were basic at pH 6.5 will be referred to as peptide "region 1", those that were neutral at pH 6.5 will be referred to as peptide "region 2", and those that were acidic at pH 6.5 as peptide "region 3". All three enzymes, IA, IB and IC (Figs. 23, 24, 25) generate some peptides which are unique to only one enzyme. IA, when compared with IB, generates one unique peptide in the peptide "region 1", (Rf 0.77), one unique peptide in "region 2" (Rf 0.87), and three unique peptides in "region 3" (Rf 0.74, 0.56 and 0.3). Enzyme IB, when compared with IA, generated one unique spot in "region 1" (Rf 0.68) and one spot in "region 3" (Rf 0.53). Three other faint spots could be visualized in "region 1" of IB which did not occur in IA but it should be stressed that these spots are questionable. By a similar comparison it can be

FIGURE 29

A Composite ε-amino-^{14}C-acetyl Lysine Peptide Map of 17α-Hydroxysteroid Dehydrogenases

This map shows only those peptides which are unique to each protein. The spots with dotted lines for enzyme IB are questionable.



COMPOSITE
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
IC
IA
IB?
III
IA
III
IA
IIC
IA
IC
IID
III
IC
IA
IB?
IC
IA
IB?
IC
IA
III
III
IIC
IA
IID
R_f
2nd DIMENSION
ORIGIN
1st DIMENSION

determined that each of the three enzymes IA, IB and IC generate peptides which are unique to each enzyme. A comparison of enzymes IIC and IID also reveals the occurrence of peptides which are unique to only one enzyme (Fig. 26, 27). To allow a comparison of each of the enzymes to the remaining five a composite map was drawn (Fig. 29) which contains only those spots which are unique to each enzyme. Each of the enzymes is represented by two or more spots. However, the three spots shown for enzyme IB are questionable and it may very likely be that IB does not generate peptides which are unique when compared to the remaining five enzymes. Although the dissimilarities of the peptide profiles of the different enzymes have been stressed, it should also be emphasized that the peptide profiles of the six 17α-HSDases also show major similarities in their patterns.

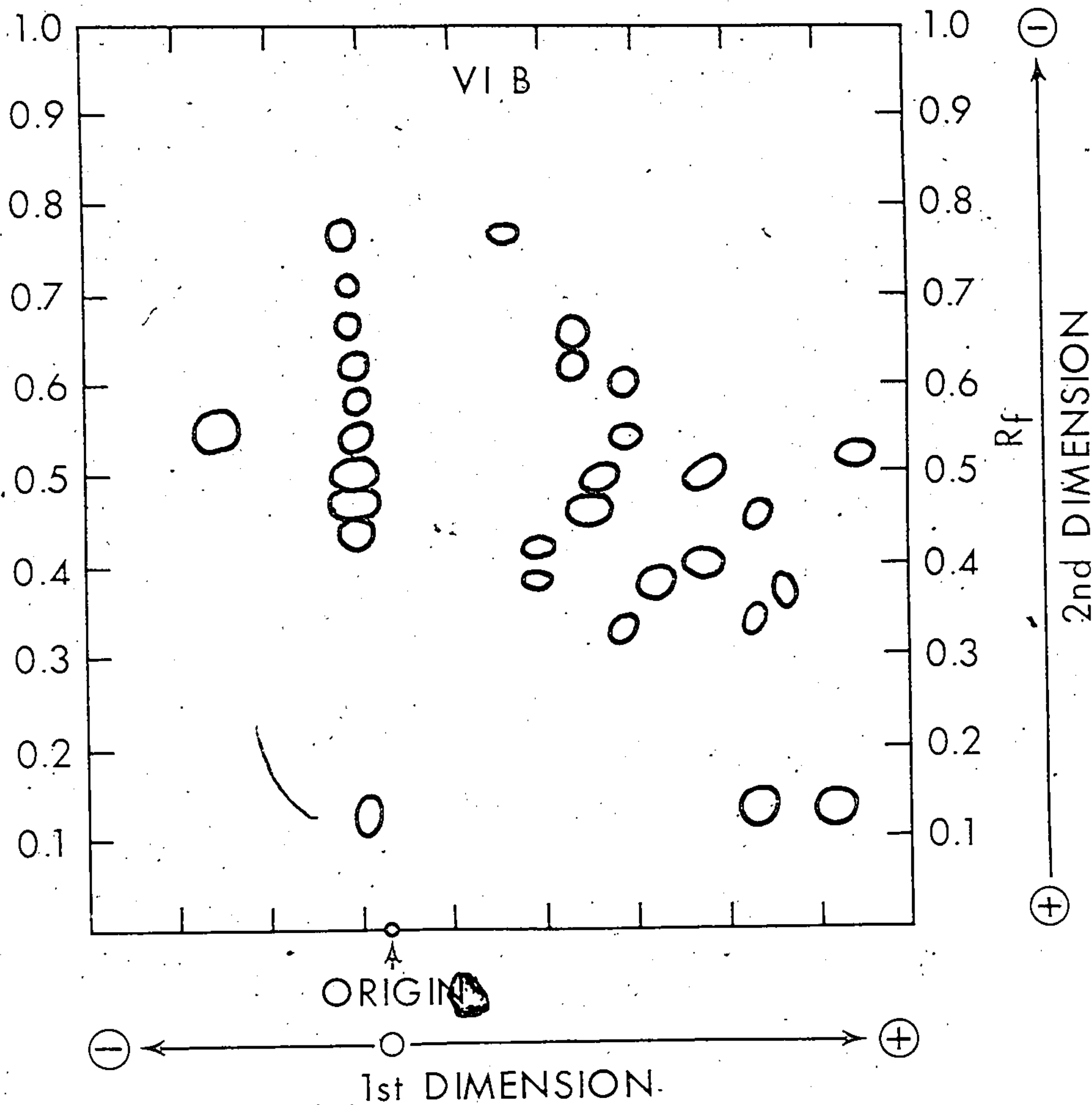
The peptide profile of the 17β-HSDase (VIB) (Fig. 30) is distinctly dissimilar from any of the 17α-HSDases mentioned above. This enzyme generated fewer peptides which were basic at pH 6.5 and also generated peptides that were relatively more acidic at that pH.

FIGURE 30

The ε-amino-^{14}C-acetyl Lysine Peptide Map of

Enzyme VIB (17β-HSDase)

See Fig. 23 for details.



VI B
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Rf
2nd DIMENSION
ORIGIN
1st DIMENSION

DISCUSSION

The data on the substrate specificities, Vmax and Michaelis constants suggests that enzymes IA, IB, IID and III have both a greater affinity and a higher rate of reaction for the non-aromatic steroid, epitestosterone, than for 17α-estradiol. Although enzyme IIC has the same Vmax for both 17α-estradiol 3-glucuronide and epitestosterone, the apparent Km is almost 10 fold lower for the androgen. The greater affinity of enzyme IIC for the androgen explains the higher specific activity of this enzyme for epitestosterone when compared with 17α-estradiol 3-glucuronide. Enzyme IC exhibits similar substrate specificities for both the androgen and 17α-estradiol 3-glucuronide. This is supported by the fact that similar Vmax and Km values were obtained for both steroids.

When the 17α-HSDases IA, IB and IID were incubated with phenolic steroids, these enzymes showed almost similar specificities for 17α-estradiol, and its glucuronyl and glucosyl conjugates. However the galacturonide conjugate did not successfully replace the other estrogens. Therefore the substrate binding sites of enzymes IA, IB and IID more readily recognize the stereospecificity of glucuronic acid at position 4 of the sugar, than that of galacturonic acid when conjugated with 17α-estradiol. Enzymes

IC and IIC have a higher specificity for 17α-estradiol 3-glucuronide than for any of the other estrogens tested. This difference is not due to a greater affinity for 17α-estradiol 3-glucuronide by the enzyme but instead due to a faster rate of reaction. Therefore 17α-estradiol 3-glucuronide may be oriented on the enzyme in a manner which significantly reduces the activation energy for the reaction. Since the glucosyl conjugate also exhibited a lower specificity when compared with 17α-estradiol 3-glucuronide, perhaps the charge associated with the carboxyl function of the glucuronide may be important in the observed increase in the rate of catalysis of the glucuronide conjugate.

When estrone, estrone glucuronide or androstenedione were incubated with 17α-HSDases, in each case the specific activity for the reduction reaction was lower than the oxidation reaction. This can be explained by the fact that the free energy required to reduce is much higher than that required to oxidize (69).

The relatively higher specific activities of the 17α-HSDases with epitestosterone as substrate may suggest the importance of androgen detoxification in female rabbit liver. However the fact that these enzymes can be inhibited by

epitestosterone at concentrations as low as 2 μM indicates that relatively low levels of androgens are processed by the female rabbit liver at any time. It is interesting to note that Thaler-Dao et al. (9) did not observe a similar substrate inhibition of their rabbit liver 17β-HSDase at concentrations of testosterone higher than 2 μM. However the kinetic behaviour of a (3 and 17) β-HSDase activity from Pseudomonads testosteroni (16) and 17β-HSDase activity from mature male rat testes (68) also exhibited substrate inhibition. In both cases substrate inhibition was observed with testosterone but not with 17β-estradiol.

There is only one report in the literature documenting the molecular weight of 17α-HSDase. On the basis of the elution of rabbit liver 17α-HSDase activity from a Sephadex G-200 column, Breuer (10) calculated a molecular weight of 20000 daltons. This value is exactly one half of the value calculated for the 17α-HSDases in this thesis by SDS-polyacrylamide gel electrophoresis. This discrepancy is probably due to the large error involved in determining the molecular weight of such a small protein on the basis of its elution from a G-200 column. A more accurate estimate would have been achieved by gel filtration on either Sephadex G-100 or G-75. The molecular weights reported for the 17α-HSDases in this thesis are more consistent with the molecular weight

estimations for the 17β-HSDases from the following sources; guinea pig kidney, 31000 (25); human placenta (subunit), 33000 (6); rabbit liver, 35000 (9).

The amino acid composition of only one other 17-HSDase, human placental 17β-estradiol dehydrogenase, has been reported in the literature (61). A comparison of the amino acid compositions of this enzyme and the rabbit liver 17α-HSDases reveals distinct dissimilarities for a majority of the amino acids. However, very close similarities between the 17α- and 17β-HSDase are evident for the amino acids serine, glutamic acid, proline, methionine, phenylalanine and histidine.

The multiple nature of the rabbit liver 17α- and 17β-HSDase activity raises the difficult question of the integrity of these enzymes. Are these multiple enzymes artifacts of the purification procedure? That is, could one enzyme possibly have been modified during the purification procedure to generate multiple active forms of 17α-HSDase. Are these multiple forms true isozymes and if they are, are they single or multiple gene products (63)?

The first possibility can be eliminated on the basis of the following argument. If artifactual modification of one protein occurred to give rise to up to eight forms of 17α-HSDase, then one would expect that storage of the crude

rabbit liver supernatant for different periods of time would produce different enzyme activity profiles. If the modification was due to proteolysis (by carboxypeptidase), then by increasing the length of time for storage a completely different enzyme activity pattern would be generated if the enzyme was not completely inactivated. This has not been observed. However even if a few carboxyl terminal amino acid residues are removed from an enzyme, it does not usually result in changes in substrate specificity or electrophoretic mobility (56). There is in the literature one unusual example of removal of the carboxyl terminal amino acid by carboxypeptidase, which generated two enzymes with significantly different substrate specificities (64, 65).

Another possible method of artifactual modification of a protein is deamidation. Such a phenomenon has been observed with Neurospora cytochrome c (66). However, whether this variation is caused by deamidation during purification or by a genetic phenomenon is yet not clear (56). If the multiple 17-HSDases arise from deamidation, this process must occur by a genetic phenomenon within the cell. It is unlikely that the multiple 17-HSDases arise from deamidation during the purification procedure because of the consistently similar enzyme activity profiles obtained. If the process of deamidation was non-specific and occurred during the purification

procedure, one would expect to obtain inconsistent enzyme activity profiles. Deamidation could not possibly explain the presence of all eight 17α-HSDases since several of these enzymes have overlapping iso-electric points.

Binding of small molecules such as substrate, coenzyme, and salts may also result in the formation of multiple forms (56). This possibility can also be ruled out by the fact that the 17α-HSDase pattern obtained from different animals is consistent after gel filtration, ion-exchange chromatography or isoelectric focussing.

The frequency of reports in the literature on the heterogeneity of not only a large number of steroid dehydrogenases (9, 32, 33, 34; 59) but other enzymes such as bovine adrenal estrogen sulphotransferase (62) mouse aldolase (65) and carboxypeptidase (67) to name only a few, suggests that this is not a unique phenomenon.

The differences in the peptide maps of the thermolysin digests of each of the purified 17α-HSDases suggest that these enzymes may have small differences in their primary sequence. From this evidence, together with the other arguments given above, it is most unlikely that the multiple 17α-HSDases of rabbit liver are artifacts of the purification procedure. Since the differences in the peptide maps are small and the

amino acid compositions almost identical, there are two possible explanations for the occurrence of the multiple forms of 17α-HSDase. The multiple forms may arise from multiple genes, which are themselves products of gene duplications, or a single gene product is modified by a specific gene directed process to produce more than one form (63).

CONCLUSIONS

The 17-hydroxysteroid dehydrogenases in the soluble fraction of rabbit liver consist of two groups of enzymes, namely the 17α-HSDases and the 17β-HSDases. Although these enzymes oxidize stereospecifically either the 17α- or the 17β-hydroxyl group respectively, they can act on a variety of steroids, including estrogens and androgens, which possess these groups. It is apparent from the data presented in this thesis that there are at least eight forms of the 17α-HSDase activity. This is the first report not only of the purification of the 17α-HSDases, but also of the multiplicity of these enzymes in animal tissues. The 17β-HSDase activity also exists as multiple enzyme forms, as described in this thesis and reported by Thaler-Dao et. al. (9). This is also the first report of the purification of one of the 17β-HSDases from rabbit liver cytosol. However, evidence of the multiplicity and purification of 17β-HSDases from other animal tissues has been presented by several groups (32, 33, 34, 59).

One of the purified 17α-HSDases (enzyme IC) is particularly interesting because it is highly specific for 17α-estradiol 3-glucuronide when compared to other estrogen substrates. This first report of the purification of a highly active 17α-

HSDase, which is much more active on a conjugate than the free steroid, allows an interesting speculation of a possible pathway for estrogen metabolism. On the basis of the oxidation rates of the various multiple forms for the different estrogens tested, it is likely that conjugation with glucuronic acid at position three of the estrogen takes place before reduction which occurs at position seventeen.

The purification of the multiple 17α-HSDases poses an interesting dilemma. Are these multiple forms artifacts or are they true intracellular enzymes? If these enzymes are artifacts of the purification procedure they should be very similar in their function and structure. The most common feature is their molecular weight of approximately 39600. The amino acid compositions of these enzymes are stikingly similar except for a few minor differences. However, on the basis of their substrate specificities, their Km and Vmax values, their isoelectric points and their adsorption properties on DEAE-cellulose they are quite different. The final and most sensitive proof of the integrity of these enzymes presented in this thesis was achieved by a new technique of peptide mapping, with micro quantities of enzyme, by derivatizing ε-amino groups of lysine residues by ^{3}H-acetic anhydride. Radioautography of the derivatized lysine-containing peptides after electrophoresis suggested that there are small but

discrete differences in the primary structures of the purified multiple forms. Therefore, on the basis of the evidence presented it is concluded that the multiple forms are most probably not artifacts but, instead, are true intracellular enzymes. However, further characterization of the differences between these enzymes is required before a definitive statement can be made on whether these enzymes are actually isoenzymes, or multiple forms.

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