Purification and Properties of the Soluble 17β-Hydroxysteroid Dehydrogenase of Rabbit Uterus

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17 β-Hydroxysteroid dehydrogenase activity towards estradiol-17 β has been demonstrated in the 105,000 × *g* supernatant of rabbit uterus. Hydroxylapatite chromatography of the enzyme activity isolated by ammonium sulfate precipitation, gel filtration and DEAE-cellulose chromatography yielded a single 17 β-hydroxysteroid dehydrogenase activity. Further purification of the enzyme preparation by isoelectric focusing resulted in multiple peaks of activity. The molecular weight of the enzyme, calculated from mobility data on Sephadex gel, is approximately 64,000.

Some properties of partially purified 17 β-hydroxysteroid dehydrogenase activity have been studied.

Estradiol-17 β reacts at a faster rate than testosterone. The K_m for estradiol is 4.16×10^{-5} mol/l for the NAD-linked enzyme activity and 4.37×10^{-5} mol/l when NADP as cofactor was used. The ratio of the maximal velocity for NADP to that for NAD was 1.42. The pH-optimum for estradiol appears between 9.5 and 10.5 and for estrone between 5.5 and 6.5. The enzyme appears to be of the sulfhydryl type.

Introduction

The work of Jütting [1, 2] demonstrated that rabbit uterus is capable of transforming estradiol-17 β into estrone and *vice versa.*

Since estrogen receptors in the uterine tissue have been demonstrated, the metabolism of estrogens in this tissue has been of particular interest, because of the effects of several possible estradiol metabolites, for instance estrone, on the estradiol binding capacity of the estradiol receptor.

In this paper, purification of the soluble 17 β-hydroxysteroid dehydrogenase of pregnant rabbit uterus and some properties of the cytoplasmic enzyme are reported. The most extensively studied 17 β-hydroxysteroid dehydrogenase is that in human term placenta [3–10], which was named estradiol dehydrogenase owing to its high specificity for this hormone. The 17 β-hydroxysteroid dehydrogenase of uterine tissue is interesting for two reasons: firstly, it catalyses the interconversion of estradiol and estrone, a biologically active estrogen and a relatively inactive one (estrone could compete with estradiol for binding sites in the uterine receptor); and secondly, it is a good tool for studying steroid-protein interactions.

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Experimental procedures

Materials

[4-^{14}C]Estradiol (58 mCi/mmol), [4-^{14}C]estrone (58 mCi/mmol), [4-^{14}C]testosterone (58.2 mCi/mmol) and [4-^{14}C]androstenedione (60 mCi/mmol) were purchased from the Radiochemical Centre, Amersham, England. The radiochemical purity was verified by thin-layer chromatography on silica gel using benzene-methanol (19 : 1, v/v) and cyclohexane-ethyl acetate (1 : 1, v/v). Unlabelled steroids were obtained from Schering AG, Berlin, Germany, and used as supplied.

Carrier ampholytes came from LKB Instruments, Stockholm, Sweden. Sephadex G-200 was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. DEAE-cellulose 23 SH, phenazine methosulfate, acrylamide and methylenebisacrylamide were purchased from Serva, Heidelberg, Germany. DEAE-cellulose was treated with 1 N HCl and 1 N NaOH before washing and adjustment to the desired pH.

All organic solvents were redistilled analytical reagents. All other chemicals, analytical grade, were purchased from commercial sources.

Tissue preparation

Pregnant hare rabbits were obtained from Schering AG, Berlin, Germany. The day of mating was

designated as day zero of pregnancy. The uterus was excised on day 30, the products of conception were removed, and endometrial tissue was scraped off with a scalpel.

Enzyme purification:

All of the steps were performed at 4 °C. The myometrial tissue was suspended in 5 vol. (w/v) of 10 mmol/l Tris/HCl-buffer, 1 mmol/l EDTA, 12 mmol/l mercaptoethanol, 200 g glycerol/l, pH 7.4 (buffer A), containing 0.25 mol/l sucrose, and homogenized with an Ultra Turrax homogenizer (5 × 5 s). It was then further homogenized by 5 up-down transits with a glass-in-Teflon Potter-Elvehjem homogenizer at 1,000 rev./min for 30 s. The homogenate was filtered through four layers of cheese-cloth. The cytosol was obtained by centrifugation at 105,000 × *g* for 90 min.

Step 1

Saturated ammonium sulfate solution was added to the supernatant to 30% saturation of the salt, and the solution was stirred for at least 4 h. The inactive precipitate was removed by centrifugation at 27,000 × *g* for 30 min. Ammonium sulfate was then added to 50% saturation, and the solution was stirred for at least 4 h. The active precipitate was collected by centrifugation at 27,000 × *g* for 30 min. The pH was adjusted during any of the ammonium sulfate steps. A dark red precipitate was obtained. This was dissolved in a minimal amount of buffer A, and dialyzed extensively against the same buffer.

Step 2

The enzyme solution obtained after ammonium sulfate precipitation was further purified on a Sephadex G-200 column. The column (1.5 × 90 cm) was equilibrated at 4 °C with buffer A. The column was run by reverse flow. Portions of the solution containing the enzymatic activity associated with 550 to 600 mg of protein were applied to the bottom of the column in 4 ml of buffer A. The protein was eluted with the same buffer. Enzymatically active fractions were pooled and concentrated to a protein concentration of 35 mg/ml by ultrafiltration with a PM-10 membrane.

Step 3

The enzymatically active solution was dialyzed against buffer A and then applied to a DEAE-cellulose column (1.5 × 30 cm) that had been equilibrated with buffer A. After application of the sample, elution was begun with a linear phosphate gradient between 0 and 200 mmol/l phosphate. The fractions with highest 17 β-hydroxysteroid dehydrogenase activity were pooled and concentrated to a protein concentration of 5 mg/ml by ultrafiltration with a PM-10 membrane.

Step 4

The concentrate was applied to 1.5 × 30 cm column of hydroxyl-apatite previously equilibrated with buffer A. Protein was eluted with a linear gradient of phosphate from 0 to 50 mmol/l in the equilibrating buffer at a flow rate of 40 ml/h. Fractions (3 ml) were collected and assayed for 17 β-hydroxysteroid dehydrogenase activity.

Step 5

Isoelectric focusing was conducted in an LKB 8101 electrofocusing column of 110 ml capacity using the procedure described in the manufacturer's instruction manual. Carrier ampholytes (pH 4 to 6) were used at a concentration of 3%. A stabilizing gradient of glycerol (from 5% to 60%) containing 5 mmol/l mercaptoethanol was formed stepwise. Phosphoric acid (1% in 70% glycerol) and 0.2% ethylenediamine were used as the anode and cathode solution, respectively. Approximately 15 mg of the concentrated enzyme solution after step 4 were added to the column when the gradient was one-third formed. Electrofocusing was carried out for 36 h at 4 °C, during which time the voltage was adjusted to maintain a total load of 3 watts. The column was drained by gravity and 1.25-ml fractions were collected. Enzyme activity coincided with the major protein peak at pH 5.0. The enzymatically active fractions were pooled, concentrated by ultrafiltration, and dialyzed against buffer A containing 50% glycerol. The enzyme was stored at −20 °C.

Electrophoretic procedures in gels

Isoelectric focusing was performed for 4 h at 20 °C in 7.5% photopolymerized acrylamide gels, with or without 8 mol/l urea, containing 20% glycerol and 3% ampholytes, pH 4 to 6. The sample was applied to the top of the gel, and overlaid with 50 µl of a 1% ampholyte solution, pH 4 to 6, 5% sucrose by weight. The anode electrolyte was 0.02% orthophosphoric acid and the cathode electrolyte was 0.02% ethanola-

mine. Proteins were stained according to the method of Vesterberg [11]. 17 β-hydroxysteroid dehydrogenase activity was detected by incubating the gels in the dark at room temperature with a solution containing 5 μmol of estradiol-17 β, 15 μmol of NADP, 3.1 μmol of Nitro Blue tetrazolium, 0.8 μmol of phenazine methosulfate, 0.5 ml of ethanol in a total volume of 10 ml 0.1 mol/l glycine/NaOH buffer, pH 9.4 according to the method of Schulz *et al.* [12]. Gels stained for either protein or enzyme activity were scanned at either 500 or 650 nm with a Gilford Spectrophotometer 250.

Analytical polyacrylamide disc gel electrophoresis was performed according to the method of Davis [13] (acrylamide monomer concentration, 7.5%, containing 8 M urea, Tris-glycine buffer system at pH 8.3, containing 7 nmol/l dithiothreitol). Enzyme (50 μg) was incubated in 100 μl of 8 mol/l urea containing 5 mmol/l dithiothreitol for 20 min at room temperature, applied to the tops of the gels, and overlaid with sucrose solution (5% by weight). Electrophoresis was conducted at 3 mA/tube until the tracking dye (Bromophenol Blue) was approximately 0.5 cm from the end of the gel. Proteins were detected by staining with 0.02% Coomassie Brilliant Blue in 12.5% trichloroacetic acid.

Enzyme assay

During the purification procedures, enzyme activities were calculated from the formation of [^{14}C]estrone from [^{14}C]estradiol. 0.1 μCi [^{14}C]estradiol (adjusted with unlabelled estradiol to a final concentration of 100 μmol/l) was incubated for 15 min at 37 °C in 10 mmol/l Tris/HCl-buffer, pH 7.4, containing 0.1 ml propylene glycol, 500 μmol/l coenzyme, 20% glycerol and enzyme solution in a total volume of 4.1 ml. After incubation, samples were brought into ice-water and extracted three times with 5 ml ether-chloroform (3 : 1, v/v). The pooled extracts were evaporated under nitrogen and dissolved in 0.5 ml of benzene. An aliquot (50 μl) was removed for liquid scintillation counting in order to estimate the total amount of radioactive steroids present in the extract. The benzene extracts were dried down under nitrogen and the dry residues were transferred with 0.2 ml chloroform/methanol (1 : 1, v/v) to thin-layer plates (0.25 mm with fluorescence indicator, Woelm, Eschwege, Germany) in the system benzene-methanol (19 : 1, v/v). Radioactive steroids were located by fluorescence absorption of unlabelled standards chromatographed simultaneously. Radioactivity of separated steroids was quantitated with a radiochromatogram scanner (Berthold, Wildbad, Germany) equipped with a 2 π methane gas counter and count integrator. Counting efficiency for ^{14}C was approximately 18%. The percentage of radioactivity in each sample was calculated from the initial radioactivity of [^{14}C]estradiol.

Protein determination

Protein was determined by the method of Lowry *et al.* [14] using bovine serum albumin as the standard.

Carbohydrate analysis

The enzyme preparation was assayed for neutral and amino sugars by the method of Spiro [15].

Results

Table I shows the 17 β-hydroxysteroid dehydrogenase activity of the cytosol fraction of pregnant rabbit uterus toward 17 β-estradiol, estrone, testosterone and androstenedione. The activity toward 17 β-estradiol was 4.6 times that observed with testosterone; androstenedione was reduced at 6.8 times the rate of estrone of the substrates listed in Table I; estrone was converted most rapidly.

Table I. 17β-Hydroxysteroid dehydrogenase activity of pregnant rabbit uterus 105,000 × *g* supernatant; the coenzyme was supplied by a NADP or NADPH-generating system.

Substrate	17β-Hydroxysteroid dehydrogenase [μunits/mg protein]
[4-^{14}C]Estradiol-17β	218 ± 13
[4-^{14}C]Estrone	428 ± 17
[4-^{14}C]Testosterone	47 ± 3
[4-^{14}C]Androstenedione	63 ± 5

Purification of the soluble 17 β-hydroxysteroid dehydrogenase of pregnant rabbit uterus

A summary of a representative purification is shown in Table II. A 378-fold purification with an overall yield of about 48% was usually obtained. The specific activity of the final preparation was 78 μunits/mg protein. This procedure yields 1.5 to 2 mg of

Table II. Purification of 17β-hydroxysteroid dehydrogenase of pregnant rabbit uterus.

Fraction	Total activity [nmol E_1/15 min]	Recovery over-all [%]	Specific activity [nmol E_1/ 15 min/mg protein]	Purification over-all (-fold)
Cytosol	3,842	100	3.1	–
Ammonium sulfate precipitation 0.3–0.5 saturation	3,150	82	5.58	1.8
Sephadex G-200	2,958	77	17.7	5.7
DEAE-cellulose	2,805	73	56.7	18.3
Hydroxylapatite chromatography	2,459	64	160.6	51.8
Isoelectric focusing (pH 4.8–5.0)	1,844	48	1,172	378

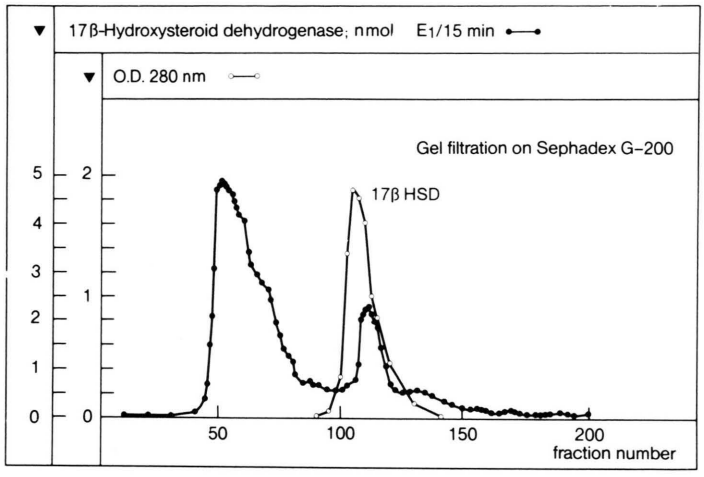


Fig. 1. Gel filtration on Sephadex G-200 of preparation containing 17β-hydroxysteroid dehydrogenase, obtained after ammonium sulfate precipitation of cytosol fraction of pregnant rabbit uterus. Gel filtration was performed as described in the "Experimental procedure" section.

pure protein from 120 g wet uteri in 7 to 9 days working time and may be scaled up. Storage in buffered 50% glycerol stabilizes the enzyme; the activity remains constant for at least 3 months at –20 °C.

Ammonium sulfate precipitation (30% to 50% saturation) resulted in a 1.8-fold purification of the 17β-hydroxysteroid dehydrogenase. Gel filtration of this fraction on Sephadex G-200 gave the elution pattern shown in Fig. 1. The highest specific activity was found in the effluent of fraction 105. The effluents from fractions 100 to 125 were pooled. The preparation contained 197 munits as total activity (almost 94% yield from the previous step) and 1.2 μunits/mg protein as specific activity. Chromatography on Sephadex G-200 resulted in 5.7-fold purification from the previous step.

The concentrated enzyme solution obtained after gel filtration was applied to a DEAE-column. Elution was begun with a linear phosphate gradient between 0 and 200 mmol/l phosphate. The distribution of protein and enzyme activity is shown in Fig. 2. The bulk of the enzyme was eluted at phosphate concentrations of approximately 80 and 110 mmol/l. This step resulted in a 18.3-fold purification and an essentially quantitative recovery from the previous step.

The enzyme recovered from DEAE-cellulose chromatography was applied to a hydroxylapatite column. After application of the sample, elution was begun with a linear phosphate gradient between 0 and 50 mmol/l. The results are shown in Fig. 3. The bulk of the enzyme was eluted at a phosphate concentration between 28 and 35 mmol/l, resulting in an overall 51. 8-fold purification and an 88% recovery from the previous step. – The 17β-hydroxysteroid dehydrogenase obtained by hydroxylapatite chromatography was further purified by isoelectric focusing in a linear glycerol gradient. The results are

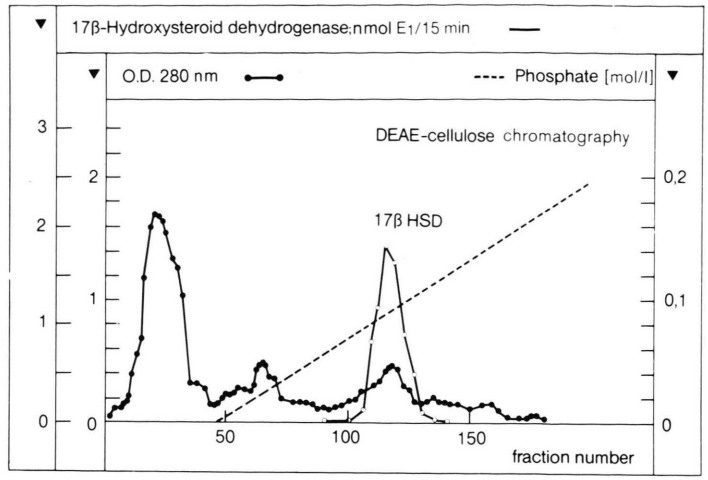


Fig. 2. DEAE-cellulose chromatography of the 17 β-hydroxysteroid dehydrogenase. The pooled eluate of Sephadex G-200 chromatography was added to the column and chromatographed with a phosphate linear gradient. For detail see under "Experimental procedure".

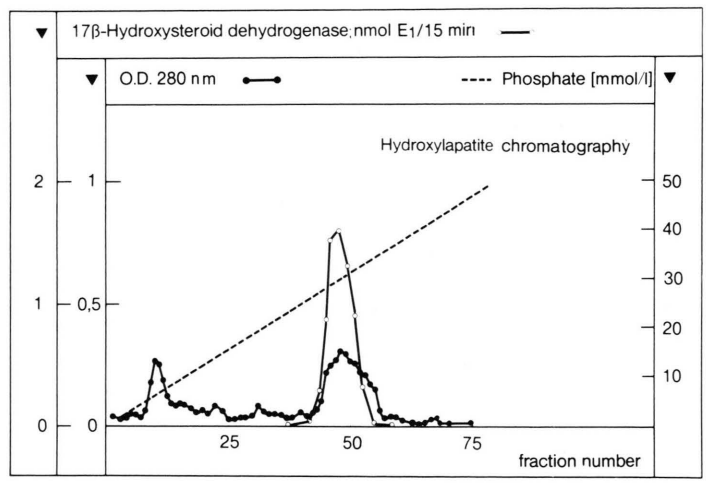


Fig. 3. Hydroxylapatite chromatography of pooled enzyme active fractions from Fig. 2. For detail see under "Experimental procedure".

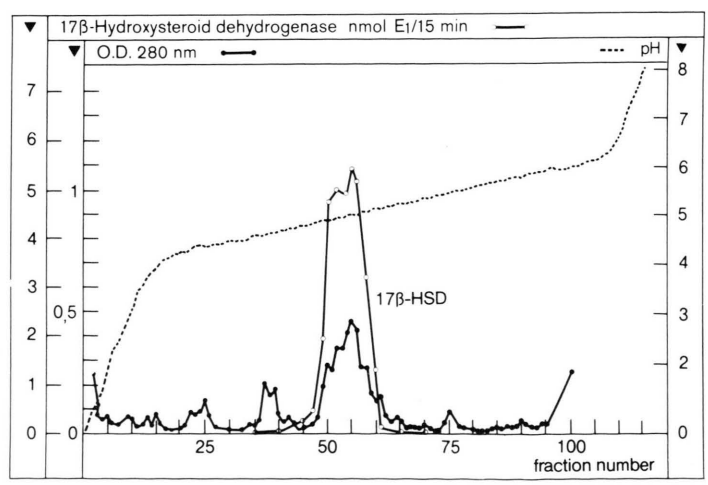
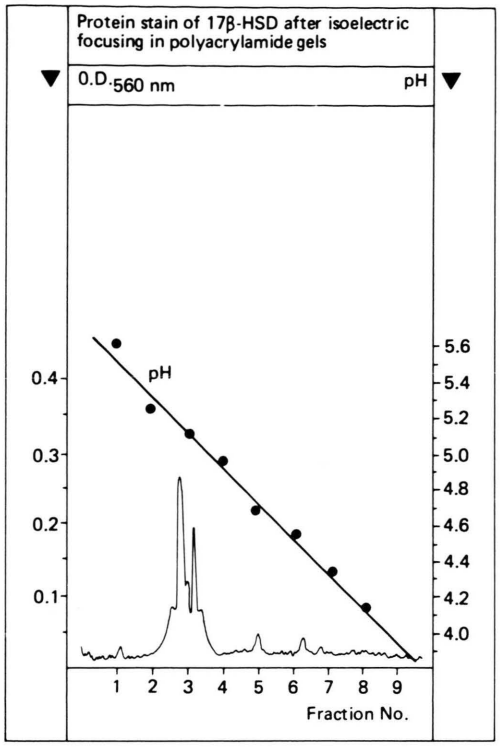


Fig. 4. Isoelectric focusing of 17 β-hydroxysteroid dehydrogenase in a pH 4 to 6 gradient. Electrofocusing was carried out as described in the text. Fractions were tested for pH, protein content and enzyme activity.



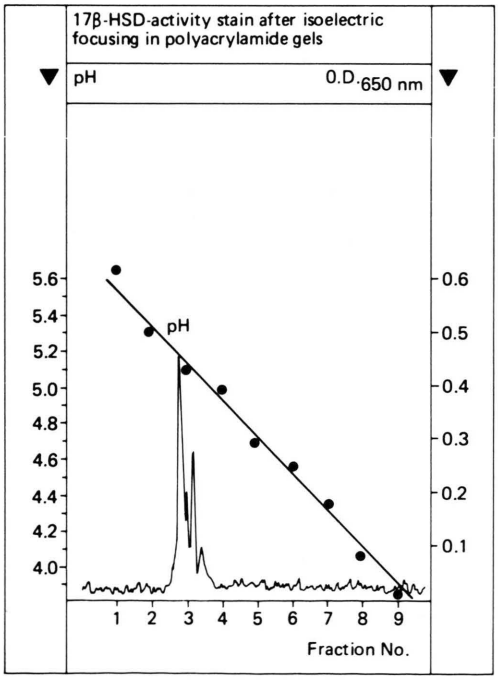


Fig. 5. Isoelectric focusing of purified native 17 β-hydroxysteroid dehydrogenase in polyacrylamide gel containing 20% glycerol. For detail see under "Experimental procedure".

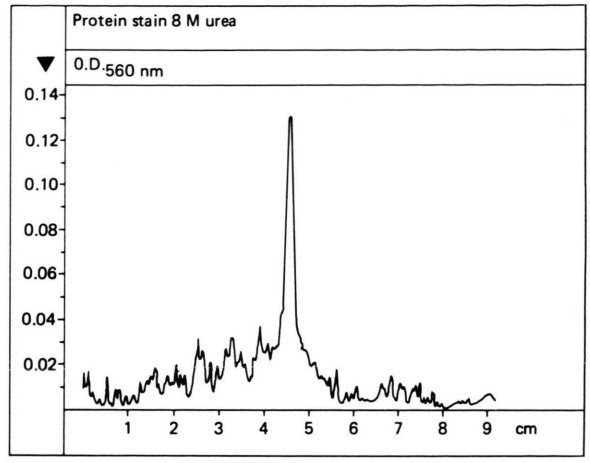


Fig. 6. Polyacrylamide disc gel electrophoresis of purified 17 β-hydroxysteroid dehydrogenase in 8 mol/l urea. For detail see under "Experimental procedure".

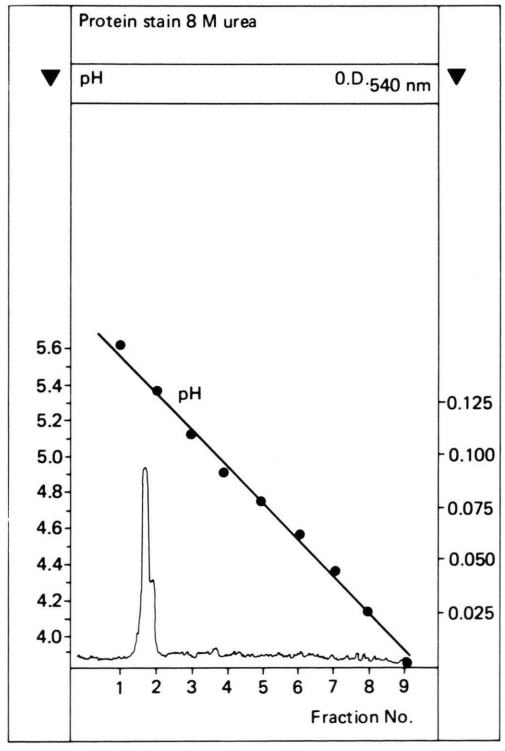


Fig. 7. Isoelectric focusing of purified 17 β-hydroxysteroid dehydrogenase in 8 mol/l urea. For detail see under "Experimental procedure".

shown in Fig. 4. Two distinct main peaks of enzymatic activity were focused around pH 5. The overall purification achieved in these peaks was 378-fold (75% yield of the previous step). – Preparative isoelectric focusing of the 17 β-hydroxysteroid dehydrogenase indicated a strong heterogeneity of this enzyme. In order to explore further the subunit structure of the enzyme, the pooled and concentrated enzyme preparation obtained after preparative isoelectric focusing was analyzed by isoelectric focusing in polyacrylamide gel over a small pH-range, a technique that has very high resolving power. Isoelectric focusing (pH 4 to 6) of the native enzyme in polyacrylamide gels containing 20% glycerol yielded a pattern of two prominent and three faint bands upon staining for protein (Figure 5 a). This pattern was congruent with that obtained by staining for 17 β-hydroxysteroid dehydrogenase activity (Figure 5 b). Analytical polyacrylamide disc gel electrophoresis in the presence of 8 mol/l urea gave only one band (no enzymatic activity remained), as shown in Figure 6.

Enzyme preparation subjected to electrophoresis in 10% polyacrylamide gels containing 0.1% sodium dodecyl sulfate gave a single band corresponding to molecular weight 31,000 ± 500, as judged by comparison with proteins of molecular weight (not shown).

Isoelectric focusing between pH 4 and 6 on polyacrylamide gels in the presence of 8 mol/l urea gave two protein components (Fig. 7).

The observed heterogeneity did not appear to arise from procedural artifacts because (1) after removal of each band as a gel slice and electrophoresis under the same conditions, each component gave a single band that stained for protein and had the same relative mobility as that of the parent band in the first electrophoresis, (2) carbohydrates could not be detected in the highly purified enzyme preparation, (3) interactions of the proteins with the ampholytes could be excluded, (4) heterogeneity resulting from proteolysis during the purification procedure was unlikely since freshly prepared cytosol on gel electrophoresis gave the same multiple banding pattern of enzyme activity as the purified enzyme.

The plot of the logarithm of molecular weight against fraction number after gel filtration on Sephadex G-200 of proteins of known molecular weight relative to data for the crude 17 β-hydroxysteroid dehydrogenase activity is shown in Fig. 8. A value of 64,000 was obtained for the molecular weight.

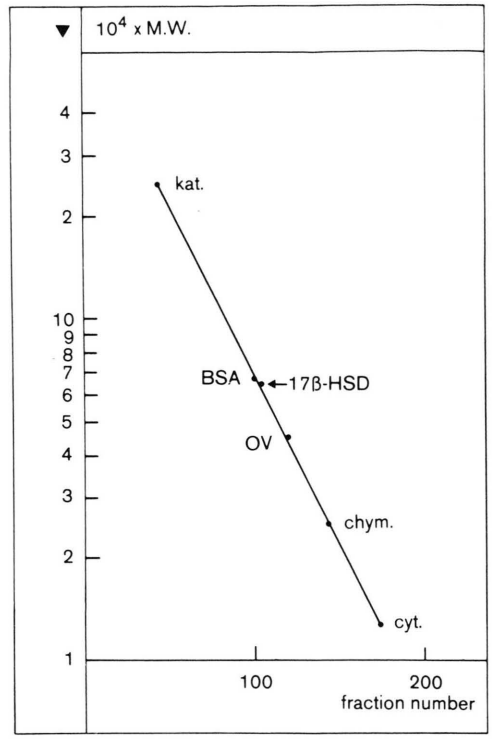


Fig. 8. Estimate of mol. wt. of the partially purified 17 β-hydroxysteroid dehydrogenase (after ammonium sulfate precipitation) by gel chromatography on Sephadex G-200. Marker proteins were cytochrome c (cyt.), chymotrypsinogen A (chym.), ovalbumin (OV), bovine serum albumin (BSA) and catalase (cat.).

Kinetic analysis

In order to establish the optimal incubation time for subsequent studies, a rate study of estradiol oxidation was performed measuring the formation of [^{14}C]estrone from [^{14}C]estradiol incubated with crude cytoplasmic fraction. As shown in Fig. 9, the rate of estradiol oxidation is relatively constant at 37 °C up to 15 min. The rate of estrone formation by soluble 17 β-hydroxysteroid dehydrogenase was determined at incubation temperatures between 15 °C and 60 °C. The reaction rate increased exponentially with increasing temperature from 15 °C to 43 °C, and drastically decreased at temperatures exceeding 43 °C. The maximal rate was achieved at 43 °C.

Activity of the enzyme at different pH-values is plotted in Fig. 10. The pH-optimum appears for estradiol between 9.5 and 10.5, and for estrone between 5.5 and 6.5. The pH-optima depended upon the kind of buffer used. The effect of increasing con-

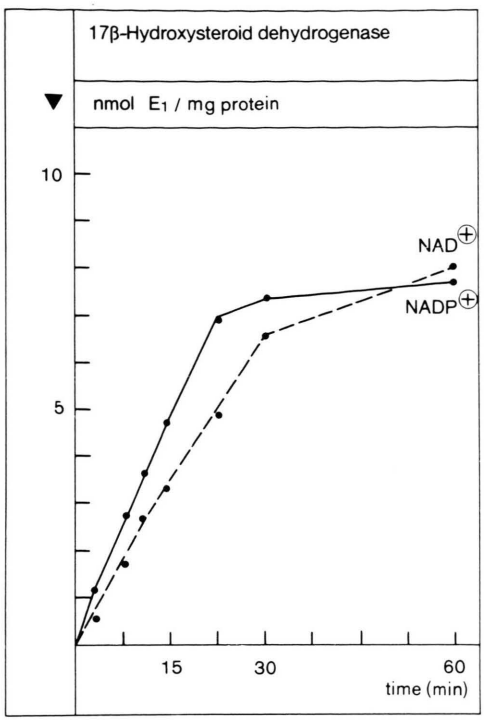


Fig. 9. Dynamic studies on the production of [^{14}C]estrone from [^{14}C]estradiol by cytosol fraction of pregnant rabbit uterus.

centrations of the sulfhydryl reagents *p*-chloromercuribenzoate or iodoacetamide on the activity of 17 β-hydroxysteroid dehydrogenase was examined.

At a concentration of 0.1 mmol/l, *p*-chloromercuribenzoate caused 78% to 92% inhibition of the dehydrogenase activity. When iodoacetamide was used as the sulfhydryl reagent, a 40% to 60% inhibition was obtained. When the soluble enzyme, pre-incubated in 1.0 mmol/l *p*-chloromercuribenzoate, was exposed to the sulfhydryl-protecting reagents, dithiothreitol, cysteine or glutathione at concentrations of 10 mmol/l prior to the addition of $NADP^+$ and [^{14}C[estradiol, the inhibition of the dehydrogenase due to *p*-chloromercuribenzoate could not be reversed.

$CuSO_4$, $ZnCl_2$ and $MgCl_2$ inhibited the activity of the 17 β-hydroxysteroid dehydrogenase to various degrees. The most effective heavy metal was Mg^{2+}; addition of $CuSO_4$ and $ZnCl_2$ during pre-incubation resulted in a similar inhibition of the enzyme activity (Fig. 11).

For further characterization, Michaelis-Menten constants (K_m values) for the conversion of estradiol into estrone were estimated. A double reciprocal plot of concentration of estradiol and reaction velocity allowed the K_m value to be calculated at 4.16×10^{-5} mol/l for the NAD-linked enzyme activity and 4.37×10^{-5} mol/l when NADP as cofactor was used (Fig. 12 a).

The V for estradiol was determined as 1.81 nmol × min^{-1} × mg protein^{-1} (with NADP as cofactor) and 1.28 nmol × min^{-1} × mg protein^{-1} (with NAD as cofactor). The ratio of the maximal velocity for NADP to that for NAD was 1.42. The effect of NAD and NADP concentrations on the rate of estradiol oxidation (estradiol was kept constant at 10 μmol/l) is presented in Fig. 12 b. Duplicate determinations gave similar K_m values of 3.57×10^{-4} mol/l for NAD and 3.7×10^{-4} mol/l for NADP. The V for

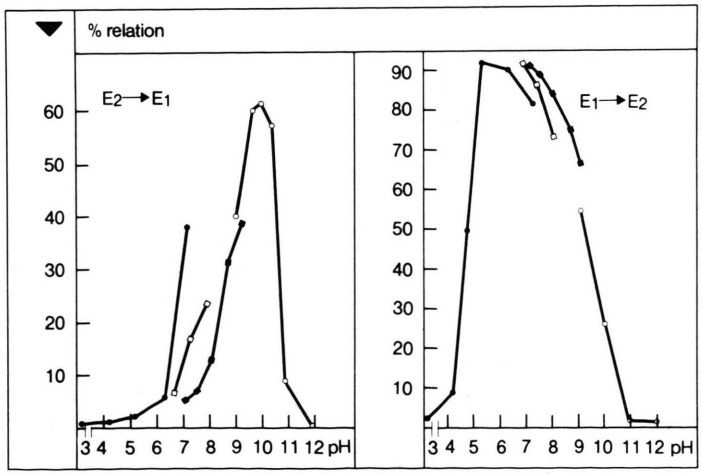
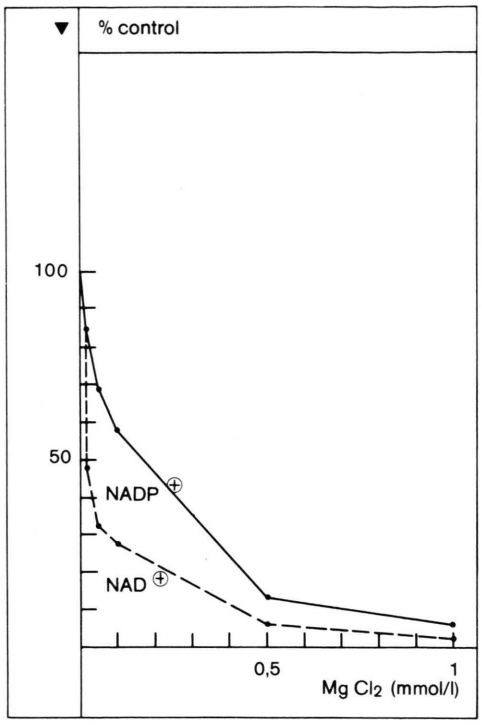


Fig. 10. The pH-dependence of the 17 β-hydroxysteroid dehydrogenase activity and the influence of buffer solution on the hydrogenation of estrone in cytosol fraction of pregnant rabbit uterus. ●—● 0.2 mol/l Na_2HPO_4/citrate; □—□ 0.15 mol/l phosphate buffer; ■—■ 0.2 mol/l Tris/HCl; ○—○ 0.2 mol/l sodium-borate/NaOH.



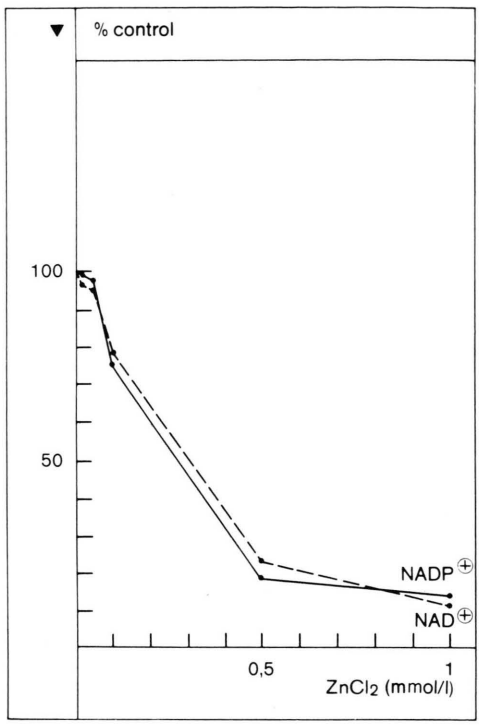
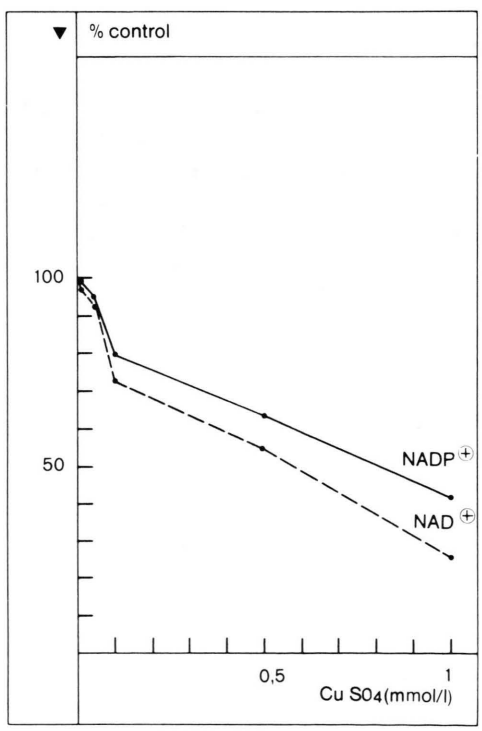


Fig. 11. Effect of increasing concentrations of heavy metals on 17 β-hydroxysteroid dehydrogenase.



NADP and NAD were determined to be 0.67 nmol × min^{-1} × mg $protein^{-1}$ and 0.22 nmol × min^{-1} × mg $protein^{-1}$, respectively.

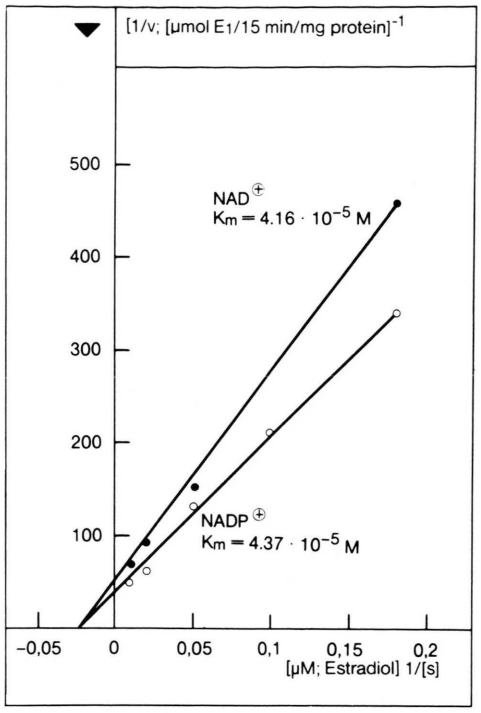
A repeat experiment for the determination of K_m of estradiol (cofactor NADP), but in the presence of 500 μmol/l of various steroid hormones, indicated that steroid hormones used were extremely weak competitive inhibitors.

Inhibitor constants (K_i) were 5.67×10^{-3}, 3.55×10^{-3}, 4.0×10^{-3}, 3.76×10^{-3} and 3.35×10^{-3} mol/l for estradiol, estrone, testosterone, androstenedione and progesterone, respectively.

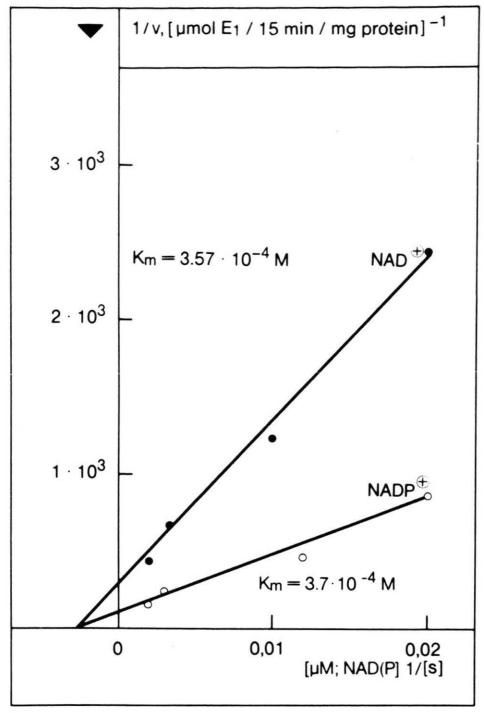
Discussion

The presence of an enzyme in rabbit myometrium which catalyzes the interconversion of estradiol to estrone has been known since 1967 from the work of Jütting *et al.* [1, 2].

This paper describes the partial purification and some properties of NAD(P)-dependent 17 β-hydroxysteroid dehydrogenase from pregnant rabbit uterus.



a



b

Fig. 12 a/b. a) Determination of Michaelis-Menten constants and maximal velocity for estradiol. Increasing concentrations of [4-^{14}C]estradiol were incubated with 17 β-hydroxysteroid dehydrogenase preparation (178 µg cytosol protein per 4.1 ml) in the presence of a constant concentration NAD or NADP (500 µmol/l) for 10 min. b) Determination of Michaelis-Menten constants and maximal velocity for NAD and NADP. [4-^{14}C]estradiol (100 µmol/l) was incubated with 178 µg cytosol protein in the presence of various amounts of the cofactor.

A procedure was developed for the purification of the 17 β-hydroxysteroid dehydrogenase by means of which large amounts of the highly purified enzyme may be prepared. The enzyme preparation seemed to be pure by the following criteria:

(1) after analytical isoelectric focusing of native enzyme all detectable proteins possessed the expected enzymatic activities;

(2) polyacrylamide gel electrophoresis in the presence of 8 mol/l urea produced a single band.

The purification was achieved in four steps with an over-all recovery and a specific activity of enzyme obtained by this procedure higher than the values obtained by the procedure of Jütting *et al.* [1, 2], using gel filtration. The present procedure yields a 378-fold purification from the 105,000 × *g* supernatant and is reproducible. The isoelectric focusing experiments indicate that the purified 17 β-hydroxysteroid dehydrogenase, like many other dehydrogenases, is composed of multiple forms. This heterogeneity could be caused by the combination of different polypeptides, or by polypeptides with a single amino acid sequence, but differentially modified, for example by covalently bound carbohydrate. The absence of carbohydrate in purified enzyme preparation rules out the possibility of carbamylation as the source of heterogeneity.

The existence of five enzyme stain bands after isoelectric focusing of the native enzyme in polyacrylamide gel is consistent with the observation of two different monomers present in unequal amounts after analytical isoelectric focusing in the presence of 8 mol/l urea. The monomers can interact to form five higher molecular forms (tetramers?). The observation that the two monomers have similar molecular weights but different isoelectric points implies that each monomer arises from a distinct genetic locus and that the multiple electrophoretic forms of

the native enzyme observed by analytical isoelectric focusing in polyacrylamide gels are probable isoenzymes. This result is similar to that reported by Engel and co-workers [7, 8] for the soluble 17 β-estradiol dehydrogenase of human term placenta and to those previously described for 17 β-hydroxysteroid dehydrogenase of human endometrium [16], 17 β-hydroxy-C_{19}-steroid dehydrogenase of the guinea pig kidney and 3(17)β-hydroxysteroid dehydrogenase of *Pseudomonas testosteroni* [17–20].

A molecular weight of 64,000 was obtained for the ammonium sulfate precipitated enzyme by gel filtration technique, but it should be emphasized that calculation of molecular weights by the use of gel filtration is only an approximation, since the calculation is based on the assumption that the molecular shape of the proteins in question is very similar to those of the proteins used as standards. The molecular weight of the 17 β-hydroxysteroid dehydrogenase of rabbit uterus is the same magnitude as that found by Hagerman [21], Jarabak and Sack [22], and Burns *et al.* [23] for the human placental estradiol dehydrogenase, using several different methods.

Some of our kinetic parameters are different from those found previously by Jütting *et al.* [1, 2]. They found that the most reactive substrate for this enzyme was the C_{18}-steroid estradiol-17 β, which has an aromatic A ring; non-aromatic steroids had much lower reaction rates (testosterone < 1%) than estradiol. The results of our study with both NAD- and NADP-linked activities suggest that there is no absolute requirement for an aromatic ring A. Jütting *et al.* [1, 2] found that the enzyme required NAD or NADP as cofactor, but NADP gave a 20% lower activity than an equimolar amount of NAD. However, our findings demonstrate that the ratio of the maximal velocity for NADP to that for NAD was 1.42.

The K_m value for estradiol estimated in the present experiments was approximately ten times higher than that described by Jütting *et al.* [1, 2] but was on the same order of magnitude as those of the 17 β-hydroxysteroid dehydrogenase of erythrocytes and porcine testes [24, 25].

Whether the discrepancies in the reported values (substrate and coenzyme specificity, K_m values) of rabbit uterine cytosol 17 β-hydroxysteroid dehydrogenase result from the presence of other 17 β-hydroxysteroid dehydrogenases in crude enzyme preparation (Jütting *et al.* used the 11,000 × *g* supernatant, not the cytosol fraction, for enzyme preparation), variations of the kinetic parameters due to differences in the conditions of the assays, or other, as yet undiscovered factors, cannot be determined at the present time.

The 17 β-hydroxysteroid dehydrogenase of rabbit uterus is probably of the sulfhydryl type. Addition of typical SH-blocking agents such as p-chloromercuribenzoate and iodoacetamide or metal ions such as $MgCl_2$, $ZnCl_2$ and $CuSO_4$ to the incubation mixture resulted in various degrees of inhibition of 17 β-hydroxysteroid dehydrogenase activity. Dithiothreitol, cysteine or glutathione were unable to reverse the inhibition of the enzyme activity. Similar observations were made by Talalay and Dobson [26] using purified 17 β-hydroxysteroid dehydrogenase of *Pseudomonas testosteroni.* In contrast to the uterine dehydrogenase, the placental enzyme has been reported to be activated by zinc.

The biological significance of the presence of the 17 β-hydroxysteroid dehydrogenase in rabbit uterus remains open to question. We can only speculate about the physiological relevance of 17 β-hydroxysteroid dehydrogenase activity in the cells of target organs for estradiol.

The early steps in estradiol action involve the binding of the steroid hormone to a specific cytoplasmic receptor protein, followed by an alteration in the properties of the steroid receptor complex, which leads to its association with the nucleus.

It is therefore possible that a high enzyme activity leads to a drastic reduction of the actual intracellular estradiol concentration, thus reducing the "sensitivity" of the target organ for estradiol.

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