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THE PARTIAL PURIFICATION OF 17α- AND 17β-ESTRADIOL DEHYDROGENASE ACTIVITIES FROM CHICKEN LIVER*

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SUMMARY

The partial purification of two estradiol dehydrogenases from chicken liver is described. Fifty-fold concentration of an $NADP^+$-requiring 17α-hydroxysteroid dehydrogenase and thirty-fold concentration of an $NADP^+$-requiring 17β-hydroxysteroid dehydrogenase have been achieved by ammonium sulfate fractionation and ion-exchange chromatography. The stoichiometry of the reactions was established and the product identified as estrone.

INTRODUCTION

A search for a 17α-estradiol dehydrogenase as a basis for further investigations of the enzyme-catalyzed dehydrogenations at C-17 in the estradiol molecule was prompted by the partial purification of a 17β-estradiol dehydrogenase from human placenta[1] and by subsequent kinetic studies of LANGER, ALEXANDER AND ENGEL[2], and of ADAMS, JARABAK AND TALALAY[3] who used a highly purified preparation.

There was no reported purification of a 17α-estradiol dehydrogenase when this work was undertaken. Tissues from animals known to metabolize or to excrete 17α-estradiol (VELLE[4]) were examined as possible sources of that enzyme. Recently OZON AND BREUER[5] and DÖLLEFELD AND BREUER[6] have reported the presence of 17α-estradiol dehydrogenase activity in chicken liver and horse placenta respectively.

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Abbreviations etc.: The following non-standard trivial names and abbreviations have been used: epitestosterone, 17α-hydroxyandrost-4-en-3-one; androstenedione, androst-4-ene-3,17-dione; $NAD(P)^+$, the cofactor specificity of an enzyme preparation which uses either pyridine nucleotide; 17α-estradiol dehydrogenase, 17α-hydroxysteroid:NAD(P) oxidoreductase; 17β-estradiol dehydrogenase, 17β-hydroxysteroid:NAD(P) oxidoreductase.

MATERIALS AND METHODS

Reagents

All reagents were of certified or analytical quality. (Fisher Scientific Company, Fairlawn, N.J.). "Special enzyme grade" $(NH_4)_2SO_4$ was supplied by Mann Research Laboratories, Inc., New York, N.Y.

Solvents

Glass-redistilled or distilled water passed through a Barnstead Standard deionizing column (Barnstead Still and Sterilizer Co., Boston, Mass.) was used for all aqueous solutions. Organic solvents were of analytical grade (redistilled).

Steroid substrates

Stock solutions of recrystallized steroids (2 mM) prepared in absolute ethanol and stored at 4–5° were used in most assays of enzyme activity. The purity of steroid substrates was tested and the tentative identification of reaction products from incubation mixtures and spectrophotometric assays was made by thin-layer and paper chromatography as described below.

Radioactive steroids

[6,7-$^3H_2$]estrone, 40.6 C/mmole (New England Nuclear Corporation, Boston, Mass.) was purified by paper chromatography.

Cofactors

The β forms of NAD^+, $NADP^+$ and the reduced compounds were obtained from Sigma Chemical Company, St. Louis, Mo. The stated purity of the oxidized cofactors was 98% (corrected for 4.5 and 3 moles of H_2O respectively); this was confirmed by enzymatic reduction with glucose-6-phosphate dehydrogenase (Sigma Type V) in a modification of the assay of HORECKER AND KORNBERG[7]. No contaminants were found in 1.0 mM solutions of NAD^+ and $NADP^+$ by examination on poly(ethyleneimine)cellulose in 0.2 M aqueous lithium chloride[8].

Ion-exchange celluloses

DEAE- and CM-celluloses (Brown Co., Berlin, N.H.) were washed with 0.1 M HCl and 0.1 M NaOH followed by repeated washing in distilled water before use. The capacities of the exchangers (approx. 0.7 mequiv./g and 0.6 mequiv./g respectively) were unaffected by this treatment.

Dialysis tubing (Arthur H. Thomas Co., Philadelphia, Pa.) was soaked in aqueous EDTA (5 g per 100 ml) for at least 48 h before use; it was then washed thoroughly in deionized water and in the dialysis buffer. Dialysis was carried out at 4–5°.

Determination of radioactivity

Counting was done on randomized samples in a Packard Tri-Carb liquid-scintillation spectrometer (Model 314 Ex.) The efficiency for ^3H was about 23%, sufficient counts were accumulated to ensure an accuracy of about 1%.

Estimation of estrone

Estrone was measured spectrofluorimetrically[9] on randomized samples by means of a Farrand Model-A fluorimeter.

Thin-layer chromatography

Plates were prepared with silica gel G (Merck, according to Stahl). Mobilities of steroid reaction products were compared with those of reference compounds in the system, ether–benzene (2:1, v/v). Before spraying with 95% ethanol–conc. sulfuric acid (1:1, v/v) the plates were examined in ultraviolet light. In visible light, 17*α*-estradiol was seen as a bright yellow spot immediately after treatment with fresh reagent. On heating at 100° for 5–10 min, estrone and the epimeric estradiols appeared as orange spots which became dull on exposure to air.

Paper chromatography

Steroids were partitioned on Whatman No. 1 paper equilibrated by the rapid method of BUSH AND CROWSHAW[10]. Samples and reference compounds were washed to the start line with ethyl acetate–methanol–chloroform (1:1:1, v/v/v) and the paper was dried in air. The procedure was repeated twice. The paper was then soaked in ether–75% methanol (4:1, v/v) and placed in the system, ligroin–toluene–70% methanol (2:1:3, v/v/v) at 29°. Phenolic steroids were detected with $FeCl_3$–$K_3Fe(CN)_6$ (ref. 11).

Countercurrent distributions

Four- and eight-tube countercurrent distributions were carried out in separatory funnels with stripping[12]. 100 transfer distributions were made in a 100-tube all-glass apparatus (H. O. Post Scientific Co., New York) calibrated for 10 ml lower phase. The system was carbon tetrachloride–methanol– water (2:1:1, v/v/v); equal volumes of upper and lower layer were used. On completion of the distributions the contents of selected tubes were evaporated under reduced pressure, the residues dissolved in 5 ml ethanol (U.S.P.) and 10-*μ*l samples removed at the same time for ^3H counting and fluorescence assay. Statistical analyses and calculation of the curves that best fitted the data were carried out using the computer programme described by PURDY, GOLDMAN AND RICHARDSON[13].

Tissue incubations

Whole tissue homogenates were prepared in a Virtis homogenizer in 0.1 M sodium phosphate, Tris or sodium bicarbonate–carbonate buffers to give a pH range of 6–9.5. The homogenates were centrifuged twice at 34 000 × *g* for 30 min. Incubation mixtures usually consisted of 5 ml of the second supernatant fraction, 5 *μ*moles of substrate in 0.1 ml ethanol and 25–62.5 *μ*moles of cofactor in 0.1 or 1.0 ml of aqueous solution. All incubations were carried out in air in a Dubnoff incubator for 30 min at 37°. The mixtures were quickly cooled to 4°, the pH adjusted to 7.4–8.0, and the steroids extracted with three 5-ml portions of methylene chloride. The incubation mixtures *plus* organic solvent were centrifuged at 3000 rev./min for 10 min to break any emulsion and to separate the aqueous and organic phases. Any tissue present was compressed into a disk at the interface and was removed before withdrawing the organic solvent. Extracts were evaporated under reduced pressure, the residues were

dissolved in 0.5 ml ethanol and 30-μl samples were examined by thin-layer chromatography.

Localization of enzyme activity

Trimmed sheep, chicken and turkey liver were each ground and homogenized in a Virtis homogenizer at reduced speed using 1 ml sucrose–sodium phosphate buffer (0.25 M–0.1 M; pH 7.2) for each g (wet weight) of tissue. Subcellular fractions were prepared by differential centrifugation[14]. The mitochondrial fractions were sedimented between 900 and 9000 $\times$ *g*, washed with the same buffer solution and centrifuged at 15 000 $\times$ *g*; this procedure was repeated twice. The microsomal preparations were collected by centrifugation of the supernatant fraction at 105 000 $\times$ *g* and washed twice. Phase-contrast microscopy of supernatant and debris showed no intact cells after homogenization. The microsomal fraction was heavily contaminated with mitochondria after the first centrifugation, but most were removed in subsequent washes.

Enzyme assay

A spectrophotometric assay for both 17α- and 17β-estradiol dehydrogenase activities in the presence of saturating amounts of substrate and cofactor was based upon that used by LANGER AND ENGEL[1] for the 17β-estradiol dehydrogenase of human placenta. The assay system was composed of: 0.2 μmole steroid in 0.1 ml ethanol; 300 μmoles $NaHCO_3$–Na_2CO_3 buffer (pH 9.5 at 20°); 0.4 μmole $NADP^+$ in 0.1 ml distilled water; 0.3 ml enzyme preparation, and brought to a total volume of 3 ml with distilled water. The temperature was 37°, the final pH was 9.2. In all instances the reaction was initiated by the addition of $NADP^+$ and the rate of change of absorbance at 340 mμ was measured in a Zeiss PMQ II spectrophotometer against a blank cell containing all components of the assay system except steroid. Readings were taken 20 sec after the addition of cofactor and every 10 sec thereafter for 1.5–2 min. Velocities were calculated from the slopes of the zero-order portion of the curves plotted as absorbance against time and were corrected for the absorbance change in the blank cuvette. A unit of 17α- or 17β-estradiol dehydrogenase activity was defined as the amount that catalyzes the conversion of 1 μmole of substrate per min per ml under the assay conditions specified above. Because of the low enzyme concentrations in the tissues studied, all activities are reported in munits. Specific activity was expressed in munits of enzyme activity per mg of protein determined either by the method of LOWRY *et al.*[15], or by the ratio of absorbance at 280 mμ to 260 mμ using Adam's nomograph (Calbiochem) based on the data of WARBURG AND CHRISTIAN[16].

Estrone from 17β-estradiol

37 assays were run for 1 min using a dialyzed 0–50% $(NH_4)_2SO_4$ precipitate. The rate of change of absorbance was measured at 340 mμ and the reaction mixtures were quickly transferred to ethanol at 4°. Preliminary experiments showed that the rate of formation of NADPH was constant for at least 2 min. It was therefore possible to correct the absorbance reading for the time taken to raise the lid of the cuvette holder, remove the cuvette and discharge the reaction mixture. Each cuvette was rinsed twice with 0.5 ml ethanol; the resultant pool was centrifuged to remove the

precipitated protein which was resuspended and washed three times with fresh ethanol. The combined washings were evaporated under reduced pressure. Chromatographically pure [6,7-$^3H_2$]estrone ($3.18 \cdot 10^6$ disint./min) was added to the dry residue and, after evaporation of the solvent, the residue was subjected to a four-tube countercurrent distribution with stripping using 150 ml ethyl acetate as upper phase and 30 ml distilled water as lower phase. The aqueous extracts were discarded.

The organic extracts were pooled, evaporated under reduced pressure, then partitioned between hexane–90% aqueous methanol (1:1, v/v) in an eight-tube countercurrent distribution with stripping. The aqueous methanol extracts were combined, dried and the residue subjected to a 100-transfer countercurrent distribution.

Estrone from 17α-estradiol

The above procedure was modified. 25 assays were run for 1 min and the reactions quenched in methanol at 4°; to this pool was added $3.07 \cdot 10^6$ disint./min of chromatographically pure [6,7-$^3H_2$]estrone. The remainder of the experiment was carried out as described above.

Enzyme purification

Unless otherwise stated, all manipulations were carried out at 4–5°. Chicken liver obtained within 1 h of slaughter was dissected free of fatty tissue and minced in a meat grinder. 3 ml of a 0.1 M sodium phosphate buffer (pH 7.8), 10 mM in both nicotinamide and EDTA, was used for each g (wet weight) of tissue and a homogenate was prepared in a Waring blendor at reduced speed (Variac set at 80 V). 1.5 kg of tissue yielded 7.8 l of homogenate; this was centrifuged at 14 600 × *g* for 45 min and the residues discarded. Phase-contrast microscopy of supernatant and centrifuged debris showed that practically all the cells were ruptured under these conditions.

Solid $(NH_4)_2SO_4$ (231 g/l) was slowly added to the supernatant with constant stirring, the pH was adjusted with 1 M HCl to 6.8 and maintained at that value with 3 M NH_4OH. The mixture was stirred for 30 min after the $(NH_4)_2SO_4$ had dissolved. The protein precipitate was sedimented by centrifugation at 15 000 × *g* for 30 min and discarded. It usually contained about 0.2 munits/mg protein of 17β-estradiol dehydrogenase activity after dialysis. No 17α-estradiol dehydrogenase was ever detected in this fraction.

A second $(NH_4)_2SO_4$ fraction was collected using 451 g/l. The precipitate was centrifuged at 15 000 × *g* and this fraction, when suspended in a minimal volume of 5 mM sodium phosphate buffer (pH 7.2) containing glycerol (50%, v/v) constituted the stock preparation. 700 ml was obtained from 1.5 kg tissue. The $(NH_4)_2SO_4$ fractions corresponded to 0–30% and 30–80% saturation calculated for 0° using TAYLOR's[17] equation.

Chromatography on DEAE-cellulose

Further purification of the enzyme activities in the stock preparation was effected on DEAE-cellulose. A jacketed glass column (diameter 5 cm) was packed with this anion-exchanger at room temperature in sodium phosphate buffer (5 mM, pH 7.2) which was 1 mM in EDTA and contained glycerol (20%, v/v). The 5 cm × 40 cm

DEAE-cellulose column was gradually cooled to 5° by circulating refrigerant from a Buchler refrigerated fraction collector and before application of the sample, was equilibrated at 5° with at least 1500 ml of the same buffer mixture.

Approx. 40 ml of the stock preparation was dialyzed for a total of 3 h against four 4-l volumes of the buffer described above. The contents of the dialysis bag (approx. 60 ml) were centrifuged at 35 000 × *g* for 20 min and the small amount of precipitate was discarded. The enzyme activities of the supernatant were measured before chromatography; specific activities of 17α- and 17β-estradiol dehydrogenases were of the order of 0.4 and 0.9 munits/mg protein respectively.

30 ml of this supernatant was applied to the column and eluted directly with the equilibrating buffer; an L.K.B. fixed-wavelength (253 mμ) recorder was used to monitor protein content. Fractions (80 drops) were collected. A typical elution pattern is shown in Fig. 1.

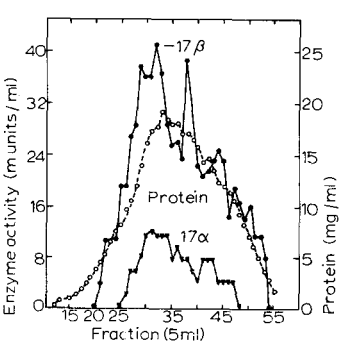


Fig. 1. Chromatography on DEAE-cellulose. Approx. 40 ml of stock $(NH_2)_2SO_4$ precipitate was dialyzed against 5 mM sodium phosphate buffer (pH 7.2) containing EDTA (1 mM) and glycerol (20%, v/v). The non-diffusible material was centrifuged and 30 ml of supernatant applied to a 5 cm × 40 cm column of DEAE-cellulose. 510 ml of effluent was collected before the elution of protein. Enzyme assays and protein determinations were performed in duplicate on each fraction. ●—●, 17β-estradiol dehydrogenase; ▼—▼, 17α-estradiol dehydrogenase; ○— — —○, protein.

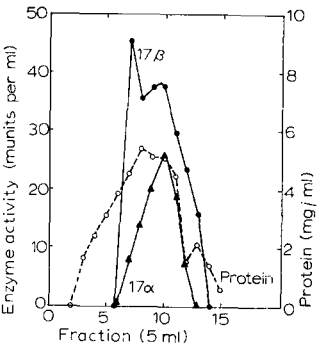


Fig. 2. Chromatography on CM-cellulose. Fractions that contained the most 17α-estradiol dehydrogenase activity after chromatography on DEAE-cellulose were pooled and 30 ml was applied to a 5 cm × 20 cm column of CM-cellulose, equilibrated and eluted with the phosphate–EDTA–glycerol mixture described under Fig. 1. A typical elution pattern is depicted; 240 ml of effluent was collected before elution of protein. Enzyme assays and protein determinations were carried out in duplicate on each fraction. ●—●, 17β-estradiol dehydrogenase; ▲—▲, 17α-estradiol dehydrogenase; ○— — —○, protein.

Chromatography on CM-cellulose

Fractions from the DEAE-cellulose chromatogram that contained 17α-estradiol dehydrogenase activity were pooled and a 30-ml portion was applied to a 5 cm × 20 cm column of CM-cellulose. The column was equilibrated with the same buffer mixture as that used for chromatography on DEAE-cellulose (Fig. 2).

Gel filtration on Sephadex G-200

A partial separation of both estradiol dehydrogenase activities has been obtained by gel filtration on Sephadex G-200 (Fig. 3).

Portions of stock $(NH_4)_2SO_4$ precipitate dialyzed for 18 h against running tap-water at 13° were centrifuged at approx. 34 000 × *g* for 20 min. The residue was dis-

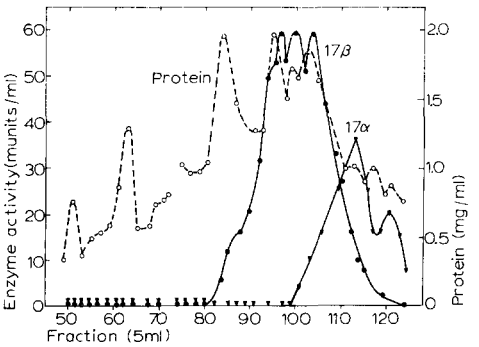


Fig. 3. Gel filtration on Sephadex G-200. 2 g of a lyophilized preparation of 17α- and 17β-estradiol dehydrogenase activities was applied to a 3 cm × 120 cm column of Sephadex G-200 in 6.5 ml 50 mM sodium phosphate buffer, (pH 6.7) at 5°. The void volume was 410 ml determined with Blue Dextran. Elution was carried out with the same buffer and assays were done in duplicate at 42°. ●—●, 17β-estradiol dehydrogenase; ▼—▼, 17α-estradiol dehydrogenase; ○— — —○, protein.

carded and the supernatant shell-frozen and freeze-dried. This material (8 g) was ground in a mortar with 50 ml 100 mM sodium phosphate buffer (pH 6.8) shaken gently with 100 ml suspension of CM-Sephadex in the same buffer, and centrifuged at 12 000 × *g* for 20 min. The CM-Sephadex was washed twice with fresh phosphate buffer and the combined washings (150 ml) were shell-frozen and freeze-dried.

Gel filtration was carried out on a 3 cm × 120 cm column of Sephadex G-200 in 50 mM sodium phosphate buffer (pH 6.7) at 5°. The void volume determined with Blue Dextran 2000 (Pharmacia) was 410 ml. The enzyme preparation applied to the column consisted of 2 g freeze-dried material in 6.5 ml 50 mM sodium phosphate buffer (pH 6.7). The specific activities of the 17α- and 17β-estradiol dehydrogenases were 0.7 munits and 5 munits per mg protein respectively. Elution was carried out with the same buffer. Assays were performed in duplicate at 42°. The 17β-estradiol dehydrogenase began to be eluted after one void volume.

RESULTS

Tissue incubations

Sheep- chicken- and turkey-liver homogenates catalyze the reversible oxidation of the estradiol epimers to estrone; the apparent cofactor required was $NADP^+$. Similar results were obtained with rabbit liver homogenates in the presence of NAD^+ or $NADP^+$; in the presence of either pyridine nucleotide calf-liver was found to oxidize 17β- but not 17α-estradiol.

Localization of enzyme activity

Incubation of subcellular fractions of sheep, chicken and turkey liver at pH 7.0–9.0 showed that the enzymes which reversibly oxidized 17α- and 17β-estradiol to estrone were located almost exclusively in the cell supernatant and that $NADP^+$ was the preferred cofactor.

No conversion of the estradiol epimers to estrone occurred when enzyme preparation was omitted from the incubation or spectrophotometric assay mixture or when a boiled enzyme preparation was used.

Chicken liver was chosen as starting-material for large-scale preparations because of its relatively abundant activity and ready availability.

Characterization of product

The product formed from both estradiol epimers in incubations and pooled spectrophotometric assays was provisionally identified as estrone by thin-layer and paper chromatography. The reduction products of estrone in incubation experiments were also tentatively identified as 17α- and 17β-estradiol by their mobilities on thin-layer chromatograms.

When spectrophotometric assays were run with stored precipitates or when new methods of purification were attempted, it was the practice to pool several assay mixtures in ethanol or methanol at 4°, remove precipitated protein by centrifugation, resuspend and extract the precipitate with fresh solvent and examine the steroid reactants and products by thin-layer chromatography.

TABLE I

STOICHIOMETRY OF OXIDATION OF EPIMERIC ESTRADIOLS

Substrate	*NADPH* formed (μmoles)*	*Estrone** formed (μmoles)*	*NADPH per estrone*
17β-Estradiol	3.03	3.04	1.00
17α-Estradiol	1.81	1.56	0.86

* Calculated from total change in absorbance at 340 mμ.
** Calculated from countercurrent distribution.

Stoichiometry and identification of estrone

The stoichiometric relations between steroid alcohol oxidation and pyridine nucleotide reduction are shown in Table I.

The identification of estrone as the oxidation product of the two estradiol epimers is depicted in Table II. Statistical analysis of the countercurrent distributions provided satisfactory evidence for congruence of the mass and radioactivity curves.

Purification

A 50-fold purification of the 17α-estradiol dehydrogenase activity and a 30-fold purification of the 17β-estradiol dehydrogenase activity was obtained by the procedure described. In a typical preparation, the crude homogenate after centrifugation had specific activities of 0.1 munit 17α- and 0.4 munit 17β-estradiol dehydrogenase (per mg of protein), whereas the specific activities after chromatography on CM-cellulose were 5 and 12 munits/mg of protein respectively. The overall yields were 190 and 150%. Increases in total activity occurred chiefly after chromatography on DEAE-cellulose. It is possible that an inhibitor of both dehydrogenations, an NADPH-oxidizing system or a nucleotidase present in the initial homogenate and in the $(NH_4)_2SO_4$ precipitate after dialysis, was removed during ion-exchange chromatography. The 17β-estradiol dehydrogenase activity was always found in 2–3 times the quantity of the 17α-estradiol dehydrogenase activity.

TABLE II

ANALYSES OF COUNTERCURRENT DISTRIBUTIONS OF ESTRONE PREPARED BY ENZYMATIC OXIDATION OF 17α- AND 17β-ESTRADIOL

	Estrone from 17α-estradiol			*Estrone from 17β-estradiol**		
	Value	*t*	*P*	*Value*	*t*	*P*
Standard errors of difference:						
Coefficients of linear terms	0.00814	2.29	>0.1	0.00924	1.97	>0.1
Coefficients of quadratic terms	0.00557	1.58	>0.2	0.00632	0.75	>0.5
Mean tube number for ^3H	23.5			24.5		
Mean tube number for estrone	22.8			24.9		
Standard error of difference	0.23	2.66	>0.05	0.29	1.39	>0.2
$K^{**}/(K+1)$ for ^3H	0.237			0.248		
$K/(K+1)$ for estrone	0.231			0.252		
Standard error of difference	0.0023	2.66	>0.05	0.0028	1.39	>0.2

* Degrees of freedom = 3.

** K is partition coefficient (concentration of solute in upper phase/concentration of solute in lower phase).

Preliminary experiments with $(NH_4)_2SO_4$ fractionation, at 10, 15 or 25% increments of saturation showed that the 17β-estradiol dehydrogenase activity was distributed throughout the 25–75% fractions whereas the other enzyme activity was largely confined to the precipitate collected between 30 and 50% saturation. In large-scale preparations, fractions were taken at 30 and 80% saturation to minimize loss of 17α-estradiol dehydrogenase activity and to facilitate speedy fractionation.

The yields of 17α- and 17β-estradiol dehydrogenase activities were not enhanced by the addition of either cysteine–HCl (1 mM), nicotinamide (10–100 mM) or glycerol (10 or 20%, v/v) to the buffer used for homogenization. Salt fractionation in glycerol–buffer mixtures was unsatisfactory.

Although the estradiol dehydrogenase activities of crude homogenates survived heating at 60° for 30 min, heating to 50° followed by rapid cooling proved impractical as a means of purification because of poor recoveries.

Stability (Table III)

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

Centrifuged homogenates made at pH 7–8 showed no loss of activity when stored for 24 h at 4°. The activities of the stock $(NH_4)_2SO_4$ preparation were stable for at least 4 months when stored in 5 mM sodium phosphate buffer containing glycerol (50%, v/v). While there was no loss of the 17β-estradiol dehydrogenase activity during that time, the recovery of the 17α-estradiol dehydrogenase activity varied from 75–100%.

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

TABLE III

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

Column I lists enzyme activities assayed within 4 h of preparation. Column II lists enzyme activities assayed after storage.

	Specific activity (munits/mg protein)			
	17α		*17β*	
	I	*II*	*I*	*II*
Centrifuged homogenate after 24 h at 5°	0.1	0.1	0.3	0.3
30–80% $(NH_4)_2SO_4$ precipitate kept for 4 months at −10° in glycerol (50%, by vol.) and dialyzed	0.4	0.4	0.9	0.9
Eluate of DEAE-cellulose column after 24 h at 5° (without glycerol)	0.4	0.2	3.0	3.0
Eluate of DEAE-cellulose column containing glycerol (20%, by vol.) after 7 days at 5°	3.0	3.0	6.0	6.0
Eluate of DEAE-cellulose column containing glycerol (20%, by vol.) after 3 days at 19–23°	3.0	0	6.0	6.0
Preparation obtained by chromatography on DEAE-cellulose and CM-cellulose stored at 5° for 3 days in 5 mM sodium phosphate buffer containing glycerol (20%, by vol.)	5.0	5.0	12.0	12.0

estradiol dehydrogenase activity in these preparations was also stable at 19–23° for at least 3 days; the 17α-estradiol dehydrogenase activity was almost completely lost within 12 h under the same conditions.

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

pH optima (Fig. 4)

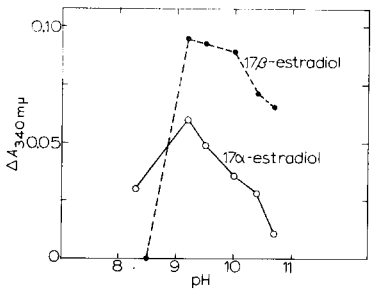
The initial rates of dehydrogenation of both estradiol epimers were determined over the pH range of 6.4–10.7. Comparable results were obtained when glycine–NaOH buffer was used instead of sodium bicarbonate–carbonate. The maxima in reaction rate between 9–9.5 are similar to those reported for the 17β-estradiol dehydrogenase of human placenta.

TABLE IV

APPARENT VALUES FOR K_m AND v_{max} FOR 17α-ESTRADIOL DEHYDROGENASE ACTIVITY AT 25°

In column 1 substrate refers to the compound under test. $(NH_4)_2SO_4$ precipitates taken between 30 and 80% or 40 and 60% saturation were dialyzed and chromatographed on DEAE-cellulose in 5 mM sodium phosphate buffer (pH 7.2) containing glycerol (20%, v/v). Each cuvette contains 20 mg protein. Assays were carried out in duplicate or triplicate at each substrate concentration.

Substrate	K_m *(μM)*	v_{max} *(mμmoles substrate per min per mg protein)*
17α-Estradiol	12	1.0
$NADP^+$	3.8	2.6



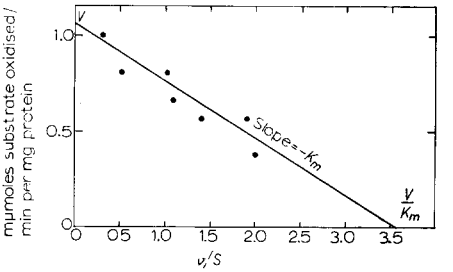


Fig. 4. The pH dependence of 17α- and 17β-estradiol dehydrogenase activities. An $(NH_4)_2SO_4$ precipitate taken between 0–50% saturation was stored for 6 days at −14° in glycerol buffer and dialyzed against 5 mM sodium phosphate buffer (pH 7.2), 1 mM in EDTA and in cysteine–HCl. The non-diffusible material was centrifuged at 34 600 × *g* for 30 min and 0.5 ml supernatant was assayed in duplicate for each substrate against a series of buffers. The assay mixtures for each substrate at each pH were pooled and extracted. The steroids were examined by thin-layer chromatography to convrm that the product was estrone.

Fig. 5. Determination of apparent K_m for 17α-estradiol dehydrogenase by the method of WOOLF[17]. $(NH_4)_2SO_4$ precipitates taken between 30 and 80% or 40 and 60% saturation were dialyzed and chromatographed on DEAE-cellulose in 5 mM sodium phosphate buffer (pH 7.2) containing glycerol (20%, v/v). Assays were carried out in duplicate or triplicate at each substrate concentration. The graphical representation is that described by WOOLF[17] where the initial velocity (v) is plotted against the ratio v/S (S = substrate concentration). The line is the regression fitted by unweighted least squares. K_m (substrate, 17 α-estradiol) = 12 μM.

A linear increase in absorbance at 340 mμ was observed in assays carried out at pH 8.0 or below. The reaction mixtures became turbid on standing for a few minutes at room temperature. Examination of extracts of pooled mixtures by thin-layer chromatography showed that no estrone was formed in these circumstances.

Kinetic measurements

Apparent kinetic constants for both estradiol dehydrogenase activities were determined at 25° using eluates of DEAE-cellulose columns (Tables IV and V). Values of v_{max} (maximal velocity) and K_m (Michaelis constant) were obtained by the linear method of WOOLF[18] advocated by HOFSTEE[19]. Regression lines were fitted by the method of least squares without weighting (Fig. 5).

TABLE V

APPARENT VALUES FOR K_m AND v_{max} FOR 17β-ESTRADIOL DEHYDROGENASE ACTIVITY AT 25°

In column 1 substrate refers to the compound under test. $(NH_4)_2SO_4$ precipitates taken between 30 and 80% or 40 and 60% saturation were dialyzed and chromatographed on DEAE-cellulose in 5 mM sodium phosphate buffer (pH 7.2) containing glycerol (20%, v/v). Each cuvette contains 20 mg protein. Assays were carried out in duplicate or triplicate at each substrate concentration.

Substrate	K_m (μM)	v_{max} (*mμmoles substrate per min per mg protein*)
17β-Estradiol	16	3.5
NADP+	8	5.2

The reactions catalyzed by 17α- and 17β-estradiol dehydrogenase activities apparently followed Michaelis–Menten kinetics with respect to the steroid substrates. The determination of apparent K_m for $NADP^+$ at 25° for both activities gave non-linear plots. Attempts to measure Michaelis constants for NAD^+ were unsuccessful because of high activity in the blank cuvette on the addition of this nucleotide.

Cofactor specificity

The estradiol dehydrogenase activities of chicken liver do not show an absolute cofactor requirement for $NADP^+$. In incubation experiments with crude homogenates and subcellular fractions NAD^+ also served as coenzyme but the yields of estrone from both epimers were appreciably greater with $NADP^+$.

Reactivity towards other steroid substrates

Eluates of DEAE-cellulose columns which oxidized 17α- and 17β-estradiol to estrone also oxidized epitestosterone and testosterone to androstenedione, provisionally identified by comparison of its mobility with that of the authentic compound in the thin-layer system previously described. The initial velocities obtained with the C_{19} substrates varied from 50–75% of those determined with the estradiol epimers. Whether or not these activities reside in the same protein molecule has yet to be determined.

DISCUSSION

Two estradiol dehydrogenase activities found mainly in the soluble fraction have been partially purified from chicken liver. The 17α-estradiol dehydrogenase activity has been concentrated about 50-fold (5 munits/mg protein) and the 17β-estradiol dehydrogenase about 30-fold (12 munits/mg protein). The activity of the latter enzyme is 2–3 times that of the former and a partial separation may be effected by $(NH_4)_2SO_4$ fractionation. Partial separation of both activities on Sephadex G-200 further supports the existence of two distinct enzymes which are pyridine nucleotide-linked and which show a marked preference for $NADP^+$.

The product of both dehydrogenations is estrone which is formed in an amount equivalent to the pyridine nucleotide reduced .

These results are in broad agreement with those of OZON AND BREUER[5] in that the enzyme activities are found mainly in the soluble fraction, the 17β-estradiol dehydrogenase activity is predominant and that a partial separation may be achieved by salt fractionation. Further comparison of pH optima and cofactor specificity is precluded by the different experimental methods employed.

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