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Human Placental 17β-Estradiol Dehydrogenase

IV. DIFFERENTIATION OF 17β-ESTRADIOL-ACTIVATED TRANSHYDROGENASE FROM THE TRANSHYDROGENASE FUNCTION OF 17β-ESTRADIOL DEHYDROGENASE*

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SUMMARY

Two estrogen-sensitive pyridine nucleotide transhydrogenase systems of human placenta have been examined and shown to be separate and distinct entities by both physical and kinetic properties. The 17β-estradiol-activated transhydrogenase of human placenta has been shown to be free from 17β-estradiol dehydrogenase activity by a direct assay procedure which utilizes 17β-estradiol-6,7-^{3}H-17α-^{2}H. Rechromatography of the 17β-estradiol-activated transhydrogenase did not cause generation of dehydrogenase activity. Its activation does not involve oxidation-reduction at C-17 of 17β-estradiol. The 17β-estradiol-activated transhydrogenase is stimulated by 17β-estradiol-17α-^{2}H without loss of ^{2}H and by diethylstilbestrol, but not by estrone or by 17α-methylestra-1,3,5(10)-triene-3,17β-diol. The transhydrogenase function of 17β-estradiol dehydrogenase involves a cyclic oxidation-reduction of the C-17 alcohol, as shown by formation of estrone and loss of ^{2}H from 17β-estradiol-17α-^{2}H. It is stimulated by 17β-estradiol and by estrone but neither by diethylstilbestrol nor by 17α-methylestra-1,3,5(10)-triene-3,17β-diol.

The separation from extracts of human term placenta of two enzyme systems capable of catalyzing the transfer of hydrogen from TPNH to DPN has been reported (1). The first system effects transhydrogenation in the presence of 17β-estradiol[1] and both the DPN- and TPN-linked 17β-estradiol dehydrogenase activities; it has been designated "substrate (17β-estradiol)-mediated transhydrogenase" (2), and will be referred to in this paper as the transhydrogenase function of estradiol dehydrogenase. The second system catalyzes transhydrogenation in the presence of estradiol and is unassociated with estradiol dehydrogenase activity. It has been designated the "estradiol-dependent transhydrogenase" (3). In the light of the new evidence to be presented in this paper we prefer the name "estradiol-activated transhydrogenase."

These results supported the inferences drawn by Hagerman and Villee (4) from the kinetic analysis of a mixture of the dehydrogenase and the transhydrogenating systems. They concluded that there are two separate transhydrogenating systems in soluble extracts of human term placenta. The absence of detectable levels of estradiol-activated transhydrogenase in heated placental extracts was in agreement with the observations of Jarabak *et al.* (5), who found that the ratio of transhydrogenase to dehydrogenase activities was unaltered over a 2500-fold purification of the estradiol dehydrogenase activity in heat-treated placental extracts.

We now report additional data obtained with an independent and direct assay procedure that confirm the absence of detectable DPN-linked estradiol dehydrogenase activity in the partially purified preparations of estradiol-activated transhydrogenase. We also report experiments that provide direct evidence for cyclic oxidation and reduction of estradiol as the mechanism for the transhydrogenase function of estradiol dehydrogenase and exclude this mechanism for the estradiol-activated transhydrogenase. Studies of the structural specificity for stimulation of the two transhydrogenase systems are in accord with these findings.

EXPERIMENTAL PROCEDURE

Materials

All melting points were taken on a Reichert-Köfler hot stage apparatus and are reported as read.

TPN and DPN were obtained from P-L Biochemicals. Tris, trisodium DL-isocitrate, disodium glucose 6-phosphate, yeast glucose 6-phosphate dehydrogenase (type V), and crystalline yeast alcohol dehydrogenase were obtained from Sigma. Spectral quality glycerol, obtained from the Matheson Company,

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[1] The non-standard trivial names used are: estradiol, 17β-estradiol; estradiol-17α-^{2}H, 17β-estradiol-17α-^{2}H; 17α-methylestradiol, 17α-methylestra-1,3,5(10)-triene-3,17β-diol.

East Rutherford, New Jersey, was used throughout. All water used was glass redistilled. All organic solvents were redistilled analytical reagents. All other chemicals, unless otherwise noted, were analytical reagent grade, Silica Gel G (Brinkmann Instruments, Inc., Westbury, Long Island, N. Y.) was used for thin layer chromatography. The spots were made visible by spraying with concentrated sulfuric acid-ethanol, 1:1, and heating for several minutes at 120°. Pig heart isocitrate dehydrogenase (TPN specific) was obtained from Calbiochem. No DPN-linked isocitrate dehydrogenase activity could be detected with an assay that would reveal as little as 0.1% contamination.

Recrystallized estrone and estradiol were found to be homogeneous by thin layer chromatography (ether-benzene, 2:1) and by gas-liquid chromatography at 210° (1% QF-1). Combined gas-liquid chromatography-mass spectrometry was carried out on an LKB 9000 instrument, with the solid injection technique of Menini and Norymberski (6). QF-1, OV-1, and OV-17 liquid phases on Gaschrom Q (Applied Science Laboratories, State College, Pennsylvania) were used in the gas chromatographic separations.

Commercial diethylstilbestrol (Nutritional Biochemicals, lot No. 3132) appeared as two separate spots of approximately equal intensity on thin layer chromatography in carbon tetrachloride-methanol, 9:1, and tetrahydrofuran-benzene-ether, 1:10:20. Five successive crystallizations from different solvents caused no significant change in the melting point of 169–171°; thin layer chromatography again showed two spots in approximately the same proportions. Freshly prepared solutions of the recrystallized diethylstilbestrol in methanol showed predominantly one spot of R_F 0.49 (benzene-methanol, 7:1), with a smaller spot of R_F 0.42. When solutions of diethylstilbestrol were allowed to stand at room temperature and portions were chromatographed, the spot of lower R_F increased in intensity, and, after 4 hours, the two spots were of equal intensity. In some cases trailing was visible between the two spots. That there was equilibration between the two substances while on the plate was shown by chromatographing freshly dissolved, diethylstilbestrol (crystallized five times) from near one corner of a plate (20 × 20 cm) successively in two directions at right angles. The same solvent system (benzene-ether, 3:2) was used in both directions. After spraying, a clear rectangular pattern was found, establishing that the two substances are interconvertible. The second spot may be the *cis* isomer of diethylstilbestrol (7). Samples of diethylstilbestrol from four different suppliers showed the same two spots. Combined gas-liquid chromatography-mass spectrometry (1% OV-17, 190°) of the material crystallized five times showed only one major peak; total impurities amounted to less than 1.5% of the main component. Mass spectra, recorded at the beginning, middle, and end of the chromatographic peak, were indistinguishable. The molecular ion, *m/e* 268 and fragmentation peaks, *m/e* 253 (—CH_3), 239 (—C_2H_5), 224 (—(CH_3 + C_2H_5)), and 210 (—$2C_2H_5$) were prominent; fragments found at *m/e* 159, 145, and 107 were also characteristic.

17α-Methylestradiol—Estrone (10 g, 37.04 mmoles) in ether (650 ml) and tetrahydrofuran (500 ml) was refluxed overnight with a 6-fold molar excess of methyl magnesium chloride (76 ml of a 3 M solution in ether) with slow evaporation of the ether. Dilution with water, extraction into ethyl acetate, washing to neutrality, and evaporation of the ethyl acetate left an oily residue, which was chromatographed on alumina (activity III). Elution with chloroform gave a mixture of estrone and 17α-methylestradiol (7 g); elution with chloroform-ether (19:1) gave 17α-methylestradiol. After crystallization from acetone it melted at 195–196° (1.78 g, 17% yield), in agreement with Nicholas (8). Gas-liquid chromatography on 1% OV-1 showed the presence of approximately 3% impurity (M^+ 268) which may be caused by partial dehydration of the tertiary alcohol in the flash heater. This impurity was not detected by thin layer chromatography. Mass spectrometry indicated that the main product had the molecular ion at *m/e* 286 as expected, and showed the fragmentation pattern *m/e* 226, 185, and 172, characteristic of the unchanged ring A:B region of estrone. The nuclear magnetic resonance spectrum (deuteriochloroform-pyridine) is consistent with the absence of the 17α-hydrogen and with the introduction of a methyl group (1.21 ppm from tetramethylsilane) on a carbon bearing an oxygen atom.

Estradiol-17α-²H—Estrone (2.1 g, 7.7 mmoles) was dissolved in tetrahydrofuran (100 ml) and a 4-fold molar excess of sodium borodeuteride (99% ^2H; 330 mg) was added. After 5 hours at room temperature, ethanol (35 ml) was added to dissolve the remaining solid borodeuteride, and the solution was left overnight. Extraction into ethyl acetate, washing, and evaporation gave a white crystalline solid. Crystallization from methanol gave estradiol-17α-²H (1.5 g, 5.5 mmoles, 71% yield) m.p. 175.5–177°, undepressed on mixing with authentic estradiol, m.p. 176–177°. The nuclear magnetic resonance spectrum differed from that of estradiol only in the absence of the 17-H triplet at 3.80, 3.93, and 4.06 ppm downfield from tetramethylsilane (pyridine solution). Combined gas-liquid chromatography-mass spectrometry on OV-17 (1.4%, 208°) showed one major chromatographic peak; the relative heights of the mass spectral peaks at *m/e* 272 and 273 indicated that the steroid contained 95.8% of monodeuterated estradiol.

A sample of the estradiol-17α-²H adsorbed on a stainless steel gauze pellet was left at 37° overnight in an atmosphere of acetic anhydride and pyridine, when it was completely converted to the acetate (9). It was examined by gas-liquid chromatography-mass spectrometry (1.4% OV-17; 228°); 0.6% of estrone acetate could be detected in the deuterated estradiol diacetate.

Oxidation of a sample with chromium trioxide (1.1 eq) under conditions known not to cause loss of enolizable hydrogen at C-16 (10) gave estrone, identified by gas-liquid chromatography-mass spectrometry, that contained in two assays 1.0% and 3.8% monodeuterioestrone in excess of natural abundance.

Estradiol-6,7-³H was obtained from New England Nuclear. Thin layer chromatography (ether-benzene, 2:1) revealed the presence of a radioactive contaminant. The steroid was purified by chromatography in this same system. The area corresponding in mobility to that of reference estradiol was scraped off and the scraping was eluted with several portions of absolute ethanol and evaporated under vacuum. The residues were dissolved in a measured amount of absolute ethanol. Aliquots were taken for counting and rechromatography on the above thin layer system, and these revealed one radioactive spot corresponding in mobility to that of reference estradiol. This material was used for all experiments and had a specific activity of 4.25×10^7 dpm per μg. Estrone was not detectable.

Radioactivity was determined by counting randomized samples (11) in a Nuclear-Chicago liquid scintillation spectrometer (model 6814). The efficiency for tritium was usually about 32%. Sufficient counts were accumulated to insure an accuracy of about 2%.

Estradiol-6,7-^{3}H-17α-^{2}H—The prepared estradiol-17α-^{2}H (450 μg) was mixed with 4.25 × 10^6 dpm (0.1 μg) of the purified estradiol-6,7-^{3}H to give a specific activity of 9.44 × 10^3 dpm per μg and a deuterium content of 95.8%. This mixture was used for all of the double isotope experiments.

Methods

Assays for the estradiol dehydrogenase, estradiol-activated transhydrogenase, and protein were performed as described previously (1). Conductivity was measured with a Radiometer model CDM 2 d conductivity meter.

In accordance with the recommendations of the Commission on Enzymes of the International Union of Biochemistry, a unit is defined as that amount which catalyzes the conversion of 1 μmole of substrate per min per 3 ml under the specified conditions of the assay. Previously, for assays employing TPN or DPN, we defined a unit as that amount of enzyme activity which caused an increase in absorbance of 0.001 per min at 340 nm. These values can be converted to Enzyme Commission milliunits by multiplying by 0.48.

Enzyme assays were usually carried out on a Gilford model 2000 recording spectrophotometer; protein assays and other spectrophotometric measurements were made on a Zeiss model PMQ II spectrophotometer. Unless otherwise stated, all assays were done at 25°.

Solutions of enzyme were concentrated by ultrafiltration on a Diaflo concentrating device (Amicon Corporation, Lexington, Massachusetts) with a UM-1 membrane.

Estrone and estradiol were measured by the method of Slaunwhite *et al.* (12) with a Farrand model A photofluorometer. When samples from countercurrent distributions were analyzed, the measurements were made in random order (11).

The assay for estradiol-activated transhydrogenase with isocitrate dehydrogenase as the TPNH-generating system was the same as the usual transhydrogenase assay, substituting equivalent amounts of trisodium DL-isocitrate and pig heart isocitrate dehydrogenase for the disodium glucose 6-phosphate and glucose 6-phosphate dehydrogenase.

Countercurrent distributions were carried out on a 100-tube, all glass apparatus (H. O. Post Scientific Company, New York).

Hydroxylapatite Chromatography—In our experience, commercial hydroxylapatite prepared by the method of Tiselius, Hjerten, and Levin (13) did not effect the separation of the two transhydrogenating systems under study, and the flow rates were extremely low. Hydroxylapatite prepared by the method of Anacker and Stoy (14) does separate the two enzyme systems, although occasional batches so prepared were ineffective and gave unacceptably low flow rates (<10 ml per hour). If the procedure below is followed rigorously, effective batches of hydroxylapatite are obtained consistently. Mixture of the hydroxylapatite with cellulose gives higher flow rates, and steeper gradients give faster elution, but poorer separations are achieved.

The procedure of Anacker and Stoy was modified slightly. The hydrolysis is carried out at 37° with enough changes of 0.05 N NaOH so that the pH of the supernatant fluid reaches 10 or 11. The precipitated hydroxylapatite is then washed with water, rejecting 15 to 30% of the material as fines, the pH was adjusted to 7.2 with 5 mM KH_2PO_4, and again washed with 4 liters of water, and stored in water at room temperature.

Columns packed by sedimentation give good flow rates. After packing, the column is equilibrated with at least 5 void volumes of 5 mM potassium phosphate, pH 7.2 (20% glycerol), and until the pH of the eluate remains at 7.2. Equilibration at pH 7.2 must be carried out for at least 24 hours. Several of the many problems of hydroxylapatite chromatography have been discussed by Hjerten (15) and Bernardi (16).

Low flow rates (5 to 10 ml per hour) at room temperature lead to destruction of the heat-labile, estradiol-activated transhydrogenase. Chromatography under slightly acid conditions (pH 6.8) leads to very poor recoveries both of the estradiol-activated transhydrogenase and estradiol dehydrogenase. Flow rates at 5–10° are 5 to 10 ml per hour. An LKB peristaltic pump has been used on several occasions to increase the flow rate to 30 to 35 ml per hour.

Preparation and Purification of Enzymes—Human term placentas were obtained immediately after delivery. They were kept in cracked ice up to the first stage of purification which usually occurred within an hour. Unless otherwise noted subsequent operations were carried out at 4°.

Homogenization—Placentas were trimmed free of connective tissue, fetal membranes, and grossly necrotic tissue. The remaining tissue was blotted, cut into small pieces, and homogenized in a Waring Blendor with 2 volumes of 10 mM phosphate buffer, pH 7.2, in 20% glycerol at full speed for 30 sec. Unless otherwise noted, all buffers used were potassium phosphate in 20% glycerol at pH 7.2. The homogenate was centrifuged for 30 min at 9000 × *g*. After removal of the top fatty layer, the supernatant fluid was poured off and the precipitate discarded.

Ammonium Sulfate Fractionation—Solid ammonium sulfate (17.6 g/100 ml of supernatant) was added slowly in portions to the supernatant fluid (approximately 30% of saturation). The pH was maintained at 7.2 with 3 M ammonia. After stirring for 30 min, the suspension was centrifuged for 20 min at 8000 × *g*. The resulting precipitate was dissolved in a minimal amount (20 to 40 ml) of 10 mM phosphate buffer in 50% glycerol (0 to 30% ammonium sulfate precipitate). The supernatant fluid from the previous ammonium sulfate precipitation step was made approximately 50% saturated with ammonium sulfate (12.7 g/100 ml) with the same procedure and centrifugation as above to obtain a 30 to 50% ammonium sulfate precipitate. This precipitate was dissolved in a minimal amount (20 to 40 ml) of 10 mM phosphate buffer in 50% glycerol. This preparation usually contained the bulk of the three enzymatic activities and was stored at −14°. At this temperature the preparation can be stored for several months without significant loss of dehydrogenase activity, and this preparation is the material used for hydroxylapatite chromatography.

Routinely, however, the supernatant fluid from the previous ammonium sulfate precipitation was made approximately 60% saturated with ammonium sulfate (6.6 g per ml of supernatant), with the same procedures outlined above, to yield a 50 to 60% ammonium sulfate precipitate. The results obtained from 10 placentas are shown in Table I.

Hydroxylapatite Chromatography—Portions of the redissolved 30 to 50% ammonium sulfate precipitate were diluted with 1.5 volumes of 10 mM phosphate buffer containing no glycerol in order to reduce the glycerol concentration to 20%. The resultant solution was applied to an equilibrated hydroxylapatite column. After the first few milliliters had entered the column, the flow rate began to slow down, giving an uneven pattern. The top few centimeters were then stirred up evenly to improve the flow rate. After the sample had entered the column bed, a

TABLE I

Purification of human placental extract

The averages of the results from 10 placentas are presented through the ammonium sulfate precipitation stage. Subsequent steps were carried out in portions; hence, the results given are only for percentage of recovery of the activities applied to the hydroxylapatite column (95 to 100%), and the specific activities of the eluted activities. These values are averages obtained from several columns run under the conditions described in the text.

	DPN dehydrogenase			TPN dehydrogenase			Transhydrogenating activity		
	Total activity	Recovery	Specific activity	Total activity	Recovery	Specific activity	Total activity	Recovery	Specific activity
	milliunits	%	*milliunits/mg*	*milliunits*	%	*milliunits/mg*	*milliunits*	%	*milliunits/mg*
Supernatant after homogenization	(42,000)[a]	(100)	(3.9)	(24,300)	(100)	(2.3)	(2,350)	(100)	(0.3)
0 to 30% $(NH_4)_2SO_4$ precipitate	2,600	6.2	4.5	1,660	6.8	2.5	225	9.6	0.5
30 to 50% $(NH_4)_2SO_4$ precipitate	22,500	53.6	14.5	12,100	49.8	8.3	1500	63.8	0.95
50 to 60% $(NH_4)_2SO_4$ precipitate	1,520	3.6	2.6	980	4.0	1.7	340	14.5	0.55
Hydroxylapatite column Eluate I (estradiol dehydrogenase and its transhydrogenase function)	[b]	(53.6)	240	[b]	(49.8)	150	[b]	[c]	43
Hydroxylapatite column Eluate II (estradiol-activated transhydrogenase)	0		0	0		0	[b]	[c]	6.9

[a] Values in parentheses are approximate and are based on assays of crude fractions.

[b] These experiments were carried out on portions of the 30 to 50% ammonium sulfate precipitate and cannot be related to the original extract in terms of total activity; recovery after hydroxylapatite chromatography is usually 95 to 100% of the applied activity.

[c] Previous values for transhydrogenating activity were obtained for both types. However, the total of both recovered activities is usually quantitative.

few milliliters of 5 mM phosphate buffer were added and elution was begun with a linear gradient beginning at 10 mM and ending at 80 mM phosphate buffer.

With columns measuring 2.2 × 50 cm (1) and a linear gradient of 5 mM per 100 ml, overlapping peaks of DPN-linked estradiol dehydrogenase and the associated transhydrogenase activity were eluted at phosphate concentrations of 20 to 30 mM. No further activity appeared until the phosphate concentration reached 45 mM. Transhydrogenase activity (estradiol-activated transhydrogenase), unassociated with estradiol dehydrogenase activity, then appeared and was completely eluted at about 55 mM phosphate. The fractions eluted between 30 and 45 mM phosphate were combined, concentrated by ultrafiltration with the Amicon membranes or by dialysis against 30% Ficoll, and reassayed for estradiol dehydrogenase and transhydrogenase activities. The highly sensitive 3-acetylpyridine adenine dinucleotide assay for estradiol dehydrogenase (5) was also used. No activity was found. The transhydrogenase activity eluted between 45 and 55 mM was similarly concentrated; no dehydrogenase activity was found by the three assays used, whereas the transhydrogenase concentration was increased. A similar elution pattern was also seen with larger columns (3.3 × 83 cm) as shown in Fig. 1.

Unless otherwise noted, the enzymes used in the following experiments were obtained after such a purification procedure. The first pool of enzymatic activity (20 to 30 mM phosphate) was the source of estradiol dehydrogenase and its transhydrogenase function, whereas the second pool of enzymatic activity (45 to 55 mM phosphate) was the source of estradiol-activated transhydrogenase.

RESULTS

Properties of Estradiol-activated Transhydrogenase

The second pool of enzymatic activity or the estradiol-activated transhydrogenase was checked for proportionality of velocity to enzyme concentration; the results of a representative experiment are shown in Fig. 2. The formation of DPNH in these transhydrogenase assays was confirmed by the addition of acetaldehyde and yeast alcohol dehydrogenase (17). An immediate decrease in absorption at 340 nm was observed, indicating oxidation of the DPNH formed in the transhydrogenase reaction.

Controls for the transhydrogenase assays which contained all of the assay components except steroid, were always run at the same time. Additional controls, run on pools of estradiol-activated transhydrogenase, contained all assay components except the TPNH-generating system, or the DPN, or both. No significant change in absorbance was observed unless the system was complete. Before hydroxylapatite chromatography, the correction for the blank (control without steroid) usually amounted to a change in absorbance of 0.0001 to 0.0002 per min; after hydroxylapatite chromatography, no correction was necessary.

In order to explore any possible effect of estradiol on the yeast glucose 6-phosphate dehydrogenase used as the TPNH-generating system in the transhydrogenase assay, isocitrate dehydrogenase was substituted. The values for estradiol-activated transhydrogenase obtained with this assay were in agreement with those obtained in the standard procedure. The manner in which the TPNH is generated appears to be immaterial for the transhydrogenase action (17). The rate of TPNH generation

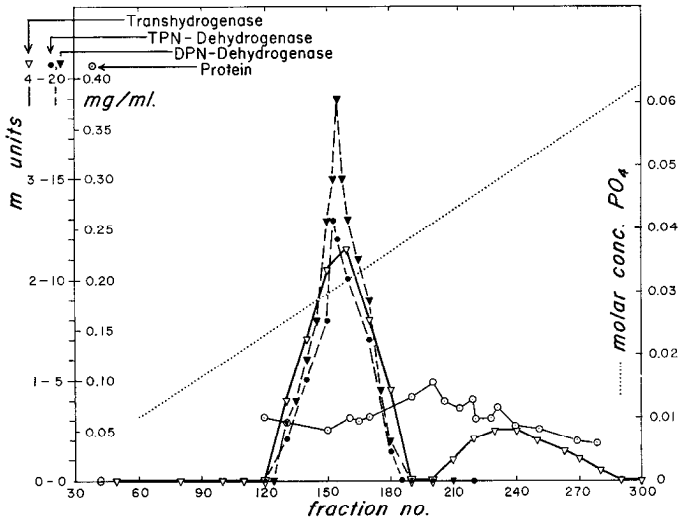

FIG. 1. Hydroxylapatite chromatography of a 30 to 40% ammonium sulfate precipitate. A preparation (40 ml), containing 118 milliunits per ml of DPN-linked estradiol dehydrogenase, 73 milliunits per ml of TPN-linked estradiol dehydrogenase, 23 milliunits per ml of transhydrogenating activity, and 15.8 mg per ml of protein, was applied to the column (3.3 × 83 cm). This preparation was obtained from a stored solution of the 30 to 40% ammonium sulfate precipitate in 10 mM phosphate buffer in 50% glycerol by dilution to 20% glycerol prior to chromatography with 1.5 volumes of 10 mM phosphate buffer (no glycerol). Fractions of 160 drops (approximately 10 ml) were collected. A linear gradient gave a change of 5 mM per 200 ml. The void volume was 530 ml. Chromatography was done at room temperature. ▽, transhydrogenase activity in milliunits per ml; ▼, DPN- and ●, TPN-linked dehydrogenase activities in milliunits per ml. ⊙, protein, as milligrams per ml, determined by measurement of the absorbance at 280 and 260 nm. -----, phosphate, molar in effluent.

should be maintained at more than twice that of transhydrogenation in order to ensure that transhydrogenation is rate-limiting.

Stability of Estradiol-activated Transhydrogenase

Storage of the estradiol-activated transhydrogenase at pH 7.2 (potassium phosphate buffer in either 20 or 50% glycerol) at 5° results in the loss of 50% of the activity after 3 weeks, and 90 to 100% after 2 months, whereas at this pH, the estradiol dehydrogenase may be stored for as long as 2 months without significant loss of activity. Raising the pH to 7.8 in either 20 or 50% glycerol increases the stability of the estradiol-activated transhydrogenase. After 1 month at pH 7.8, 15 to 40%, and after 2 months, 40 to 60% of the activity is lost. No attempt was made to study reactivation.

Rechromatography of Estradiol-activated Transhydrogenase

Rechromatography of estradiol-activated transhydrogenase on hydroxylapatite under the conditions previously described (1) results in a 2.5-fold purification. The enzyme was eluted in the same range of phosphate concentrations (40 to 50 mM), and over 95% of the enzyme activity applied to the column was recovered. Assays for estradiol dehydrogenase with acetylpyridine adenine dinucleotide (5) showed no dehydrogenase in any of the fractions.

Effect of Temperature on Activity of Estradiol-activated Transhydrogenase

The rate of the estradiol-activated transhydrogenase reaction increases with increasing temperature, reaching a maximum at

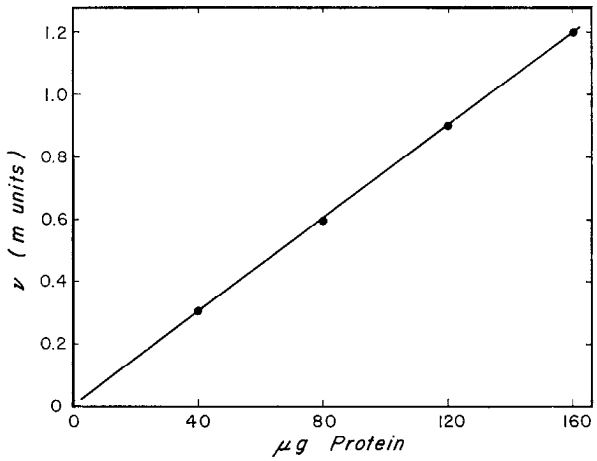

FIG. 2. Proportionality of 17β-estradiol-activated transhydrogenase concentration and velocity. The preparation had a specific activity of 7.6 milliunits per mg of protein.

45°. At 30°, 37°, and 45°, respectively, it is 1.5, 2.5, and 2.7 times faster than the rate found at 25°. Heating at 55° (5) inactivates the enzyme (1).

Assay of Purified Estradiol-activated Transhydrogenase for DPN-linked Estradiol Dehydrogenase with Aid of Estradiol-6,7-^{3}H-17α-^{2}H

Although estradiol dehydrogenase had not been detected by the spectrophotometric assay in the separated estradiol-activated transhydrogenase preparations, it seemed advisable to use a direct assay with ^{3}H- and ^{2}H-estradiol to determine whether estrone is formed and if ^{2}H is lost during the reaction. The estradiol-activated transhydrogenase was therefore incubated at 25° with doubly labeled estradiol and DPN under the conditions used for the assay of DPN-linked estradiol dehydrogenase.

Estradiol-6,7-^{3}H-17α-^{2}H (0.9 μmole, 2.31 × 10^6 dpm, 95.8% of ^{2}H) in 0.3 ml of propylene glycol was incubated with 3 ml of estradiol-activated transhydrogenase (0.45 milliunit, 1.68 mg of protein, and no detectable estradiol dehydrogenase activity by spectrophotometric assay), 0.9 ml of 1 M sodium carbonate-bicarbonate buffer, pH 9.2, 0.3 ml of a 25% solution of human serum albumin, and 4.2 ml of water. The reaction was initiated by the addition of 1.8 μmoles of DPN in 0.3 ml of water. Over a period of 6 min there was no net increase in the absorbance at 340 nm compared with the blank. The reaction was stopped after 6 min by the addition of 15 ml of ice-cold methylene chloride. The contents of the incubation flask were extracted with two additional 15-ml portions of methylene chloride and the pooled extracts were evaporated under vacuum. The residue was transferred completely to a Craig countercurrent distribution apparatus, and a 49-transfer countercurrent distribution was done in carbon tetrachloride-methanol-water (2:1:1, v/v) (18). The statistical analysis of the distribution was done by the method of Purdy, Goldman, and Richardson (11) and is summarized in Table II. The specific activity of the recovered estradiol with respect to ^{3}H was 9600 dpm per μg compared with an initial specific activity of 9440 dpm per μg. The contents of the tubes in the region of the countercurrent distribution where estrone would be present (tubes 10 to 14) were pooled and examined by gas-liquid chromatography-mass spectrometry. The results showed the presence of 0.5% estrone, compared with 0.6% found in the incubated steroid. There was no significant change

TABLE II

Analyses of countercurrent distributions of estradiol after incubation with estradiol-activated transhydrogenase

See text for experimental conditions.

	Dehydrogenase assay	Transhydrogenase assay
$K/(K+1)$ for ^3H[a]	0.698	0.700
$K/(K+1)$ for estradiol	0.700	0.705
Standard error of difference[b]	0.0028	0.0027
Degrees of freedom	2	4
Specific activity (dpm/μg)[c]	9600	9100
Standard error of specific activity (%)	3.20	2.48
Recovery of ^3H[d] (%)	97.5	90
Deuterium content[e] (%)	96.1	95.2

[a] K is partition coefficient (ratio of concentration of solute in upper phase to concentration of solute in lower phase).

[b] Dehydrogenase assay, $t = 0.55$, $p > 0.5$; transhydrogenase assay, $t = 2.03$, $p = 0.1$.

[c] Specific activity of incubated estradiol-6,7-^{3}H-17α-^{2}H = 9440 dpm per μg.

[d] These figures are the percentages of ^3H in the countercurrent distributions that were associated with estradiol. The corresponding figure for a boiled control experiment was 90%.

[e] Deuterium content of incubated estradiol-6,7-^{3}H-17α-^{2}H = 95.8%.

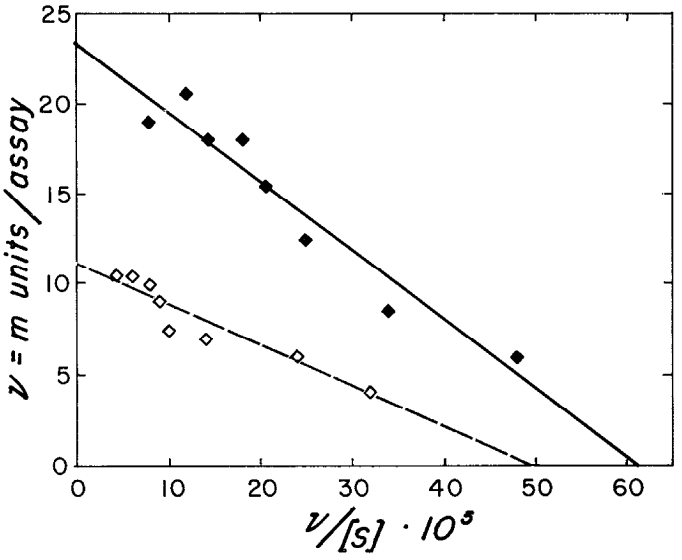

FIG. 3. Hofstee (19) plot of dehydrogenation of estradiol-17α-^{2}H (◇) and estradiol (◆) by DPN-linked estradiol dehydrogenase. Each cuvette contained 25.5 milliunits of DPN-linked 17β-estradiol dehydrogenase activity (specific activity = 350 milliunits per mg of protein). Assays were run at 25°. Human serum albumin was omitted from these assays. The steroids were added in 0.1 ml of ethanol. The regression lines were obtained by the method of least squares.

in the deuterium content of the recovered estradiol (found, 95.2% ^{2}H) from that of the incubated steroid (95.8% ^{2}H).

Comparison of Rates of Dehydrogenation and Transhydrogenation with Estradiol-17α-^{2}H

Estradiol Dehydrogenase—The rates of dehydrogenation of estradiol and of estradiol-17α-^{2}H by estradiol dehydrogenase

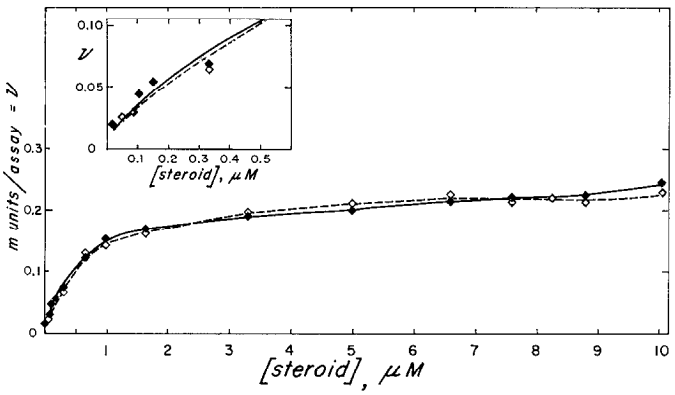

FIG. 4. Comparison of the stimulation of estradiol-activated transhydrogenase by estradiol-17α-^{2}H (◇) and by estradiol (◆). Each cuvette contained 0.2 milliunit of transhydrogenase (specific activity = 0.4 milliunit per mg of protein). Assays were run at 25°. The steroids were added in 0.02 ml of ethanol.

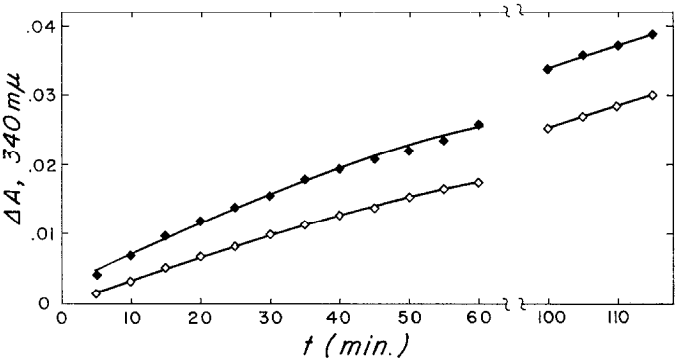

FIG. 5. Kinetic isotope effect in transhydrogenase function of estradiol dehydrogenase. Each cuvette contained 0.23 milliunit of transhydrogenase activity (specific activity = 110 milliunits per mg of protein). Assays were run at 25°. The steroids were added in 0.01 ml of ethanol. Estradiol (◆); estradiol-17α-^{2}H (◇).

(DPN linked) are shown in Fig. 3. The rate of dehydrogenation with estradiol-17α-^{2}H is 48% of that of the nondeuterated steroid. The maximal velocity (V) for the DPN-linked dehydrogenation of the unlabeled estradiol derived graphically from Fig. 3 is 23.4 milliunits, for 17α-deuterioestradiol, the corresponding value is 11.2 milliunits. The $V_H/V_{^2H}$ for the reaction is therefore 2.1, in good agreement with the value reported by Adams *et al.* (20).

Estradiol-activated Transhydrogenase—The stimulation of estradiol-activated transhydrogenase by estradiol and by estradiol-17α-^{2}H is shown in Fig. 4. For the two steroids, the rate of transhydrogenation is essentially the same.

Transhydrogenase Function of Estradiol Dehydrogenase—The rates of transhydrogenation of the mixture of the DPN- and TPN-linked estradiol dehydrogenase activities with estradiol and estradiol-17α-^{2}H, estrone, 17α-methylestradiol, and diethylstilbestrol were determined. The rate of transhydrogenation with 2.1 milliunits of enzyme in the assay was the same with estrone, deuterated, and nondeuterated estradiol. However, if only 0.23 milliunit of enzyme are used in the assay (Fig. 5), the rate in the presence of estradiol-17α-^{2}H is much slower initially, and increases until, eventually (after 1 hour), it approaches that of the nondeuterated estradiol. Diethylstilbestrol and 17α-methylestradiol do not stimulate transhydrogenation.

Is Estradiol Oxidized When It Stimulates Estradiol-activated Transhydrogenase?

The estradiol-activated transhydrogenase was incubated with estradiol-6,7-^{3}H-17α-^{2}H at 25° in order to ascertain whether estrone is formed and whether ^{2}H is lost during the transhydrogenation reaction.

The incubation mixture contained the following components: 7.5 ml of 1 M Tris-chloride, pH 7.4, 250 μmoles of glucose 6-phosphate in 5.0 ml of water, 15 units of yeast glucose 6-phosphate dehydrogenase in 7.5 ml of 50 mM phosphate buffer, pH 7.2, 33.75 μmoles of DPN in 5.0 ml, 3.8 milliunits of estradiol-activated transhydrogenase (25 ml, 0.3 milliunit per mg of protein), and 22.5 ml of water. Two 2.9-ml portions were withdrawn and the change in absorbance at 340 nm was followed for 5 min. After the addition of 10 nmoles of TPN in 0.1 ml of water to each cuvette and 230 nmoles in 2.3 ml of H_2O to the incubation flask, the reaction was followed at 340 nm until all the TPN was reduced. To the incubation flask and to one cuvette, respectively, 336 nmoles (8.68×10^5 dpm in 0.92 ml of ethanol) and 15 nmoles (3.78×10^4 dpm in 0.04 ml) of estradiol were added (total = 9.06×10^5 dpm). The substrate control received 0.04 ml of ethanol. The reaction was followed for 20 min. A total of 69.1 nmoles of DPN was reduced. The reaction mixture was worked up as described in the previous section. The results are summarized in Table III. The specific activity of the recovered estradiol was 9100 dpm per μg compared to an initial specific activity of 9440 dpm per μg; the deuterium content of the estradiol was unchanged (found, 96.1% ^{2}H at C-17). Examination of the pooled "estrone" fractions by gas-liquid chromatography-mass spectrometry showed 0.5% estrone as compared to 0.6% estrone in the steroid incubated.

Does Estradiol Undergo Cyclic Oxidation-Reduction when it Stimulates Transhydrogenase Function of Estradiol Dehydrogenase?

To ascertain if (*a*), estrone is formed and (*b*), if deuterium is lost from the remaining estradiol during the transhydrogenation reaction, the dehydrogenase was incubated with estradiol-17α-^{2}H at 25°.

The incubation mixture contained the following components: 1.8 ml of 1 M Tris-chloride buffer, pH 7.4, 60 μmoles of glucose 6-phosphate in 1.2 ml of water, 3.6 units of yeast glucose 6-phosphate dehydrogenase in 1.8 ml of 50 mM phosphate buffer, pH 7.2, 7.5 μmoles of DPN in 1.2 ml of water, 1.4 milliunits of transhydrogenase activity in 0.15 ml of water (specific activity = 110 milliunits per mg of protein), and 11.25 ml of water. Two 2.9-ml portions were withdrawn and the change in absorbance at 340 nm was observed for 5 min after the addition of 10 nmoles of TPN in 0.1 ml of water to each cuvette and 40 nmoles of TPN in 0.4 ml of water to the incubation flask. The reaction was followed at 340 nm until all the TPN was reduced. To the incubation flask and to one cuvette, respectively, 16 μg (59 nmoles in 0.08 ml of ethanol) and 4 μg (15 nmoles in 0.02 ml of ethanol) of estradiol-17α-^{2}H were added (total = 74 nmoles). The substrate control received 0.02 ml of ethanol. The reaction was followed until the change in absorbance was approximately that obtained in previous incubations with the estradiol-activated transhydrogenase (*i.e.* 20 min). A total of 15.6 nmoles of DPN was reduced. The reaction mixture was worked up as described in the previous section, except that the dried residues remaining after the methylene chloride extraction were adsorbed onto stainless steel gauzes, and left at 37° overnight in an atmosphere of acetic anhydride and pyridine, and thus converted to the acetate (9). These samples were then examined by combined gas-liquid chromatography-mass spectrometry. The results are given in Table III. A second experiment was performed in which the reaction was allowed to proceed for 2 hours. In both experiments estrone was formed and there was loss of ^{2}H from the estradiol-17α-^{2}H (Table III).

TABLE III

Transhydrogenase function of estradiol dehydrogenase

	20 min	2 hours
	nmoles	
Estradiol-17α-^{2}H[a] incubated	74	74
Estrone formed	9.5	10.5
DPNH formed	15.6	72.7
Estradiol recovered (% ^{2}H at C-17)	86	50

[a] The estradiol-17α-^{2}H incubated contained 95.8% ^{2}H at C-17.

Effect of Other Estrogens on Stimulation of the Two Transhydrogenase Systems

The estradiol-activated transhydrogenase is stimulated by diethylstilbestrol as well as by estradiol (Fig. 6), but not by estrone or by 17α-methylestradiol at all concentrations tested up to 10 μM, the limit imposed by solubility. As already noted, estrone is as effective as estradiol in stimulating the transhydrogenase function of estradiol dehydrogenase, whereas diethylstilbestrol and 17α-methylestradiol are, as might be expected, inactive.

DISCUSSION

The properties of the two soluble estradiol-sensitive, pyridine nucleotide-linked transhydrogenase systems of human placenta are listed in Table IV.

The possibility that the estradiol-activated transhydrogenase is an artifact resulting from dual pyridine nucleotide specificity of placental glucose 6-phosphate dehydrogenase has been eliminated by two experiments. In the first, it was shown that placental glucose 6-phosphate dehydrogenase is separable from

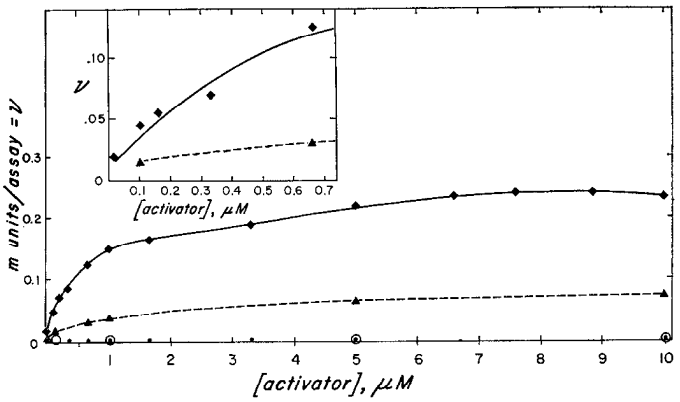

FIG. 6. Comparison of the effect of estrone (●), 17α-methylestradiol (○), and diethylstilbestrol (▲) on the stimulation of estradiol-activated transhydrogenase. Each cuvette contained 0.2 milliunit of activity (specific activity = 0.4 milliunit per mg of protein). Assays were run at 25°. The activators were added in 0.02 ml of ethanol. Estradiol (◆).

TABLE IV

Properties of the two soluble estradiol-sensitive pyridine nucleotide-linked transhydrogenase activities of human placenta

	Estradiol-activated	Transhydrogenase function of estradiol dehydrogenase
Phosphate concentration (mM) at which activity is eluted from hydroxylapatite	45–55	20–30
Presence in extract after heating to 55°	No	Yes
Effect of 1.67 M NaCl	Increased activity	Inhibition
Association with DPN (and TPN)-linked estradiol dehydrogenase activity	No	Yes
Kinetic isotope effect with estradiol-17α-^{2}H	No	Yes
Formation of estrone during transhydrogenation	No	Yes
Loss of 17α-^{2}H during transhydrogenation	No	Yes
Structural specificity for activation		
Estradiol	+	+
Estradiol-17α-^{2}H	+	+
Estrone	−	+
17α-Methylestradiol	−	−
Diethylstilbestrol	+	−

estradiol-activated transhydrogenase by hydroxylapatite chromatography.[2] In the second, in the absence of substrate (glucose 6-phosphate), transhydrogenation took place when TPNH was generated with isocitrate and isocitrate dehydrogenase.

Although it has previously been shown by two spectrophotometric assays (1) that the estradiol-activated transhydrogenase was free from DPN- or TPN-linked estradiol dehydrogenase activity, it was deemed desirable to examine this question by a direct method. Estradiol-6,7-^{3}H-17α-^{2}H was incubated with estradiol-activated transhydrogenase under the conditions employed for the estradiol dehydrogenase assay. The only steroid recovered from the reaction mixture was estradiol with the same ^{2}H and ^{3}H content as the starting material. Thus, there is no evidence for contamination of the estradiol-activated transhydrogenase with estradiol dehydrogenase activity. Furthermore, rechromatography of the estradiol-activated transhydrogenase on hydroxylapatite resulted in a 2.5-fold purification and no evidence for dissociation into dehydrogenase and transhydrogenase activities (21).

As a preliminary to studies of the mechanism of the two transhydrogenating systems, the maximal rates of dehydrogenation of estradiol and estradiol-17α-^{2}H by DPN-linked estradiol dehydrogenase were determined. In agreement with previous findings (20) the $V_H/V_{^2H}$ was found to be 2.1. The estradiol-activated transhydrogenase was stimulated equally well by estradiol and estradiol-17α-^{2}H (Fig. 4), whereas, in measurement of the transhydrogenase function of estradiol dehydrogenase, a kinetic isotope effect was observed (Fig. 5). These results suggested a fundamental difference in the mechanisms by which estradiol stimulates the two systems.

Estradiol-6,7-^{3}H-17α-^{2}H (350 nmoles) was therefore incubated with estradiol-activated transhydrogenase under the conditions used for transhydrogenase assays. Although 69 nmoles of DPN were reduced, the estradiol recovered had the same ^{3}H specific activity and ^{2}H content as the starting material (Table III). No estrone was formed. It would thus appear that *estradiol functions as a true activator* and does not undergo chemical change.

When transhydrogenation was performed with the estradiol dehydrogenase system, estradiol-17α-^{2}H gave a kinetic isotope effect (Fig. 5), estrone was formed, the ^{2}H content of the estradiol was reduced, and the amount of DPNH formed exceeded that of the estrone produced. Moreover, the relative amounts of estrone and estradiol did not alter appreciably from 20 min to 2 hours, although there was a further reduction in the ^{2}H content of the estradiol recovered (Table III). These data clearly indicate cyclic oxidation-reduction of estradiol and provide direct proof for the hypothesis proposed by Talalay, Hurlock, and Williams-Ashman (2).

Examination of the structural specificity for stimulation of the two transhydrogenase systems provided further evidence that they are indeed different. Both systems are stimulated by estradiol and estradiol-17α-^{2}H and neither by 17α-methylestradiol. As might be expected, estrone stimulates the transhydrogenation by the dehydrogenase system, but, surprisingly, it has no effect upon the estradiol-activated transhydrogenase. In this respect, the estradiol-activated transhydrogenase is similar to the estradiol-binding protein or proteins of rat uterus described by Jensen (22) and Jensen and Jacobson (23). The similarity is strengthened by the fact that the estradiol-activated transhydrogenase is stimulated by diethylstilbestrol, a substance that displaces estradiol from the uterine estradiol-binding protein (24). As expected, diethylstilbestrol does not stimulate transhydrogenation by the dehydrogenase system.

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[2] A portion of the redissolved 30 to 50% ammonium sulfate precipitate was subjected to hydroxylapatite chromatography. Fractions of the eluate were assayed for glucose 6-phosphate dehydrogenase as well as for transhydrogenase, and estradiol dehydrogenase activity. The placental glucose 6-phosphate dehydrogenase activity was eluted at phosphate concentrations from 25 to 33 mM, whereas the estradiol-activated transhydrogenase activity was eluted at 30 to 38 mM phosphate.

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