Amino Acid Composition and Subunit Structure. Human Placental 17β-Estradiol Dehydrogenase†

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ABSTRACT: Human placental 17β-estradiol dehydrogenase has been purified in quantity and found to have a molecular weight of about 68,000 in the presence of 17β-estradiol or of glycerol. The amino acid composition and a single N-terminal sequence of five residues has been determined. Since previous work (Burns *et al.* (1971), *Biochem. Biophys. Res. Commun.* *44*, 786) indicated a subunit molecular weight of about 33,500 the present results indicate that 17β-estradiol dehydrogenase is a dimer of probably identical subunits.

Estradiol[1] dehydrogenase from human placenta has been purified to apparent homogeneity by several groups (Descomps *et al.*, 1968; Jarabak, 1969; Karavolas *et al.*, 1970). Discrepancies between data obtained using the different preparations have been evident, particularly with regard to the molecular weight values reported for the enzyme. In a recent communication (Burns *et al.*, 1971) we reported studies which suggested that the enzyme is a dimer, possibly of identical subunits, with a total molecular weight of about 68,000. At about the same time Jarabak and Street (1971) reported similar results. Present studies extend this work and lend further support to this conclusion.

Materials

The sources of most of the materials have been described previously (Karavolas *et al.*, 1970). Commercial acrylamide and *N,N'*-methylenebisacrylamide were crystallized from chloroform and acetone, respectively. Spectroquality glycerol from Matheson or Eastman was used throughout. Coomassie Blue was from Mann Research Labs.

Hydroxylapatite was prepared as described previously (Karavolas *et al.*, 1969). Fibrous DEAE-cellulose (type 40, Brown Co.) was treated before use with 1 N HCl and 1 N NaOH by the method of Peterson and Sober (1962). Microgranular DEAE-cellulose (Whatman DE-52) was soaked overnight in saturated NaCl, washed with water, degassed, and equilibrated according to directions in the manufacturer's manual. Dialysis tubing (A. H. Thomas Co.) was heated on a steam bath in 10% glycerol–5 mM EDTA for 30–60 min and washed thoroughly in water before use.

Experimental Section

Purification of Estradiol Dehydrogenase. The isolation and purification of estradiol dehydrogenase was based on the procedure developed previously in this laboratory (Karavolas *et al.*, 1970). The present scheme, however, incorporates modifications designed to facilitate the handling of greater quantities of material than previously employed and is outlined below. Details of a typical purification are given in Table I.

CRUDE ENZYME PREPARATION. The collection of the placentas, homogenization, and preparation of the enzyme fraction precipitating between 30 and 50% saturation with ammonium sulfate were performed as described previously (Karavolas *et al.*, 1969).

DEAE-CELLULOSE CHROMATOGRAPHY. Redissolved ammonium sulfate precipitates from ten placentas were combined and centrifuged at 70,000*g* for 60 min. The sediment was washed by suspension in a small volume of 10 mM phosphate buffer containing 50% glycerol and centrifuged at 100,000*g* for 90 min. The supernatants were combined and dialyzed[2] thoroughly against three 5-l. changes of 10 mM phosphate buffer,[3] without mercaptoethanol. The dialyzed enzyme preparation was then applied to a 4.5 × 52 cm column of fibrous DEAE-cellulose which had been equilibrated with 30 mM phosphate buffer without mercaptoethanol. After all the enzyme had been applied, a linear phosphate gradient from 50 to 250 mM (50 mM change/l.) was started at a flow rate of 150 ml/hr. The bulk of the enzyme activity was eluted at phosphate concentrations of 70–100 mM. All chromatograms employing gradients were monitored by continuously recording conductivity in the column effluent as it passed through a 1-ml flow-through cell (Radiometer Model CDM 2d conductivity meter).

Hydroxylapatite Chromatography. Fractions from step 2

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[1] In this paper estradiol is used in place of the accepted trivial name, 17β-estradiol.

[2] All dialysis steps and storage of enzyme fractions after chromatography were carried out at 4° unless otherwise stated. Chromatography and the collection of fractions during chromatography were carried out at room temperature.

[3] Unless otherwise noted, all buffers were potassium phosphate (pH 7.2), 20% glycerol, 5 mM EDTA, and 7 mM 2-mercaptoethanol. The EDTA was added as a 100 mM solution of Na_2EDTA titrated to pH 7.0 with NaOH.

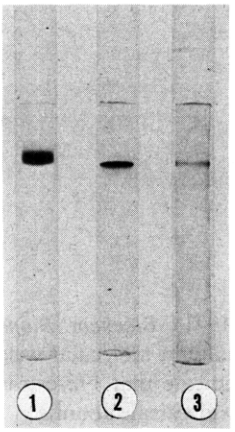

FIGURE 1: Polyacrylamide gel electrophoresis of purified estradiol dehydrogenase. (1) pH 6.9; (2) pH 8.9, stained for protein with Coomassie Blue; (3) pH 8.9, stained for dehydrogenase activity.

having the highest specific activity were pooled and dialyzed at 4° against several changes of 5 mM phosphate buffer. The dialyzed solution was then filtered through a glass fiber pad (Millipore Corp.) to remove small quantities of insoluble material, and applied to a 5.2 × 55 cm hydroxylapatite column which had been equilibrated with 5 mM phosphate buffer. The top few centimeters of the column bed were stirred gently during enzyme application to improve the flow rate (Karavolas *et al.*, 1970). After the enzyme sample had entered the column, elution was started with a linear phosphate gradient between 5 and 55 mM (12.5 mM change/l.). The flow rate was 150–200 ml/hr; most of the enzyme activity was eluted between 18 and 25 mM phosphate.

DE-52 CHROMATOGRAPHY. The most active fractions from step 3 were pooled and applied directly to a 1.7 × 29 cm column of microgranular DEAE-cellulose (Whatman DE-52) which had been equilibrated with 30 mM phosphate buffer. Elution was carried out with a linear phosphate gradient from 30 to 180 mM phosphate buffer (75 mM change/l.) at a flow rate of 25 ml/hr. The enzyme was eluted between 45 and 55 mM phosphate.

SEPHADEX GEL FILTRATION. Polyacrylamide gel analysis of the enzyme fractions isolated following step 4 showed estradiol dehydrogenase to be the major protein constituent, and to be contaminated with small amounts of other proteins. The enzyme was not purified further by a second hydroxylapatite chromatographic step as was possible with the previous procedure (Karavolas *et al.*, 1970), but the pure enzyme was obtained by Sephadex G-100 gel filtration. The enzyme fractions from step 4 were pooled, dialyzed against 5 mM phosphate buffer, and concentrated by adsorption to a small hydroxylapatite column (1 × 5 cm) followed by elution with 100 mM phosphate buffer. A 2.5-ml sample of concentrated enzyme was applied to a 1.5 × 85 cm column of Sephadex G-100 in 100 mM phosphate buffer. The column was adapted for upward flow chromatography and the flow rate was 2.5 ml/hr. Fractions of similar specific activity from different gel filtration runs were pooled, dialyzed against 10 mM phosphate buffer, and stored at 4°. A single protein band was seen on disc gel electrophoresis at pH 6.9 and pH 8.9 (Figure 1).

Analytical Procedures

Enzyme Assay. Estradiol dehydrogenase activity was defined and assayed as described previously (Karavolas *et al.*, 1970; Langer and Engel, 1958). In this work estradiol was the only substrate used. Assays were done at 22 ± 1°.

Protein Determinations. Protein concentrations were determined by the ratio of absorbances at 280 and 260 nm (Warburg and Christian, 1942). Whenever possible, pooled enzyme fractions were dialyzed thoroughly, and the absorbance was measured using the dialysis buffer as a blank.

Polyacrylamide Disc Gel Electrophoresis. Each step of the purification procedure was monitored using analytical disc gel electrophoresis. The pH 8.9–8.3 alkaline gel system of Davis (1964) was employed without sample or spacer gels. The gels contained 20% glycerol and electrophoresis was performed at 20°. Samples were made uniform with respect to volume, glycerol concentration and electrophoresis buffer concentrations. Protein in the gels was detected with Coomassie Blue according to Fairbanks *et al.* (1971) and enzyme activity with the dehydrogenase stain used by Karavolas *et al.* (1970). Gel electrophoresis in the presence of sodium dodecyl sulfate was performed as described by Weber and Osborn (1969). Gel electrophoresis was performed occasionally at pH 4.3 and 6.9, both in the presence of 20% glycerol.

Carboxymethylation. Pure estradiol dehydrogenase was dialyzed at 4° against distilled water, lyophilized, and carboxymethylated according to Crestfield *et al.* (1963). After reaction, the protein was again dialyzed against water, lyophilized, and stored at −15°.

Performic Acid Oxidation. Lyophilized estradiol dehydrogenase was oxidized with performic acid according to Hirs (1967). When the reaction was complete the reactants were (twice) diluted 40-fold with water, frozen, and lyophilized.

Amino Acid Composition Studies. A sample of carboxymethylated lyophilized estradiol dehydrogenase was dissolved in 1 ml of 10 mM phosphate buffer (pH 7.2) without EDTA, without glycerol, and without mercaptoethanol and centrifuged briefly to remove insoluble material. Three identical samples (approximately 250 μg each) were taken and each was mixed with an equal volume of concentrated HCl in glass ampoules. Residual oxygen was removed with nitrogen, the contents frozen in liquid nitrogen, and the ampoules sealed under vacuum and then incubated at 105–108° for 24, 48, and 72 hr, respectively. After incubation the ampoules were opened and the samples dried in a desiccator containing NaOH pellets. Amino acid analysis was performed using a Beckman Model 120C automatic amino acid analyzer fitted with high-sensitivity attachments. Tryptophan was determined from the ultraviolet absorption spectrum of carboxymethylated estradiol dehydrogenase dissolved in 0.1 N NaOH (Goodwin and Morton, 1946).

Carbohydrate Analysis. The procedure used was a modification[4] of that described by Clamp *et al.* (1967). A sample of approximately 500 μg of lyophilized carboxymethylated estradiol dehydrogenase and 7.5 μg of *myo*-inositol was mixed with 0.5 ml of 2 N trifluoroacetic acid and heated in a screw-capped tube (13 × 100 mm) at 100–110° for 2 hr. The sample was then dried under a stream of nitrogen at 40°, washed twice with methylene chloride to remove residual acid, and refluxed in the sealed tube with 0.5 ml of 5 M methanolic HCl held at 65° for 16 hr. Excess solvent was removed with a stream of nitrogen, and the residue acetylated by addition of pyridine (100 μl) and acetic anhydride (100 μl), followed immediately by evaporation under nitrogen at 40°. The sample was de-O-acetylated by the addition of 0.5 ml of 5 M methanolic HCl and heating at 65° for 1 hr. The residue was

[4] V. Rheinhold and R. W. Jeanloz, unpublished procedure.

TABLE I: Purification Scheme for Estradiol Dehydrogenase.

Step	Fraction	Total DPN-Linked Act. (Units)	Recovery (%) For Step	Recovery (%) For Pooled Best Fractions[a]	Sp Act. (munits/mg of Protein)	Purification (-fold) Over Preceding Step	Purification (-fold) Overall
1	$(NH_2)_2SO_4$–30–50% saturation	421			14.0		
2	DEAE-cellulose	421	95	68	170	12.1	12.1
3	Hydroxylapatite	287	95	67	1325	7.8	95
4	DE52-cellulose	193	90	65	4833	3.6	345
5	Sephadex G-100	49[b]	99	95	7800–8150	1.6–1.7	557–582

[a] The entire "best fraction pool" consisting of fractions of highest specific activity was used in the subsequent chromatographic step (except for step 5). [b] The enzyme pool following DE-52 chromatography was concentrated and divided into portions prior to Sephadex chromatography.

recovered by evaporation again, and per(trimethylsilylated)- for 1 hr at room temperature with a mixture of hexamethyldisilazane and trimethylchlorosilane (Sylon-HT, Supelco Inc., Bellefonte, Pa.) in pyridine and evaporated to dryness. The residue was extracted with heptane and aliquots of the supernatant were analyzed in a Perkin-Elmer Model 900 gas chromatograph equipped with dual-flame ionization detectors and a stainless steel column (300 × 0.3 cm) containing 0.1% OV-17 on textured glass beads (Supelco Inc.). The initial column temperature was 80°, which was increased 10° per min to 250°. The peak areas and retention times were automatically recorded with a digital integrator (Kent Instruments, Ltd., Luton, Bedford, U. K.) and the concentration of each component corrected to that of the internal standard, *myo*-inositol.

N-Terminal Sequence Analysis. A 2.5-mg sample of performic acid oxidized and lyophilized estradiol dehydrogenase was subjected to 5 cycles of degradation by the phenyl isothiocyanate procedure in an automated instrument (Beckman Sequencer Model 890) using the procedures of Edman and Begg (1967). The amino acid thiazolidone derivatives obtained at each cycle of degradation were converted to the corresponding phenylthiohydantoins by treatment with 1 N HCl for 10 min at 80° and identified by both gas–liquid chromatography (Pisano and Bronzert, 1969) and thin-layer chromatography.

Estimation of Molecular Weight by Ultracentrifugation. The procedure of Simpson and Bethune (1970) was used to measure the molecular weight of estradiol dehydrogenase in the presence of estradiol. A small volume of concentrated enzyme in 100 mM phosphate buffer was dialyzed for 70 hr at room temperature against 1 l. of 10 mM potassium phosphate buffer (without glycerol) and 8 μM estradiol. The estradiol was added last, in 1 ml of redistilled 95% ethanol, to the full volume of buffer. The addition was slow and the mixture was stirred vigorously to prevent the estradiol from precipitating.

Results

Enzyme Preparation. The yield of pure estradiol dehydrogenase from the crude enzyme ammonium sulfate precipitates was 20–25% when the purification scheme outlined in Table I was followed. The final product was homogeneous by analytical disc gel electrophoresis (Figure 1) and by sodium dodecyl sulfate disc gel electrophoresis. Its specific activity (DPN linked) was approximately 8 U/mg and was within the range of values reported previously (Descomps *et al.*, 1968; Jarabak, 1969; Karavolas *et al.*, 1970). A detailed comparison with these values was not made since the assay systems used by different groups do not always give comparable results at the same pH and temperature.

Amino Acid Composition. The amino acid composition for estradiol dehydrogenase was calculated from the analysis of samples hydrolyzed for 24, 48, and 72 hr (Table II). Previous work (Burns *et al.*, 1971) had indicated a minimum subunit molecular weight of 33,500 ± 1000 as estimated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. This molecular weight range was assumed when a preliminary amino acid composition was calculated. A final composition was based on 22 arginine residues/subunit; calculations based on 41 leucine or 13 phenylalanine residues gave similar results. We did not quantitate the total recovery of amino acids from the hydrolyzed protein sample because the limited solubility of the unhydrolyzed protein in aqueous buffer interfered with accurate protein determination. A sample of the material which did not dissolve in buffer was hydrolyzed and found to have a composition identical to that of the soluble protein. Other problems arising from the limited solubility of denatured estradiol dehydrogenase are discussed below.

An unidentified compound (Unknown, Table II) present in small and variable amounts in different hydrolysates was detected consistently during amino acid analysis. The compound was eluted ahead of lysine in the short-column analysis of basic amino acids and just after phenylalanine in the long column analysis of the neutral and acidic amino acids. This elution behavior is similar to that of glucosamine, and to a lesser extent, tryptophan, but comparison to standards demonstrated that the unknown differed from both of these compounds; it has not been identified.

Carbohydrate. Estradiol dehydrogenase was examined for the presence of carbohydrate residues by a procedure utilizing gas–liquid chromatography as the means of detection. No hexosamines were found. A small quantity of glucose was found amounting to about 1% by weight of the total protein sample. Since the final enzyme purification step involved Sephadex chromatography it is likely that the glucose arises from dextran gel.

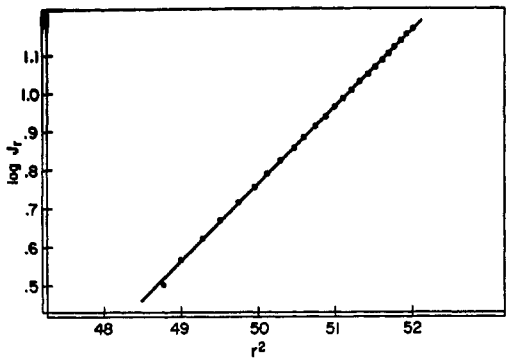

FIGURE 2: Time-lapse ultracentrifugation of estradiol dehydrogenase (1.5 mg/ml) in 10 mM phosphate buffer (pH 7.2), 7 mM mercaptoethanol, 5 mM EDTA, and 8 μM estradiol. The meniscus was depleted at 35,600 rpm for 18 hr at 20°. J_r is the fringe number at radial distance, r.

Molecular Weight Determination by Ultracentrifugation. The apparent partial specific volume for estradiol dehydrogenase of 0.74 g/ml was calculated (Cohn and Edsall, 1943) from the amino acid composition (Table II), excluding the unknown compound. This value was used in previous work (Burns *et al.*, 1971) to calculate a molecular weight of 67,700 for estradiol dehydrogenase when the enzyme, in buffer containing 20% glycerol, was examined by ultracentrifugation. In the present studies we obtained a molecular weight value of 69,000 by ultracentrifugation when the enzyme was in an aqueous buffer without glycerol but containing 8 μM estradiol (Figure 2). The agreement between these two molecular weight estimates indicates that the presence of glycerol does not greatly modify the partial specific volume of the enzyme.

An attempt was made to determine directly the partial specific volume of the enzyme in the presence of 20% glycerol by a comparison of its behavior during ultracentrifugation in the presence of either 10 mM phosphate buffer or phosphate buffer in deuterium oxide and deuterated glycerol. The attempt failed because of a disturbance of the interference fringe pattern during centrifugation, possibly arising from a gradual aggregation of the enzyme.

N-Terminal Sequence of Estradiol Dehydrogenase. The sequence of the first five residues at the amino terminus of the enzyme was determined by Edman degradation in an automatic apparatus. The sequence found was: Ala-Glu-Thr-Val-Val-. Only these residues could be determined unequivocally since, by the time the fifth cycle had been completed, quantities of the amino acids released in earlier cycles were interfering with recognition of the newly released N-terminal amino acid. This problem resulted from the limited solubility of the lyophilized, performic acid oxidized estradiol dehydrogenase in the buffer used for dissolving the protein. The major portion of the N-terminal alanine was released in the first cycle of degradation but sufficient unchanged protein was present to result in further release of alanine in the subsequent two cycles. Hence, extraneous amino acids quickly reached levels at which they interfered with interpretation of data. The possibility that the sample of estradiol dehydrogenase was substantially impure is unlikely since a previous N-terminal amino acid determination (Burns *et al.*, 1971) using fluorodinitrobenzene gave alanine with no discernible traces of other amino acids.

Solubility of Estradiol Dehydrogenase. We encountered solubility problems on several occasions when dealing with lyophilized estradiol dehydrogenase. Carboxymethylated enzyme is more soluble in aqueous buffer than is unmodified and lyophilized or performic acid oxidized estradiol dehydrogenase, but in all cases it was difficult to achieve complete solution.

TABLE II: Amino Acid Analysis of Estradiol Dehydrogenase.

	Yield Obtained (nmoles)[a]			Estimated Residues/Mole[b] Subunit
	24 hr	48 hr	72 hr	
Lys	9.71	9.31	9.31	10
His	6.04	6.44	6.67	7
Arg	22	22	22	22
$^1/_2$-Cys[c]	5.45	5.90	4.71	6
Asp	21.56	21.42	20.61	21
Thr	15.68	15.11	14.57	16
Ser	18.08	16.44	5.84	19
Glu	28.66	30.39	25.09	28
Pro	19.06	19.29	18.28	19
Gly	31.27	31.32	30.03	31
Ala	35.94	36.92	34.70	36
Val	27.01	31.31	31.95	32
Met	3.63	2.92	2.09	4
Ile	3.83	4.00	4.03	4
Leu	40.66	41.35	41.32	41
Tyr	5.63	4.64	4.16	6
Phe	12.86	12.89	12.92	13
Trp				1
Unknown[d]	2.87	1.48	3.62	
Total residues in subunit				316

[a] Duplicate samples were run for each time of hydrolysis. To assist comparison the values given are based on a constant yield of arginine as 22 nmoles. The actual yields of arginine were from 22.16 to 22.82 nmoles. [b] The values for threonine, serine, methionine, and tyrosine have been determined after correction for losses due to acid hydrolysis. The maximum values for valine and isoleucine have been used since these amino acids are released slowly during hydrolysis. Tryptophan was determined by spectral analysis (Goodwin and Morton, 1946). The amino acid composition corresponds to a minimum molecular weight of 33,659. [c] Determined as *S*-carboxymethylcysteine. [d] Yields determined from average color yield of all standard amino acids except Lys, Arg, His, Pro, and $^1/_2$-Cys.

Discussion

Previously we have suggested that estradiol dehydrogenase is a dimer, possibly of identical subunits, on the basis of the following evidence (Burns *et al.*, 1971). (i) The enzyme when examined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate gives a single protein band with an estimated molecular weight of 33,500 ± 1000; (ii) the enzyme in phosphate buffer (containing 20% glycerol) has a molecular weight of 67,700 as determined by ultracentrifugal analysis; (iii) N-terminal analysis of estradiol dehydrogenase gave alanine. In the present work we provide further evidence

to support our original conclusion. An N-terminal sequence of five amino acid residues (Ala-Glu-Thr-Val-Val-) was determined for the enzyme. The pentapeptide sequence both confirms the earlier finding of N-terminal alanine and makes it unlikely that estradiol dehydrogenase has dissimilar subunits. The finding of a single N-terminal sequence requires that if there are different subunits of about the same molecular weight, one has alanine as its N-terminal residue and the other is N blocked.

Descomps *et al.* (1968) obtained a molecular weight value of 92,000 for estradiol dehydrogenase by gel filtration in the presence of a substrate analog. Since this value was approximately that to be expected from a trimer of subunits, we investigated the effect of estradiol in the absence of glycerol on the molecular weight of the enzyme. We obtained a value of 69,000 by ultracentrifugation in 10 mM phosphate buffer, lacking glycerol but containing 8 μM estradiol. The close agreement between this value and the previously reported value of 67,700 for the enzyme in the same buffer but containing glycerol and lacking estradiol indicates that at the concentration studied the enzyme is a dimer in the presence of either glycerol or substrate.

Recently Jarabak and Street (1971) reported studies with estradiol dehydrogenase ("17β-hydroxysteroid dehydrogenase") which lead them to conclusions similar to ours. By sodium dodecyl sulfate gel electrophoresis they estimated a subunit molecular weight of 35,000 and calculated molecular weight values for the active enzyme from 72,000 to 73,000 by ultracentrifugation and in cross-linking experiments utilizing dimethyl suberimidate. The overall agreement between this work and our own is gratifying.

Two points deserve further comment. The first concerns the techniques used to perform gel electrophoresis in sodium dodecyl sulfate. An earlier publication (Karavolas *et al.*, 1970) gave an estimate of 48,000 for the molecular weight of estradiol dehydrogenase by sodium dodecyl sulfate gel electrophoresis using the procedure of Marshall and Zamecnik (1969). We subsequently found values of 33,000–34,000 for the molecular weight of native, carboxymethylated, or performic acid oxidized enzyme by the sodium dodecyl sulfate gel electrophoresis procedure of Weber and Osborn (1969) and the same value for the native enzyme by the Fairbanks *et al.* (1971) procedure. We therefore repeated the measurements using the procedure of Marshall and Zamecnik (1969) omitting sodium dodecyl sulfate from the running buffer and obtained poor protein band resolution but a molecular weight of approximately 48,000 (Burns *et al.*, 1971). We have since learned that in the procedure of Marshall and Zamecnik (1969) sodium dodecyl sulfate is normally included in the electrophoresis buffer and, in fact, had been included when the original estimate of 48,000 was obtained.[5] Since Jarabak and Street (1971) obtained a molecular weight value of 35,000 using the procedure of Marshall and Zamecnik and included sodium dodecyl sulfate in the electrophoresis buffer, we are unable to provide any satisfactory explanation for the previous estimate of 48,000.

The second point concerns a comparison of the amino acid composition of estradiol dehydrogenase determined by Jarabak and Street (1971) with that given in Table II. The composition originally published by Jarabak and Street (1972) has subsequently been amended and now agrees fairly closely with the composition we have determined. We were unable to determine the composition of the unknown compound found during amino acid analysis, but Jarabak and Street apparently did not find this substance. Other discrepancies appear to be minor and it seems clear that both groups are dealing with the same protein. This conclusion is significant in the light of apparent differences in previous determinations of substrate specificities and kinetic constants. The possibility that very small quantities of contaminating proteins could account for some differences between previous results cannot be entirely excluded.

We conclude therefore that all recent evidence (Jarabak and Street, 1971; Burns *et al.*, 1971) strongly supports the contention that estradiol dehydrogenase is a dimer, very probably of identical subunits, with an overall molecular weight of approximately 68,000.

[5] R. D. Marshall personal communication. See also legend to Figure 1 in Marshall and Zamecnik (1969).

Acknowledgments

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