Purification and Characterization of Epimeric Estradiol Dehydrogenases (17α and 17β) from Equine Placenta†

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ABSTRACT: Estradiol 17α-dehydrogenase (EC 1.1.1.148) and estradiol 17β-dehydrogenase (EC 1.1.1.62) from horse placenta have been purified to homogeneity. Both enzymes are localized in the microsomal fraction and are solubilized in 1.5% sodium cholate. The 17α- and 17β-dehydrogenases are separated by selective elution from hydroxylapatite with 0.5 and 1.0 M potassium phosphate, respectively. Subsequent purification is achieved by two affinity-absorption steps using reactive blue 2-agarose and estriol hemisuccinate–Sepharose. Homogeneous estradiol 17α-dehydrogenase has a specific activity of 10 IU/mg and has been purified 5900-fold with an 87% recovery. Homogeneous estradiol 17β-dehydrogenase has a specific activity of 10.6 IU/mg and has been purified 15000-fold with an apparent recovery of 100%. Each enzyme exhibits a single band on polyacrylamide disc gel electrophoresis and on sodium dodecyl sulfate (SDS) gel electrophoresis. The mobilities of the two on SDS gels are identical and correspond to subunit molecular weights of 33000. The apparent molecular weight of the undenatured, active enzyme, as determined by gel filtration on Sephacryl S-300, is 52000 in the case of the 17α-dehydrogenase and 68500 for the 17β-dehydrogenase. Both enzymes exhibit pH optima at 9.0–9.5; both prefer NAD^+ over $NADP^+$ but utilize both cofactors. Both are highly specific for their respective epimers of estradiol with apparent K_m values of 1.7 μM at pH 9.5.

We have purified estradiol 17α-dehydrogenase and estradiol 17β-dehydrogenase from a single source as a first step toward the ultimate goal of comparing structure and active site topography of these epimeric dehydrogenases with each other and with human placental estradiol 17β-dehydrogenase. The human enzyme has been purified and crystallized (Chin et al., 1976) and topography of its active site examined by affinity-labeling techniques in this laboratory (Chin & Warren, 1975; Chin et al., 1982; Murdock & Warren, 1982).

The presence of both enzymes has been reported in sheep (Pierrepoint et al., 1971) and horse (Dollefeld & Breuer, 1966) placenta in late pregnancy and in chicken, rabbit, sheep, and turkey liver (Renwick & Engel, 1967). Because of the quantity of tissue available and the interest in using a second mammalian placental source for comparison with the human placental enzyme, we have pursued the activities from horse placenta. Both estradiol 17α-dehydrogenase and estradiol 17β-dehydrogenase are present in horse placenta in quantities sufficient to allow structural studies.

We now report the purification to homogeneity of estradiol 17α-dehydrogenase and estradiol 17β-dehydrogenase from horse placenta and their characterization in terms of apparent molecular weight, subunit composition, substrate, and cofactor specificity and certain kinetic parameters.

Experimental Procedures

Reagents. Reagent-grade salts, inorganic acids, organic solvents, and Scintiverse were obtained from Fisher. Dithioerythritol, 17α-estradiol, 17β-estradiol, Sephadex LH-20, NAD^+, $NADP^+$, *p*-nitrophenol acetate, Coomassie Blue R250, Sephacryl S-300, sodium cholate, reactive blue 2-agarose, and other steroids were purchased from Sigma. Acrylamide, *N,N,N',N'*-tetramethylethylenediamine, *N,N'*-methylenebis(acrylamide), ammonium persulfate, sodium dodecyl sulfate (SDS), Tris, glycine, molecular weight standards, and DNA grade Bio-Gel hydroxylapatite were purchased from Bio-Rad. The gel filtration calibration kit was obtained from Pharmacia. 17β-[4-^{14}C]Estradiol and 17α-[2,4(η)-^{3}H]estradiol were obtained from Amersham. The water used throughout the procedures was deionized and double distilled.

Assays of Estradiol 17α- and 17β-Dehydrogenase Activity. In the isotope assay the production of estrone from estradiol was measured by using 17β-[4-^{14}C]estradiol or 17α-[2,4-(η)-^{3}H]estradiol to form radiolabeled estrone. Assay mixtures of 5.0 mL total volume contained 0.15 μmol of the appropriate epimer of estradiol (15000 dpm) and 1.0 μmol of NAD^+ in 0.1 M sodium pyrophosphate buffer containing 10% glycerol, pH 9.5. Samples were incubated for 10 min at 37 °C and then immersed in an ice bath. Radioactive estrone and estradiol were quickly extracted twice with 2 volumes of anhydrous ether. The ether extracts were evaporated under N_2 and the residues dissolved in 0.2 mL of ethanol and applied to a 1 × 10 cm column of Sephadex LH-20. The column was eluted with methanol–benzene, 1:9, and 15 fractions of 1.5 mL were collected. The radioactivity in each fraction was quantitated in a Model 3320 Packard TriCarb liquid scintillation spectrometer. Radioactivity in the first peak (fractions 3–6) represents the estrone formed while that in the second peak (fractions 8–14) is unreacted estradiol.

Dehydrogenase activity as reflected by the production of NADH from NAD^+ was calculated from the initial linear increase in absorbance at 340 nm. Unless otherwise specified, the assay mixture contained 0.1 μmol of 17α- or 17β-estradiol and 0.6 μmol of NAD^+ in 0.1 M sodium pyrophosphate buffer containing 10% glycerol with a total volume of 3.0 mL, pH 9.5. As described by Langer & Engel (1958) assays were conducted at 25 °C and started by the addition of enzyme. One international unit (IU) is defined as the amount of enzyme converting 1 μmol of NAD^+ to NADH in 1 min. Protein concentrations were determined as described by Lowry et al. (1951).

Assays for Esterase Activity. Esterase activity was measured by following the hydrolysis of *p*-nitrophenol acetate as

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described by Rahem & Sih (1969). *p*-Nitrophenol acetate was dissolved in methanol (1.0 mg/mL). The assay mixture contained 100 μL of the *p*-nitrophenol acetate solution and the enzyme sample in 0.05 M phosphate buffer, pH 7.0, in a total volume of 3.0 mL. The activity was then calculated from the change in absorbance at 400 nm.

Subcellular Fractionation. Villous tissue of horse placenta was homogenized in 0.05 M potassium phosphate buffer, pH 7.0, containing 20% glycerol and 1 mM dithioerythritol (buffer A; 4 °C) at a tissue:buffer ratio of 1:3 (Waring Blendor, three 20-s bursts). Glycerol was used in the homogenization buffer instead of sucrose to stabilize the enzymes. The concentration of glycerol was such that the density of buffer A (1.046) was comparable to that of 0.25 M sucrose (1.034) (Fasman, 1976). Subcellular fractions were prepared by differential centrifugation (Schneider & Hogeboom, 1950). The mitochondrial fraction was sedimented by centrifugation for 1 h at 12000*g*. The microsomal fraction was sedimented by centrifugation of the mitochondria-free supernatant, at 105000*g* for 90 min. The resulting supernatant is placental cytosol.

Purification of Estradiol 17α- and 17β-Dehydrogenase. Horse placentas were obtained from local stables within a few hours after delivery and transported to the laboratory on ice. All further purification steps were conducted at 4 °C. The villous placental tissue was homogenized as described above. The homogenate can be stored frozen for 6 months with little loss in activity. In a typical purification, 1 L of homogenate was centrifuged at 12000*g* for 1 h to remove debris and mitochondria. The resulting supernatant was then brought to 8 mM Ca^{2+} with $CaCl_2$, the pH adjusted to 7.4, and the supernatant allowed to stand for 1 h. The Ca^{2+}-aggregated microsomes were collected by centrifugation at 25000*g* for 1 h. The aggregation of microsomes with Ca^{2+} is as described by Schenkman & Centi (1978). The microsomes were suspended in buffer A containing 1.5% cholate with a final protein concentration of 5 mg/mL and stirred at 4 °C for 1 h. The suspension was centrifuged at 25000*g* for 1 h. Cholate solubilization of the resulting precipitate was repeated once. The combined supernatants were dialyzed for 16 h against buffer A.

This solubilized, dialyzed preparation was applied to a column of hydroxylapatite (4 × 4 cm). The hydroxylapatite was washed consecutively with 200 mL each of buffer A, 0.5 M potassium phosphate buffer, and 1.0 M potassium phosphate buffer (all buffers pH 7.0 and contain 20% glycerol and 1 mM dithioerythritol). The 17α-dehydrogenase elutes with 0.5 M phosphate, and the 17β-dehydrogenase elutes with 1.0 M phosphate. The fractions containing dehydrogenase activity were pooled (keeping the 17α- and 17β-dehydrogenase fractions separate) and dialyzed against buffer A.

The two dehydrogenase preparations were stirred separately with 10 mL of blue 2-agarose (previously equilibrated with buffer A) for 6 h. Each batch of blue 2-agarose was washed 5 times with 20 mL of buffer A and 5 times with 10 mL of 1 M KCl. Most of the activity elutes in the first three KCl washes. They were pooled, keeping the epimeric dehydrogenases separate from each other, and dialyzed against buffer A.

Estriol–Sepharose[1] was prepared as described by Chin & Warren (1973). The two partially purified preparations were stirred separately with approximately 5 mL of estriol–Sepharose overnight and then washed 5 times with 10 mL of buffer A. The bound 17α-dehydrogenase and 17β-dehydrogenase were eluted from the estriol–Sepharose by stirring overnight in 5 mL of buffer A containing the respective epimer of estradiol (0.1 mM) and washing 4 times with 5 mL of the steroid solution.

Polyacrylamide Gel Electrophoresis. Electrophoresis procedures were conducted in a Hoefer electrophoresis chamber with an ISCO power supply. The SDS denaturation and gel electrophoresis were conducted as described by Laemmli (1970) with 10% acrylamide and 0.1% SDS in the running gels. The electrode buffer was Tris–glycine, pH 8.3, containing 0.1% SDS. The gels were run at 2 mA/tube until the bromophenol blue reached the bottom of the tubes. Gels containing standards of phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme with molecular weights of 92 500, 66 200, 45 000, 31 000, 21 500, and 14 400, respectively, were run simultaneously with the dehydrogenase gels.

Polyacrylamide disc gel electrophoresis was carried out as described by Davis (1964) at pH 6.9. The acrylamide concentration in the running gels was 7.5%. Gels were run at 4 mA/tube until the bromophenol blue reached the bottom of the gels and for an additional 60 min. Gels used for protein staining were fixed in 12.5% (w/v) trichloroacetic acid for 1 h and stained in Coomassie Blue as described by Bertoleni et al. (1976). Staining for estradiol dehydrogenase activity was as described by Karavolas et al. (1970).

Determination of Molecular Weights. A column of Sephacryl S-300 (1.6 × 80 cm) was equilibrated with buffer A. To calibrate the column (1) a mixture of thyroglobin, catalase, ovalbumin, and ribonuclease and (2) a mixture of blue dextran, ferretin, aldolase, and chymotrypsinogen were applied separately to the column and eluted with buffer A. The elution volumes of the proteins were determined by following the absorbance at 280 nm. The flow rate of the column was 30 mL/h. In separate experiments, estradiol 17α-dehydrogenase and estradiol 17β-dehydrogenase were applied to the column (in duplicate runs and an additional run of the mixture of the two enzymes) and eluted with buffer A. Their elution volumes were determined by quantitation of activity in each fraction.

Results and Discussion

Subcellular Localization of Estradiol 17β-Dehydrogenase. The average horse placenta weighs about 1500 g and contains about 200 IU of estradiol 17α-dehydrogenase and 100 IU of estradiol 17β-dehydrogenase. The presence of glycerol in the homogenization buffer is essential for stabilizing activity. If glycerol is omitted from the homogenization buffer, 95% of both dehydrogenase activities is lost within a few hours. Consequently, all buffers used in the purification procedure contained 20% glycerol.

Subcellular fractionation of the homogenate revealed that both estradiol 17α-dehydrogenase and estradiol 17β-dehydrogenase are in the microsomal fraction (Table I). The activity remaining in the supernatant represents microsomes that are not sedimented in one centrifugation at 105000*g*. Repeated centrifugation sediments the majority of activity from the supernatant. Microsomes can be collected from the mitochondria-free supernatant either by centrifugation at 105000*g* or by aggregation with Ca^{2+} (Schenkman & Centi, 1978). Working with large volumes makes the Ca^{2+} aggregation method advantageous and effective (Table I) as it requires only centrifugation at 25000*g*. Localization of these dehydrogenases in the microsomes is in contrast to human

[1] Abbreviations: estriol–Sepharose, estriol-16-OOC$(CH_2)_2$CONH-$(CH_2)_8$NH–Sepharose; SDS, sodium dodecyl sulfate; nortestosterone, 17β-hydroxy-4-estren-3-one; estriol, 1,3,5(10)-estratriene-3,16α,17β-triol; testosterone, 17β-hydroxy-4-androsten-3-one; epitestosterone, 17α-hydroxy-4-androsten-3-one; epiestriol, 1,3,5(10)-estratriene-3,16α,17α-triol.

Table I: Subcellular Distribution of Estradiol 17α- and 17β-Dehydrogenase[a]

subcellular fraction	estradiol dehydrogenase activity (IU[b]/30 g of wet tissue) 17α	17β
homogenate	4.68	2.92
mitochondria	0	0
microsomes	4.04	2.50
soluble	0.70	0.44
Ca^{2+}-aggregated microsomes	3.80	2.19

[a] Cellular fractionation and radioisotope assay of the enzyme activity were as described in the text. [b] One international unit (IU) is the amount of enzyme converting 1.0 μmol of substrate/min.

Table II: Purification of Estradiol 17α-Dehydrogenase from Horse Placenta[a]

sample	units	protein (mg)	sp act.[b] (IU/mg)	purification (x-fold)	recovery (%)
12000*g* supernatant	38	22400	0.0017		
cholate solubilized	30	2160	0.0139	8.1	79
hydroxylapatite	22.6	290	0.078	45.9	59
blue 2-agarose	32	200	0.160	94	84
estriol-Sepharose	33	3.3	10.0	5900	87

[a] The microsomes were precipitated from the supernatant with Ca^{2+}, and the enzyme was solubilized with 1.5% sodium cholate. [b] The dehydrogenase activity of each fraction through the blue 2-agarose step was determined by the radioisotope assay, and the spectrophotometric assay was used for the homogeneous enzyme (described in the text).

placental estradiol 17β-dehydrogenase (Langer & Engel, 1959) and the epimeric estradiol dehydrogenases in chicken, rabbit, sheep, and turkey liver (Renwick & Engel, 1969), which are all cytosolic enzymes. However, microsomal localization of estradiol 17β-dehydrogenase has been described in liver, skin, placental, and testicular tissue (Aoshima & Kochakian, 1963; Davis et al., 1972; Inano & Tamaoki, 1974; Lehmann & Breuer, 1967).

Purification of Estradiol 17α- and 17β-Dehydrogenase. A summary of the purification of estradiol 17α- and 17β-dehydrogenase from the 12000*g* supernatant is presented in Tables II and III, respectively. Both are solubilized with sodium cholate. When the Ca^{2+}-aggregated microsomes were resuspended to a protein concentration of 5 mg/mL and stirred in 0.1%, 0.5%, 1.0%, and 1.5% sodium cholate for 1 h at 4 °C, increasing amounts of both dehydrogenases were extracted with increasing cholate concentrations with a plateau at 1.5% cholate. With 0.1% cholate about 30% of both activities were extracted, 0.5% cholate solubilized 45%, and 1.0% cholate solubilized 55%, while at 1.5% cholate about 60% of the dehydrogenases were extracted. A second such extraction (at 1.5% cholate) solubilized half of the activity remaining with the microsomes, bringing the total of both dehydrogenases solubilized to about 80%. This solubilized preparation does not require further addition of cholate to maintain the enzymes in solution. If the cholate-solubilized preparation is dialyzed extensively against buffer A and centrifuged at 105000*g* for 90 min, all of both activities remain in the supernatant fraction. Consequently, the cholate was routinely dialyzed out of the solubilized material before proceeding to the next purification step and was not added again. While the dialysis was extensive and no further cholate was added, it is currently unknown whether the final preparations are absolutely free of cholate. As a result of microsomal aggregation and solubilization with cholate, each dehydrogenase is purified about 8-fold.

Table III: Purification of Estradiol 17β-Dehydrogenase from Horse Placenta[a]

sample	units	protein (mg)	sp act.[b] (IU/mg)	purification (x-fold)	recovery (%)
12000*g* supernatant	16	22400	0.0007		
cholate solubilized	13	2160	0.0006	8.6	81
hydroxylapatite	11	117	0.094	134	69
blue 2-agarose	17	112	0.152	217	106
estriol-Sepharose	17	1.6	10.6	15000	106

[a] The microsomes were precipitated from the supernatant with Ca^{2+}, and the enzyme was solubilized with 1.5% sodium cholate. [b] The dehydrogenase activity of each fraction through the blue 2-agarose step was determined by the radioisotope assay, and the spectrophotometric assay was used for the homogeneous enzyme (described in the text).

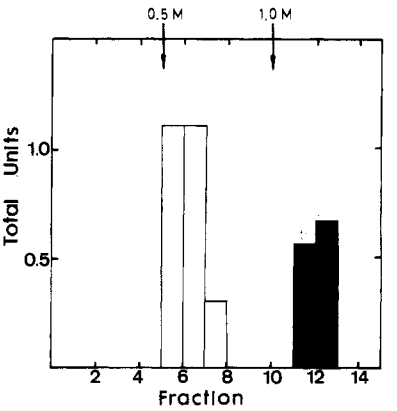

FIGURE 1: Separation of the epimeric dehydrogenases on hydroxylapatite. A cholate-solubilized, dialyzed preparation containing 2.8 IU of 17α-dehydrogenase and 1.4 IU of 17β-dehydrogenase was applied to a 1 × 6 cm column of hydroxylapatite. The column was washed 5 times with 5 mL of buffer A, 5 times with 5 mL of 0.5 M potassium phosphate buffer, and 5 times with 5 mL of 1.0 M potassium phosphate buffer. (All buffers were pH 7.0 and contained 20% glycerol and 1 mM dithioerythritol.) Eluant fractions of 5 mL were collected and aliquots of each assayed by the isotope assay (described under Experimental Procedures) for both estradiol 17α-dehydrogenase activity (open bars) and estradiol 17β-dehydrogenase activity (closed bars).

The 17α-dehydrogenase can be separated from the 17β-dehydrogenase by chromatography on hydroxylapatite, confirming that they are, indeed, separate enzymes. This was discovered by applying a cholate-solubilized, dialyzed sample containing 2.8 units of estradiol 17α-dehydrogenase and 1.4 units of estradiol 17α-dehydrogenase to a column (1.0-cm diameter) containing 5 mL of hydroxylapatite. While both dehydrogenases adhered to the gel when applied in buffer A, estradiol 17α-dehydrogenase eluted in 0.5 M potassium phosphate buffer while estradiol 17β-dehydrogenase eluted with 1.0 M potassium phosphate buffer. As shown in Figure 1, estradiol 17α-dehydrogenase activity was found only in fractions 6–8 and estradiol 17β-dehydrogenase activity was found only in fractions 12 and 13. Although the primary accomplishment is separation of the two dehydrogenases, the conditions as employed in the optimum purification scheme (described under Experimental Procedures) yield a 5.7-fold purification of estradiol 17α-dehydrogenase and 15.6-fold purification of estradiol 17β-dehydrogenase with a recovery

of each approximating 80% (Tables II and III).

The purification of each of these two enzymes to homogeneity is accomplished by two affinity-adsorption steps. Their affinity for NAD^+ is exploited with blue 2-agarose. Final purity is achieved by chromatography on the estriol–Sepharose gel. Following dialysis of the 0.5 and 1.0 M potassium phosphate fractions against buffer A, each is stirred with blue 2-agarose overnight. Although some 50% of each enzyme adheres to the blue agarose in 1 h, optimal adsorption of about 90% is obtained in 6 h. Both dehydrogenases can be eluted from the blue 2-agarose in buffer A containing either 1 mM NAD^+ or 1.0 M KCl but not with buffer A containing 0.1 mM 17α-estradiol, 17β-estradiol, or estrone. Elution with NAD^+ yields purified preparations with specific activities of over 1.0 IU/mg. However, this elution requires stirring of the blue 2-agarose in the NAD^+ for 4 h to elute 70% of the adsorbed activities. Elution with KCl does not require prolonged stirring and elutes more of the activity but yields preparations with much lower specific activities (Tables II and III). Either type of preparation (NAD^+ or KCl eluant) adheres equally well to the estriol–Sepharose, and the final preparation from either, after elution from the steroid affinity gel, is homogeneous. Hence, KCl elution from blue 2-agarose was adopted.

Use of blue 2-agarose prior to estriol–Sepharose is essential. Early attempts at purification on estriol–Sepharose without the prior step failed; neither dehydrogenase adhered to the gel. It was subsequently found that 1.0 mL of the cholate supernatant hydrolyzed 0.15 μmol of *p*-nitrophenol acetate/min. These esterases may have cleaved the affinity arm of the estriol–Sepharose, thus precluding any interaction between the gel and the dehydrogenases. After treatment on blue 2-agarose, preparations were free of detectable esterase activity.

Adsorption on blue 2-agarose with elution in 1.0 M KCl results in esterase-free preparations, a 2-fold purification of the estradiol 17α-dehydrogenase, and a 1.6-fold purification of the estradiol 17-β-dehydrogenase with apparent recoveries of 141% and 151%, respectively. The recovery may be due to the removal of an inhibitor.

Estriol–Sepharose has been successfully used in the purification of the human placental estradiol 17β-dehydrogenase (Chin & Warren, 1973). When the partially purified horse dehydrogenases were stirred separately with estriol–Sepharose overnight, essentially all of each dehydrogenase adhered to the gel. Estradiol 17α-dehydrogenase was eluted in buffer A containing 0.1 mM 17α-estradiol, and estradiol 17β-dehydrogenase was eluted with 0.1 mM 17β-estradiol. As compared to the 12000*g* supernatant, the 17α-dehydrogenase is purified 5900-fold to a specific activity of 10 IU/mg with an 87% recovery; the 17β-dehydrogenase is purified 15 000-fold to a specific activity of 10.6 IU/mg with an apparent recovery of 106%.

In the purification of human placental estradiol 17β-hydrogenase (Chin & Warren, 1973), we eluted the enzyme from the steroid-affinity gel by cleavage of the steroid–ester linkage with hydroxylamine. However, the epimeric estradiol dehydrogenases from horse placenta were unstable in hydroxylamine, even at neutral pH. Nicholas et al. (1972) reported elution of the human placental enzyme with a 0.3 mM estrone hemisuccinate and with 0.1 mM estradiol in 3.0 M urea, but not with 0.1 mM estradiol alone. Nevertheless, estradiol 17α- and 17β-dehydrogenases from horse placenta can be eluted from estriol–Sepharose in 0.1 mM 17α-estradiol and 17β-estradiol, respectively.

The purified enzymes can be stored at 4 °C in phosphate buffer, pH 7.0, containing 1 mM dithioerythritol for at least one month with little loss of activity.

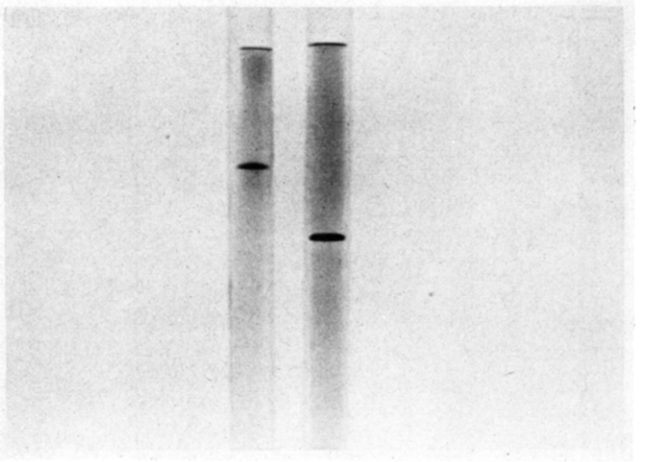

FIGURE 2: Polyacrylamide gel electrophoresis of purified horse placental estradiol 17α-dehydrogenase (left) and estradiol 17β-dehydrogenase (right). The electrophoresis and staining were conducted as described under Experimental Procedures.

Gel Electrophoresis of the Purified Dehydrogenases. The enzymes are each homogeneous as judged by gel electrophoresis (Figure 2). Each exhibits one band; estradiol 17β-dehydrogenase migrates further toward the anode than does estradiol 17α-dehydrogenase at pH 6.9. When simultaneously electrophoresed gels were stained for estradiol 17α- and 17β-dehydrogenase activities, the activity bands corresponded to the protein bands of the homogeneous preparations. Each enzyme exhibits one band on SDS electrophoresis; the positions of the two are identical, corresponding to a molecular weight of 33 000.

In that estradiol 17α- and 17β-dehydrogenases exhibit different mobilities on disc gel electrophoresis at pH 6.9, with the 17β-dehydrogenase migrating further toward the anode, suggests that the 17β-dehydrogenase is more acidic than the 17α-dehydrogenase. This is also suggested by their behavior on hydroxylapatite, where the 17α-dehydrogenase elutes at a lower potassium phosphate concentration than the 17β-dehydrogenase. Nevertheless, these apparent differences in charge may relate only to a difference in the number of cholate molecules bound. As mentioned before, it is currently unknown whether the homogeneous preparations are absolutely free of cholate.

Molecular Weight Determination on Sephacryl S-300. When the 17α- and 17β-dehydrogenases are applied to a column of Sephacryl S-300, either separately or together, estradiol 17β-dehydrogenase elutes slightly ahead of estradiol 17α-dehydrogenase. When their elution volumes are compared to elution volumes of proteins of known molecular weight, the apparent molecular weight of the 17α-dehydrogenase is 52 500, and the apparent molecular weight of the 17β-dehydrogenase is 68 500 (Figure 3).

The apparent molecular weights of the purified dehydrogenases are similar to each other and to that of human placental estradiol 17β-dehydrogenase. The subunit molecular weight of both horse placental dehydrogenases is 33 000 as indicated by SDS gel electrophoresis (average of two determinations). The subunit molecular weight of human placental estradiol 17β-dehydrogenase is 34 000 (Burnes et al., 1971). Considering the SDS gel electrophoresis data, and presuming the native enzymes may be dimers, the molecular weights should be about 66 000. This corresponds reasonably well to the molecular weight of 68 500 obtained for estradiol 17β-dehydrogenase on gel filtration. However, an actual molecular weight of 66 000 for estradiol 17α-dehydrogenase would re-

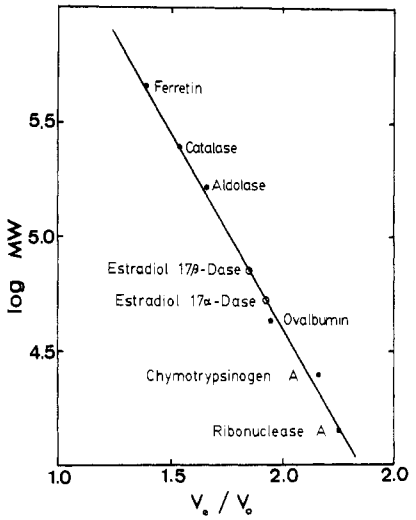

FIGURE 3: Molecular weight determination on Sephacryl S-300. Purified estradiol 17α- and 17β-dehydrogenases were applied separately to a 1.6 × 80 cm column of Sephacryl S-300 and eluted with buffer A at 30 mL/h (2-mL fractions). In separate experiments, proteins of known molecular weight were similarly applied and eluted from the column. The void volume (V_0) was determined as the elution volumes of blue dextran. Elution volumes (V_e) were determined for each protein as described under Experimental Procedures, and the V_e/V_0 was plotted against the log of the molecular weights of the standards.

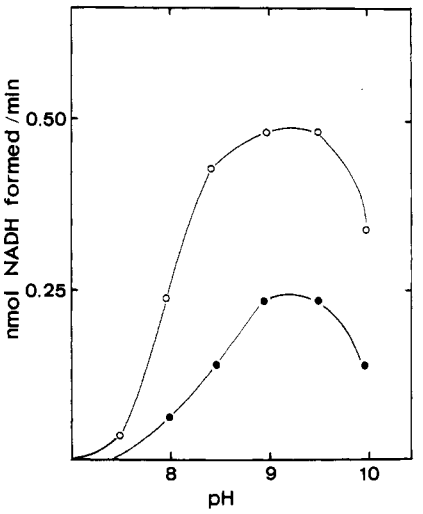

FIGURE 4: pH optima for estradiol 17α-dehydrogenase (○) and 17β-dehydrogenase (●). Assay mixtures using sodium pyrophosphate buffers with pH varied from 7.0 to 10.0 were used to measure the rate of production of NADH from NAD^+ by following the change in absorbance at 340 nm as described under Experimental Procedures.

quire that the value obtained by gel filtration be in error by 20%. Elution volumes in gel filtration can be affected by factors other than molecular weight, including shape, temperature, dissociation, and adsorption to the gel, causing variations in the molecular weight estimation by as much as 50% of that determined by other methods (Squire, 1964). Human estradiol 17β-dehydrogenase is a dimer with a molecular weight of 68 000 (Lehmann & Breuer, 1967). Thus, it is attractive to conclude tentatively that all three placental estradiol C-17 dehydrogenases are dimers having reasonably similar molecular weights.

pH Optima of the 17α- and 17β-Dehydrogenases. Assay mixtures were prepared as described under Experimental Procedures except that the sodium pyrophosphate buffer was varied from pH 7.0 to pH 10.0. The rate of production of NADH from NAD^+ was determined by linear change in absorbance at 340 nm. The pH optima for both dehydrogenases is 9.0–9.5 (Figure 4).

The pH optima for the dehydrogenases from horse placenta are comparable to those from other sources. Both the estradiol 17α-dehydrogenase and estradiol 17β-dehydrogenase exhibit pH optima at 9.0–9.5, as do those from chicken liver (Renwick & Engel, 1967). Human placental estradiol 17β-dehydrogenase has a pH optimum at 10.0 (Langer & Engel, 1958).

Kinetic Constants of the Dehydrogenases and Substrate Specificity. Double-reciprocal plots were constructed by measuring the initial rate of increase in absorbance at 340 nm with reaction mixtures containing a range of estradiol concentrations (Figure 5). The apparent K_m values of both dehydrogenases with their respective epimer of estradiol as substrate were 1.7 μM.

Estradiol 17α-dehydrogenase was assayed spectrally as described by using as potential substrates 0.3 μmol of 20α-hydroxy-4-pregnen-3-one, 20β-hydroxy-4-pregnen-3-one, epitestosterone, epiestriol, or 17β-estradiol. When 16 μg of homogeneous enzyme was added to these assay mixtures, no change in absorbance was seen. The assays were conducted such that reactivity could be seen if the rates of reaction of

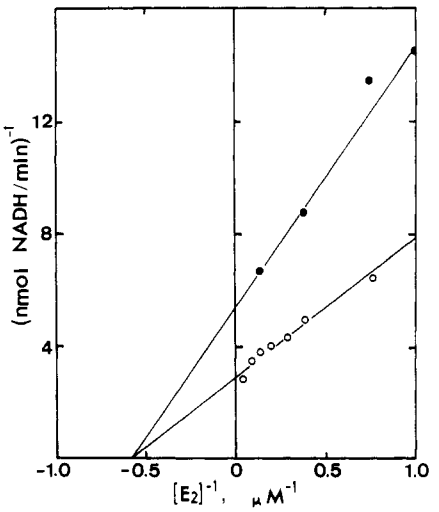

FIGURE 5: Determination of the apparent K_m value for estradiol 17α- and 17β-dehydrogenase with 17α-estradiol and 17β-estradiol, respectively. Reaction velocities were measured as described under Experimental Procedures. Assay mixtures with estradiol 17α-dehydrogenase (○) contained 4 milliunits of enzyme and those with estradiol 17β-dehydrogenase (●) contained 2 milliunits of enzyme. Each point represents the average of duplicate assays.

any of the above steroids were 1% of that observed with the 17α-estradiol as substrate. Similarly, estradiol 17β-dehydrogenase was assayed with reaction mixtures containing 0.3 μmol of testosterone, 19-nortestosterone, 20α-hydroxy-4-pregnen-3-one, 20β-hydroxy-4-pregnen-3-one, estriol, or 17α-estradiol. Again no activity was observed.

Human placental estradiol 17β-dehydrogenase shows a reactivity with testosterone and 19-nortestosterone at less than 5% of that with 17β-estradiol (Langer & Engel, 1958) while the rate with 20α-hydroxy-4-pregnen-3-one was 0.04% that with 17β-estradiol (Strickler & Tobias, 1980). Estradiol 17β-dehydrogenase from chicken liver shows reactivity with 17α-estradiol, testosterone, and epitestosterone at rates of 3%, 13%, and 9.7%, respectively, of that with 17β-estradiol (Renwick et al., 1981). The wider range of steroids accepted as substrates by these enzymes calls for further investigation with the horse placental dehydrogenases to ascertain whether any of the above steroids are substrates with catalytic rates below the sensitivity of our experiments.

The 17α- and 17β-dehydrogenases were also assayed by using $NADP^+$ instead of NAD^+ as cofactor (both cofactors were 0.2 mM). In the case of estradiol 17α-dehydrogenase, the rate of reaction with $NADP^+$ is approximately 40% of that with NAD^+. Estradiol 17β-dehydrogenase has a reaction rate with $NADP^+$ approximately 10% of that seen with NAD^+. The human enzyme utilizes $NADP^+$ at a rate of 50% that of NAD^+ (Langer & Engel, 1958). In contrast, the chicken liver dehydrogenase prefers $NADP^+$ to NAD^+ (Renwick & Engel, 1967).

Horse placental estradiol 17α-dehydrogenase and 17β-dehydrogenase appear similar to each other and to human placental estradiol 17β-dehydrogenase in terms of pH optima, cofactor preference, and molecular weights. The purification scheme defined in this presentation will make available quantities of the horse enzymes sufficient to allow further structural comparisons in this laboratory.

Registry No. Estradiol 17α-dehydrogenase, 9044-91-1; estradiol 17β-dehydrogenase, 9028-61-9; 17α-estradiol, 57-91-0; 17β-estradiol, 50-28-2.

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