Estradiol-17β Dehydrogenase from Chicken Liver*

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NADP⁺-linked estradiol-17β dehydrogenase has been purified 300- to 400-fold from cell-free extracts of chicken liver in a 20 to 30% yield by ammonium sulfate precipitation, ion exchange chromatography, and gel filtration. The enzyme is stable for at least 3 months when stored at −20°C in buffer containing glycerol (50%, v/v). Two forms, with molecular weights of 43,000 and 97,000, are present; these show one major band (Rm = 0.27) and one minor band (Rm = 0.25) on polyacrylamide disc gel electrophoresis. (Rm is defined as the ratio of the distance migrated by the protein band to that of the tracking dye.)

The species of lower molecular weight is the more active, with apparent Km values for estradiol-17β of 25 and 17.3 μM in the presence and absence, respectively, of bovine serum albumin in the assay medium. The apparent Km for NADP⁺ is 7.7 μM, and the optimum pH for dehydrogenation is 9.9. The lower molecular weight form has a λmax at 280 nm, a shoulder at 290 nm, and an A500 of 12.1 at 280 nm. The fluorescence spectrum corresponds to that of a tryptophan-containing protein with λmax at 288 nm. Isoelectric focusing in gel at pH 5 to 8 shows three major bands of pI 6.9, 6.8, and 6.0. Cross-linking with dimethyl suberimidate followed by electrophoresis reveals five bands. The enzyme is affected by thiol reagents and possesses no associated estradiol-sensitive transhydrogenase activity.

Homogenates and slices of chicken liver interconvert estradiol-17α, estradiol-17β, and estrone (1, 2). Subsequent experiments by Renwick and Engel (3) showed that high speed supernatant fractions of chicken, rabbit, sheep, and turkey liver catalyze the reversible oxidation of the estradiol epimers to estrone, and the partial purification of the estradiol-17α and -17β dehydrogenases (EC 1.1.1.148 and 1.1.1.62) was described. Both enzymes require NADP⁺ as cofactor. Their common oxidation product was identified as estrone and the stoichiometry of the reactions which they catalyze was established. George et al. (4) later found that the stereochemistry of hydrogen transfer from the estradiol epimers to NADP⁺ in the presence of these dehydrogenases involves the 4-pro-S proton of the pyridine nucleotide. This study reports the purification and some of the properties of an estradiol-17β dehydrogenase from chicken liver.

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EXPERIMENTAL PROCEDURES

Materials

Unless otherwise stated, all chemicals were of analytical grade and were obtained from British Drug Houses Ltd., Poole, Dorset, England. Pyridine nucleotides, nitroblue tetrazolium, phenazine methosulphate, steroid substrates, all Sephadex preparations, and all protein standards used as markers in gel filtration and electrophoresis were bought from Sigma Chemical Co., St. Louis, Mo.

DEAE-cellulose (DE52) from W. & R. Balston Ltd, Maidstone, Kent, England, was used in accordance with the manufacturer's instructions. Carrier ampholytes (Ampholine pH 5 to 9) were obtained from LKB-Produkter AB Sweden.

Tissue

Fresh chicken livers were bought from Fairday Poultry Processors, Ltd., Henderson, Auckland, transported in ice, and used within a few hours of slaughter. Tissue from the same source, when stored at −20°C for up to 6 months, was also satisfactory.

Purity of Steroids and Cofactors

Purity of steroids was ascertained by thin layer chromatography in the following systems: ether/benzene (2:1), ethyl acetate/benzene (2:1), and chloroform/acetone (18:15). Chromatograms were examined at 254 nm before spraying with ethanol/H₂SO₄ (specific gravity, 1.84)(1:1). Steroids were recognized as colored spots after the plates were heated at 110°C for 5 to 10 min. Compounds were recrystallized where necessary.

Pyridine nucleotides were chromatographed on polyethyleneimine-cellulose in aqueous solutions of lithium chloride (5).

Enzyme Assays

Steroid dehydrogenase assays were modifications of those described by Renwick & Engel (3) for enzymes extracted from chicken liver. The reaction mixture (3 ml) contained 300 μmol of KClO₃/K₂CO₃ buffer (pH 9.9 at 20°C), 0.4 μmol of NADP⁺, 0.17% (w/v) bovine serum albumin, and 10 to 200 μl of enzyme preparation. The reaction was initiated by the addition of 0.4 μmol of steroid in 0.1 ml absolute ethanol. The linear increase in absorbance at 340 nm at 37°C was measured in a Unicam SP1800 ultraviolet spectrophotometer over a period of 3 to 4 min against a blank cell containing all except steroid substrate. Estradiol-17β-dependent transhydrogenase (EC 1.6.1.1) was determined by the method of Jarabak et al. (6).

One unit of enzyme activity is defined as the formation of 1 μmol NADPH/min under the stated conditions of assay, or the oxidation of 1 μmol NADPH/min where estrone was used as substrate. All activities are expressed as milliunits. Specific activity is defined as milliunits enzyme activity/mg of protein determined by the method of Lowry et al. (7).

Identification of Steroid Products of Reaction

Testative identification of the products of enzyme-catalyzed steroid transformations was carried out from time to time by combining reaction mixtures, extracting steroids with organic solvents, and then comparing their mobilities against those of reference compounds in appropriate thin layer systems described above. In the case of estrogen, further characterization was achieved by analysis of acetylated derivatives on 3% OV-1/20 at 260°C in a Pye series 104 gas-liquid chromatograph fitted with dual flame ionization detectors.
**Measurement of pH**

A Radiometer (Copenhagen) pH meter, model CDM-2K, was used to determine pH against reference buffers.

**Electrophoretic Procedures**

Polyacrylamide disc gel electrophoresis was carried out in 9-cm running gels at pH 7.9 and 8.4 in systems similar to those described by Davis (8) and Hedrick & Smith (9), respectively. Electrophoresis was performed at 2 mA/tube at 4°C until the tracking dye (bromphenol blue) was approximately 0.1 cm below the stacking gel. The current was then increased to 4 mA/tube until the maker was approximately 0.5 cm from the end of the gel. Proteins were detected by staining with 1% (w/v) Amido black in 7% (v/v) acetic acid for 15 min followed by repeated washing with 7% (v/v) acetic acid at 37°C for 24 h. Results are expressed as Rf values, i.e., the ratio of distance migrated by the protein band to that of the tracking dye.

Gels stained for estradiol-17β dehydrogenase activity always contained glyceral (20%, w/v), an essential addition for the protection of the enzyme. Dehydrogenase activity was detected by incubating the gels in the dark at ambient temperature (-18–20°C) for 24 h. Results are expressed as milliunits/mg.

**Cross-linking of Subunits—Estradiol-17β dehydrogenase (about 5500 milliunits/g wet weight) was present in cell-free extracts of fresh or frozen chicken liver contain a relatively high concentration of a soluble, NADP+-linked estradiol-17β dehydrogenase (about 5500 milliunits/g wet weight of tissue).

**Ultraviolet and Fluorescence Spectra**

Ultraviolet absorption and fluorescence spectra were obtained in a Unicam SP1800 ultraviolet spectrophotometer and an Aminco-Bowman spectrophuorimeter against appropriate reference cells.

**Isolation and Purification of Enzyme**

All steps were carried out between 0 and 4°C except chromatography, which was performed at room temperature (-15–20°C). Fractions containing enzyme were concentrated by dialysis against a linear gradient of KC1, 0 to 0.2 M in Buffer E (Fig. 2). Fractions containing enzyme activity which was eluted at 4°C were concentrated and stored at -20°C until required.

**RESULTS AND DISCUSSION**

**Purification of Estradiol-17β Dehydrogenase Present in Cell-free Extracts of Chicken Liver**—It was confirmed that cell-free extracts of fresh or frozen chicken liver contain a relatively high concentration of a soluble, NADP+-linked estradiol-17β dehydrogenase (about 5500 milliunits/g wet weight of tissue). A summary of a representative purification is shown in Table II. A 300- to 400-fold overall purification of total estradiol-17β dehydrogenase activity was usually obtained in a 20 to 30% yield. Preparations of higher specific activity (about 600 milliunits/mg) could also be achieved by repeated gel filtration and concentration. The enzyme is stable for at least 3 months when stored in buffered glycerol (50%, v/v) at -20°C.

The second ammonium sulfate step resulted in an apparent increase in the specific activity of the enzyme, which was eluted at 4°C. The concentration of enzyme was determined by the absorbance at 280 nm. The enzyme is stable for at least 3 months when stored in buffered glycerol (50%, v/v) at -20°C.

**Table I**

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Constituents</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>50 mM sodium phosphate, pH 7.2</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>25 mM sodium phosphate, 50% glycerol (v/v), pH 7.2</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>5 mM sodium acetate, 1 mM EDTA, 5 mM 2-mercaptoethanol, pH 5.0</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>5 mM sodium phosphate, 1 mM EDTA, 5 mM 2-mercaptoethanol, pH 7.2</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>10 mM Tris/HCl, 1 mM EDTA, 5 mM 2-mercaptoethanol, pH 8.2</td>
<td></td>
</tr>
</tbody>
</table>

**Step 1: Preparation of Cell-free Extract**—Fresh or frozen chicken liver (105 g, wet weight) was chopped and homogenized in a Sorvall Omni-Mixer with Buffer A using a tissue/buffer ratio of 1:2 (w/v). The homogenate was centrifuged at 27,000 × g for 30 min, and then the pellet was resuspended in an equal volume of the same buffer and centrifuged as before. The supernatant fractions were combined.

**Step 2: First Ammonium Sulfate Precipitation**—Crystalline ammonium sulfate (231 g/liter) was added to the combined supernatant fractions, and the pH of the solution was maintained at 7.2 by the dropwise addition of 3 M ammonium hydroxide. Stirring was continued for 30 min after dissolution of the salt and the precipitate which formed was collected by centrifugation at 10,000 × g for 30 min. The pellet was suspended in a minimal volume of Buffer B and was stored at -20°C until required.

**Step 3: Second Ammonium Sulfate Precipitation**—The supernatant fraction obtained after the first ammonium sulfate precipitation was similarly treated using 451 g of salt/liter. This precipitate was suspended in Buffer B, stored at -20°C, and used as the source of enzyme in the steps that follow.

**Step 4: Dialysis Against Acetate Buffer**—Samples of the second ammonium sulfate precipitate were dialyzed for 20 h against 20 volumes of Buffer C, and the brown precipitate that formed during dialysis was removed by centrifugation at 10,000 × g for 20 min. The supernatant fraction was then dialyzed against Buffer D and concentrated by ammonium sulfate precipitation (500 g/liter).

**Step 5: CM-Sephadex Chromatography**—The precolumn from Step 4 was dialyzed against Buffer D and centrifuged at 10,000 × g for 20 min, and then the supernatant fraction was applied to a column (2.5 × 29 cm) of CM-Sephadex (C-25-120) equilibrated in the same buffer. Chromatography was carried out under starting conditions before application of a linear gradient of KC1, 0 to 0.2 M in Buffer D (Fig. 1). Fractions containing enzyme activity (which was eluted in the linear gradient at 7.2) were combined and concentrated.

**Step 6: DEAE-cellulose Chromatography**—The concentrated preparation from Step 5 was dialyzed against Buffer E, centrifuged at 10,000 × g for 20 min, and then applied to a column (25 × 30 cm) of DEAE-cellulose equilibrated in the same buffer. Elution was begun with 200 ml of the starting buffer before application of a linear gradient of KC1, 0 to 0.1 M in Buffer E (Fig. 2). Fractions containing enzyme activity were concentrated and stored at -20°C after the addition of an equal volume of glycerol.

**Conclusion**

The dehydrogenase was purified from chicken liver by a combination of ammonium sulfate precipitation, ultracentrifugation, and DEAE-cellulose chromatography. The purified enzyme was stable for at least 3 months when stored in buffered glycerol (50%, v/v) at -20°C.

Future work will focus on characterizing the purified dehydrogenase, including its substrate specificity and mechanism of action.
TABLE II

Purification of estradiol-17β dehydrogenase from chicken liver

A cell-free extract was prepared from 105 g of fresh chicken liver and fractionated as described in the text. Estradiol-17β dehydrogenase activity was determined by the rate of formation of NADPH at 340 nm. One unit of activity is defined as the formation of 1 nmol of NADPH/min under the conditions of assay given under "Experimental Procedures." The figures listed under "Purification" are given to the nearest whole number. The letters under "Volume" refer to separated peaks of enzyme activity.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Total protein</th>
<th>Total activity</th>
<th>Specific activity</th>
<th>Purification</th>
<th>Yield</th>
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<tbody>
<tr>
<td>Cell-free extract</td>
<td>480</td>
<td>84,000</td>
<td>577</td>
<td>6.9</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Second (NH₄)₂SO₄ precipitate</td>
<td>192</td>
<td>44,000</td>
<td>276</td>
<td>6.3</td>
<td>1</td>
<td>48</td>
</tr>
<tr>
<td>Dialysis at pH 5.0</td>
<td>575</td>
<td>15,100</td>
<td>690</td>
<td>46</td>
<td>7</td>
<td>119</td>
</tr>
<tr>
<td>Third (NH₄)₂SO₄ precipitate</td>
<td>85</td>
<td>4,300</td>
<td>640</td>
<td>149</td>
<td>22</td>
<td>111</td>
</tr>
<tr>
<td>CM-Sephadex</td>
<td>240</td>
<td>2,200</td>
<td>350</td>
<td>159</td>
<td>23</td>
<td>61</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>60 (A)</td>
<td>600</td>
<td>140</td>
<td>233</td>
<td>34</td>
<td>24</td>
</tr>
<tr>
<td>15.5 (C)</td>
<td>500</td>
<td>56</td>
<td>112</td>
<td>16</td>
<td>9.7</td>
<td></td>
</tr>
<tr>
<td>Sephadex G-100 of A + B</td>
<td>5 (D)</td>
<td>120</td>
<td>47</td>
<td>392</td>
<td>57</td>
<td>8.1</td>
</tr>
<tr>
<td>3 (E)</td>
<td>190</td>
<td>55</td>
<td>289</td>
<td>42</td>
<td>9.5</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. Chromatography of estradiol-17β dehydrogenase on CM-Sephadex. The enzyme obtained by dialysis at pH 5.0 followed by (NH₄)₂SO₄ precipitation (Step 4) was chromatographed on a column (2.5 × 29 cm) of CM-Sephadex at 18–20°C. When the bulk of the enzyme activity had been eluted under starting conditions, a gradient of KCl (0 to 0.2 M in Buffer D) was applied from Fraction 45. Fractions (10 ml) were assayed for estradiol-17β dehydrogenase activity (○), protein content (□), and conductivity (△–△). Details are given in the text.

Chromatography on DEAE-cellulose at pH 8.2 (Fig. 2) gave the first indication of two forms of estradiol-17β dehydrogenase in extracts of chicken liver. It was repeatedly shown that most of the enzyme (80% A + B) was not bound to the anion exchange resin and this fraction was purified 30- to 40-fold). The remaining enzyme (Fraction C) was eluted in association with hemoglobin in a gradient of KCl (0 to 100 mM). Repeated chromatography of Fraction C on a second column of DEAE-cellulose showed that no protein was eluted until the application of a linear gradient of KCl (0 to 50 mM in Buffer E). There was no significant purification.

Gel filtration of Fraction C on Sephadex G-100 (Fig. 3) removed associated impurities. Protein appeared in the void volume and the estradiol dehydrogenase (Peak F) was eluted in a volume identical with that of Preparation D (270 ml). Furthermore, the peaks of enzyme activity and protein content were found to be coincident when the experiment was repeated on Sephadex G-75 (Fig. 3, inset). This second estradiol-17β dehydrogenase was labeled G.

The combined fractions (Fig. 2, A + B) were concentrated and applied to a column of Sephadex G-100 (Fig. 4) which separated two estradiol-17β dehydrogenases (Preparations D and E). These were stored in Buffer B at −20°C until required. Further evidence for the second form of estradiol-17β dehydrogenase was obtained on disc gel electrophoresis at pH 8.5.

Fig. 2. Chromatography of estradiol-17β dehydrogenase on DEAE-cellulose. Active fractions obtained by chromatography on CM-Sephadex were combined and dialyzed against Buffer E. The sample was applied to a column (2.5 × 30 cm) of DEAE-cellulose equilibrated in the same buffer at 18–20°C. Elution was begun under starting conditions and a linear gradient of KCl 0 to 100 mM in the same buffer was applied as indicated by the vertical arrow. Fractions (10 ml) were assayed for estradiol-17β dehydrogenase activity (○), protein content (□), and conductivity (△–△). Details containing enzyme were pooled, labeled A, B, and C, and concentrated. Details are given in the text.

Fig. 3. GEL filtration on Sephadex G-100 and G-75. Estradiol-17β dehydrogenase (Fig. 2, Fraction C) was concentrated, dialyzed against Buffer D, and applied to a column (2.5 × 75 cm; V₀ = 180 ml) of Sephadex G-100 equilibrated and eluted in the same buffer. Fractions (10 ml) were collected at a flow rate of 32 ml/h. Estradiol-17β dehydrogenase (○) was determined at 340 nm, and protein (□) was determined at 280 nm. Inset: Active fractions (Peak F) from gel filtration on Sephadex G-100 were combined, concentrated, and applied to a column (2 × 55 cm) of Sephadex G-75 equilibrated and eluted in Buffer D.
FIG. 4. Chromatography on Sephadex G-100. Fractions A and B obtained by chromatography on DEAE-cellulose (Fig. 2) were combined, concentrated, dialyzed against Buffer D, and applied to a column (2.5 x 75 cm) of Sephadex G-100 (fine) equilibrated and eluted in the same buffer. The void volume was 190 ml. Fractions (10 ml) were collected at a flow rate of 32 ml/h. Estradiol-17β dehydrogenase (●) was determined as described in the text. Protein (○) was measured at 280 nm. Fractions 21 to 29 and 30 to 36 were pooled and labeled D and E.

TABLE III
Substrate specificity of the estradiol-17β dehydrogenase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Concentration NADP⁺</th>
<th>Specific activity NAD⁺ NAD⁺ NADPH NADH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estradiol-17β</td>
<td>0.133</td>
<td>370 77</td>
</tr>
<tr>
<td>Estradiol-17β</td>
<td>0.096</td>
<td>340</td>
</tr>
<tr>
<td>Estradiol-17α</td>
<td>0.133</td>
<td>10 0</td>
</tr>
<tr>
<td>Estradiol</td>
<td>0.133</td>
<td>48 0</td>
</tr>
<tr>
<td>Testosterone</td>
<td>0.133</td>
<td>36 0</td>
</tr>
<tr>
<td>Epitestosterone</td>
<td>0.066</td>
<td>102-110 12</td>
</tr>
</tbody>
</table>

The rates of oxidation of the steroid alcohols catalyzed by estradiol-17β dehydrogenase (Fig. 4, Preparation D) were determined according to the text. Reduction of estrone was measured in a total volume of 3 ml containing 300 μmol of Tris/HCl buffer, pH 7.4, 0.2 μmol of estrone in ethanol, 0.2 μmol of NADPH or NADH, and 3 mg of bovine serum albumin. The temperature was 37°C. All assays were carried out in duplicate, and mean values are shown.

Effect of pH—The pH optimum of the NADP⁺-linked enzymic oxidation of estradiol-17β was 9.9, and the rate of oxidation at this value was 3 to 4 times faster than that of the reverse reaction at pH 7.4; this was reflected by differences in apparent V and specific activities (Tables III and IV). The pH versus activity profile was very similar to that reported for the partially purified enzyme by Renwick & Engel (3), who determined enzyme activity at 25°C, in contrast to 37°C used in our experiments. Activity could not be measured at pH values below 7.5 because of increasing turbidity when the pH was lowered below that point, an observation reported earlier (3). The pH dependence of estradiol dehydrogenation by the enzyme from chicken liver closely resembles that described by Langer & Engel (13) for the estradiol-17β dehydrogenase of human placenta in the presence of NAD⁺. Indeed, the optima are identical at close to pH 10.

Substrate Specificity and Kinetic Constants—The relative rates of oxidation of several 17-hydroxysteroids are listed in Table III. Estradiol was the most reactive substrate, whereas its epimer, estradiol-17α, was oxidized very slowly at about 3% of the rate obtained with estradiol-17β. Estriol, which bears a third hydroxyl group in the 16α configuration, did not react. These observations recall earlier experiments on the substrate specificity of the estradiol-17β dehydrogenase of human placenta by Langer et al. (14), who found that neither estradiol-17α nor estriol was oxidized. We were unable to examine reactivity towards 16α-hydroxyestrone, a substrate which was reduced to estriol by the placental enzyme, to the surprise of Engel & Groman (15).

In our studies, the C19-steroids, testosterone and epitestosterone, were poor substrates; these epimers differ from estradiol-17β and -17α only in ring A. Throughout the purification described in this paper, the relative enzymic activities towards the epimeric estradiols and testosterones were constant. Al-
though the estradiol-17β dehydrogenase activity was NADP+-linked, there was a very low level (~1%) of NADP-linked activity that was not due to the presence of a contaminating alcohol dehydrogenase. No transhydrogenase activity in the presence of low concentrations of estradiol-17β was found, unlike that first described by Talalay & Williams-Asman (16) and Talalay et al. (17) for the estradiol-17β dehydrogenase of human placenta which from which it was subsequently separated by Karavolas & Engel (18).

The back reaction of the avian enzyme (i.e. the reduction of estrone at pH 7.4) was predominantly NADPH-linked. This was found to be a moderately greater activity than with NADH. It was noted that assays for estrone reduction were only practicable when the steroid concentration was not greater than 66 μM because of the low solubility of estrone in the assay system.

Apparent Michaelis constants and maximum velocities were determined according to Lineweaver & Burk (19) and are shown in Table IV. Whether or not albumin was present in the assay system, the apparent \( K_m \) for NADP+ was the same (7.7 μM), and little difference was found in \( V \) (418 and 455 milliunits/mg of protein, respectively). However, the apparent \( K_m \) for estradiol-17β was significantly higher in the presence of albumin (25 μM) than in its absence (17.3 μM). Apparent \( K_m \) values for \( V \) for estrone and NADPH were 1.96 μM.

Karavolas et al. (20) published kinetic constants for the estradiol-17β dehydrogenase activity of human placenta from which it was subsequently separated by Karavolas & Engel (18). The apparent \( K_m \) for NADP+ was the same (9.3 μM) as that obtained in the absence of estradiol (Fig. 5). Polyacrylamide disc gel electrophoresis of the low molecular weight (specific activity, 550 to 600 milliunits/mg of protein) and high molecular weight (specific activity, ~60 milliunits/mg of protein) preparations revealed one major protein band (\( R_m = 0.27 \)) and a minor band (\( R_m = 0.25 \)) as shown in Fig. 5 (Gels 3, 4, and 5). Under these conditions, formation of nonreactive aggregates persisted which were not distinguishable by their mobilities on polyacrylamide disc gel electrophoresis. It is possible that the net charge of the aggregate compensated for its apparently double molecular size. In consequence, the relative mobility of the protein band was similar to the active 43,500-dalton species in the electrophoresis system.

Examination of samples of Preparation D in a double sector synthetic boundary system with high speed meniscus depletion (by courtesy of Dr. G. Midwinter, Massey University),

![Fig. 6. Gel filtration on Sephadex G-150. Estradiol-17β dehydrogenase was mixed with estradiol-17β (0.2 μmol) and stored at 4°C for 48 h in Buffer D containing 0.1 M KC]. The sample (0.75 ml) was applied to a column (2.5 x 75 cm; \( V_I = 190 \) ml) of Sephadex G-150 equilibrated and eluted in the same buffer. Fractions (10 ml) were collected at a flow rate of 15 ml/h. Estradiol-17β dehydrogenase was measured at 340 nm as described in the text, and protein was determined at 280 nm. Inset: Fractions 25 to 30 from the first filtration on Sephadex G-150 were combined, concentrated, and dialyzed against Buffer D before reapplication to the same column. ---, enzyme activity (milliunits/ml); ---, \( A_{280} \).
revealed the presence of a nondialyzable, low molecular weight component (about 15,000 to 20,000), and the meniscus could not be depleted. Whether this is a contaminant or a component of a monomer = dimer = tetramer equilibrium has yet to be determined.

Electrophoresis in Polyacrylamide Gels—When disc gel electrophoresis of the estradiol-17\beta dehydrogenase was carried out at pH 8.3 to 8.4 by the method of Davis (8) and Hedrick & Smith (9), three components were found that stained coincidently for protein and enzyme activity (Fig. 5, Gels 1 and 2). This heterogeneity was quite frequently not seen when the gels were overloaded, and this condition obtained when the gels were stained for dehydrogenase activity. The nature of these multimers (or isomers) was investigated by electrophoresis of the native enzyme at different gel concentrations, and graphs of relative mobilities versus gel concentration generated parallel lines (Fig. 7). Such Ferguson (23) plots indicated that the species were molecular isomers, i.e. they were of similar size but different net charge. This finding is not unique, in that Schultz et al. (24) described multiple molecular forms of the bacterial hydroxysteroid dehydrogenase whereas the estradiol-17\beta dehydrogenase of human placenta was shown to be homogeneous by disc gel electrophoresis (25).

Isoelectric Focusing—Isoelectric focusing of the native enzyme in 6% polyacrylamide gel in the pH range 5 to 8 gave a pattern of three major bands when stained for protein (Fig. 5, Gel 9). The isoelectric points were 6.9, 6.8, and 6.0. Attempts to stain the gels for enzyme activity were unsuccessful. Using different experimental conditions, Schultz et al. (24) found at least 14 enzymically active bands in their studies of the bacterial enzyme.

Amidination—Davies and Stark (10) have shown that polymers of oligomeric proteins can be cross-linked with dimethyl suberimidate and that a series of protein bands will be resolved by SDS\(^1\) gel electrophoresis with molecular weights equivalent to integral multiples of the protomer. Cross-linking of the estradiol-17\beta dehydrogenase from chicken liver with dimethyl suberimidate for 20 h showed the presence of five bands (Fig. 8, Gels 1 and 2). The major fast-moving band had the same mobility as the native enzyme denatured with SDS and examined in the same system (Fig. 8, Gel 3). When this band was designated monomer (M, = 22,000), a plot of log molecular weight versus mobility of the bands (expressed as Rm) gave a straight line (Fig. 9), indicating putative M, values of 22,000, 32,000, 40,000, 46,000, and 59,000.

**Fig. 7. Relationship between electrophoretic mobilities of native estradiol-17\beta dehydrogenase and concentration of polyacrylamide gel.** Native estradiol-17\beta dehydrogenase (Fig. 4, Preparation D) was subjected to electrophoresis at different concentrations of polyacrylamide gel. Each point represents the mean of two determinations. Inset: Electrophoresis of Preparation D in 4.3% polyacrylamide gel showing three bands.

**Fig. 8. Cross-linking of estradiol-17\beta dehydrogenase.** SDS-polyacrylamide gels (5%, w/v) of native estradiol-17\beta dehydrogenase (Fig. 4, Preparation D). Samples 1 and 2 were cross-linked with dimethyl suberimidate at 18–20°C for 20 h as described in the text. Sample 3 was native enzyme before cross-linking.

**Fig. 9. Cross-linking of native enzyme.** Solutions of native enzyme (Fig. 4, Preparation D) were amidinated with dimethyl suberimidate as described in the text. Samples of the native and cross-linked enzyme were denatured with 0.1% (w/v) SDS and analyzed by gel electrophoresis in 5% (w/v) polyacrylamide.

\(^1\) The abbreviation used is: SDS, sodium dodecyl sulfate.
Estradiol-17\(\beta\) Dehydrogenase from Chicken Liver

**Fig. 10.** Inhibition of estradiol-17\(\beta\) dehydrogenase by p-chloromercuribenzoate (A) and reactivation by diithioerythritol (B). Native enzyme (Fig. 4, Preparation D) was dissolved in 0.1 m sodium phosphate buffer containing glycerol (25%, v/v). The concentration of enzyme protein was 3.2 mg/ml. Samples were incubated at 18-20°C with 0.4 mM (●) or 0.04 mM p-chloromercuribenzoate (▲), standardized by spectrophotometric titration with 2-mercapto- ethanol, and freshly prepared in the same buffer. Estradiol-17\(\beta\) dehydrogenase was measured in samples withdrawn at the time intervals shown, and at the end of the incubation period diithioerythritol in a final concentration of 1 mM was added as indicated by the arrows. Enzyme activity is expressed as a percentage of that of the untreated native enzyme. Control solutions without p-chloromercuribenzoate retained full enzyme activity for up to 50 h at 18-20°C.

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