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PARTIAL PURIFICATION AND EVIDENCE FOR HETEROGENEITY OF THE CYTOPLASMIC 17β-HYDROXYSTEROID DEHYDROGENASE (17β-HSD) FROM NORMAL HUMAN ENDOMETRIUM AND ENDOMETRIAL CARCINOMA

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SUMMARY

This study was designed primarily to purify the 17β-HSD from human endometrium.

The cytoplasmic 17β-HSD of secretory endometrium was partially purified by ammonium-sulphate precipitation, gel filtration, ion-exchange chromatography and isoelectric focusing. During purification procedures 17β-HSD activity was determined by a radiochemical method.

Activity patterns of the enzyme(s) from normal and neoplastic endometrium were obtained by isoelectric focusing in polyacrylamide gels and subsequent staining of the gels for enzyme activity. Estradiol (E_2) and testosterone (T) served as substrates, NAD and NADP as cosubstrates. All solutions and gels contained glycerol.

A 40-fold purification of the 17β-HSD from secretory endometrium was achieved. The overall recovery was 10%.

Isoelectric focusing in sucrose of the 17β-HSD from secretory endometrium yielded three enzymatically active bands with both E_2 and T as substrates (focused around pH 5). In contrast, the activity patterns from endometrial carcinoma gave 2, 4 or 7 bands depending on the substrate and the cofactor.

These findings may indicate that in normal and neoplastic endometrium the 17β-HSD exists in different molecular forms.

INTRODUCTION

17β-Hydroxysteroid dehydrogenases (17β-HSD) from different species have been shown, by electrophoretic techniques, to exist in several enzymatically active forms. Kochakian *et al.*[1–3] reported that disc gel electrophoresis of kidney cytosol from guinea pigs gave five bands which possessed both NAD- and NADP-linked 17β-HSD activities. Isoelectric focusing of the soluble 17β-HSD from human placenta in polyacrylamide gels resulted in five enzymatically active bands [4] indicating heterogeneity of this enzyme.

This paper reports the heterogeneity of the soluble (i.e. cytoplasmic) 17β-HSD from normal human endometrium and endometrial carcinoma and it describes the partial purification of the enzyme from secretory endometrium.

MATERIALS AND METHODS

Materials. [4-^{14}C]-Estradiol (58 mCi/mmol) was purchased from The Radiochemical Centre (Amersham, England). Unlabelled steroids were a gift from Schering AG (Berlin, Germany). NAD and NADP were purchased from Boehringer (Mannheim, Germany). Carrier ampholytes (Ampholine, pH 3–10) came from LKB Instruments (Stockholm, Sweden). Sephadex G-200 and DEAE-Sephadex A-50 were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Phenazine methosulphate, acrylamide and methylenebisacrylamide were from Serva (Heidelberg, Germany). Organic solvents and other chemicals (all analytical grade) were products of E. Merck (Darmstadt, Germany).

Tissues. Human secretory endometrium was obtained by curettage and endometrial carcinoma were excised from uteri removed at total hysterectomy. All tissues used were subjected to histological analysis.

Enzyme purification. All of the steps were performed at 4°C. Within 60 min after surgery the tissues were suspended in 5 vol. (w/v) of TEM-glycerol buffer* containing 0·25 M sucrose and homogenized by 5 up-down transits with a glass-in-Teflon Potter–Elvehjem homogenizer (B. Braun, Melsungen, Germany) at 1000 rev./min for 30 s. The cytosol was obtained by centrifugation at 105,000 *g* for 90 min and the enzyme precipitated from the cytosol at 35–50% saturation of ammonium sulphate (first step of purification).

Sephadex G-200 filtration. The ammonium-sulphate precipitate was dissolved in TEM-glycerol buffer and

* TEM-glycerol buffer: 10 mM Tris–HCl, 1 mM EDTA, 12 mM mercaptoethanol, pH 7·0, 20% glycerol, v/v.

applied to a column of Sephadex G-200 (1·5 × 90 cm.) previously equilibrated with the same buffer. Elution was carried out at 25 ml/h. Samples (0·5 ml) of each fraction (3 ml) were assayed for 17β-HSD activity and protein content (second step of purification).

The column was calibrated with the following standards (molecular weights in parentheses): cytochrome c (12,400), chymotrypsinogen A (25,000), ovalbumin (45,000), BSA (67,000), aldolase (158,000), catalase (240,000) all from Boehringer, Mannheim, Germany, and "Blue Dextran" (Pharmacia, Sweden). A plot of the elution volumes of the standards against the logarithms of their molecular weights was used to estimate the molecular weight of the soluble 17β-HSD from human secretory endometrium.

Ion-exchange chromatography. Fractions with major 17β-HSD activity were collected from the previous step and the enzyme was precipitated with ammonium sulphate. The precipitate was dissolved in 0·005 M potassium phosphate buffer (pH 7·0) containing 12 mM mercaptoethanol, 1 mM EDTA and 20% (v/v) glycerol, and dialysed against the same solution. The DEAE-Sephadex column was equilibrated with the same buffer and 5 ml of enzyme solution (98 mg protein) were applied to a 1·5 × 30 cm. column of DEAE-Sephadex. Elution was carried out with 150 ml of the equilibration buffer containing a linear phosphate gradient (0·005–0·2 M). Fractions (1·5 ml) were collected at a flow rate of 60 ml/h. Samples (0·5 ml) of each fraction were assayed for 17β-HSD activity and protein content. The conductivity of the fractions was monitored with a LKB-conductivity-meter (third step of purification).

Isoelectric focusing. The fractions containing most of the enzyme activity from the third step were pooled, dialysed against TEM-glycerol buffer and concentrated. The separation of 17β-HSD activities by isoelectric focusing (LKB column, 110 ml) in a linear sucrose gradient containing 20% (v/v) glycerol was performed by the method of Vesterberg and Svensson [5]. A final potential of 800 V was applied for 36 h at 4°C to the column containing 28 mg protein and 1% carrier ampholytes (pH 3–10). Fractions of 3·0 ml were collected and measured for 17β-HSD activity, protein content and pH (fourth step of purification).

Gel isoelectric focusing. Isoelectric focusing in polyacrylamide gels was modified from the methods of Wrigley [6] and Finlayson and Chrambach [7] and carried out in a conventional Shandon analytic disc electrophoresis chamber. The run was conducted at 20°C and started by constant current at 1·0 mA/tube until a voltage of 200 V was reached, at which point the mode was altered to constant voltage. Focusing was continued for 3 h during which the current passively fell to 0·2 mA/tube. Higher voltage or longer focusing produced migration of the pH gradient to the cathode. The gels containing 5% acrylamide, 0·2% bisacrylamide, 20% (v/v) glycerol and 1% ampholine (pH 3–10). Polymerization was catalyzed by riboflavin. Portions of 0·5 ml cytosol were placed on top of the polyacrylamide gels and overlayered with 1% ampholine solution. The solution at the anode was 0·02% orthophosphoric acid and the solution at the cathode was 0·02% ethanolamine. Gels were stained for enzyme activity at 37°C for 2 h in the dark in a solution of 5 ml H_2O, 2 ml 0·1 M glycine/NaOH buffer (pH 9·4), 0·5 ml NAD(P) (5 mg/ml), 0·25 ml phenazine methosulfate (0·25 mg/ml), 1·5 ml nitro blue tetrazolium (1·7 mg/ml) and 0·4 ml steroid (2·7 mg estradiol or testosterone/ml ethanol).

Assays of 17β-HSD activity and protein content. During 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 10 μM) was incubated for 30 min at 37°C in a total vol. of 4·1 ml Tris–HCl (pH 7·4) containing 0·1 ml propylene glycol, enzyme and 500 μM coenzyme. Under these conditions, a linear relationship was obtained between the amount of estrone formed and the concentration of enzyme [14]. After incubation, samples were brought into ice–water and extracted three times with 5 ml of ether–chloroform (3:1 v/v). The extracts were chromatographed on silica gel 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 radio-chromatogram scanner (Berthold, Wildbad, Germany) equipped with

Table 1. Scheme of purification of the cytoplasmic 17β-HSD of human secretory endometrium

Treatment	Total protein (mg)	Total activity (nmol/min)	S.A. (pmol/min/mg protein)	Recovery over-all (%)	Purification over-all (n-fold)
Cytosol	1300	12·13	9·3	100	—
35–50% $(NH_4)_2SO_4$ precipitate	448	9·7	21·7	80	2·3
Sephadex G-200 effluent	77	3·1	40·3	25·6	4·3
DEAE-Sephadex effluent	33	2·3	69·7	18·9	7·5
Isoelectrofocusing pH 3·0–10					
Peak A	3·6	1·3	361·1	10·7	38·8
Peak B	2·1	0·48	228·6	4·0	24·6
Peak C	0·9	0·11	122·2	0·9	13·1

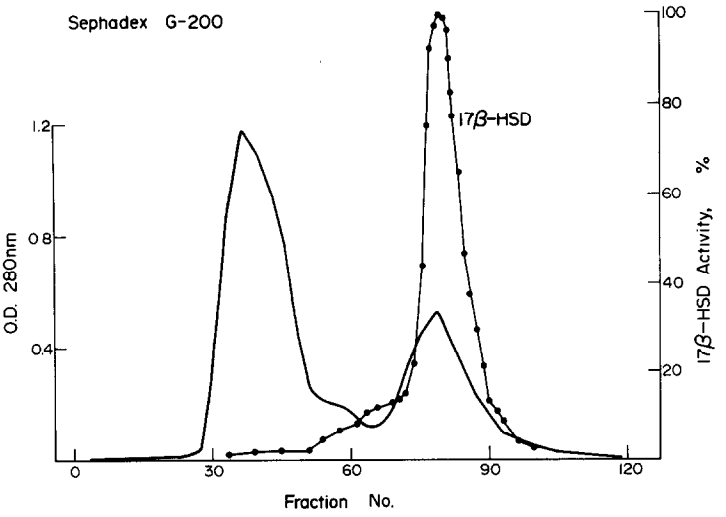

Fig. 1. Gel chromatography of an ammonium sulfate precipitate (35–50% saturation) from cytosol of secretory endometrium. The precipitate (dissolved in 3·5 ml of TEM-glycerol buffer*) was applied to a 1·5 × 90 cm. column of Sephadex G-200 and eluted with TEM-glycerol buffer. Fractions of 3 ml were assayed for 17β-HSD activity ●—● and protein content ——.

* TEM-glycerol buffer: 10 mM Tris–HCl, 1 mM EDTA, 12 mM mercaptoethanol, pH 7·0, 20% glycerol, v/v.

a 2 π methane gas counter and count integrator. Counting efficiency for ^{14}C was approximately 18%.

Protein concentrations were determined by the method of Lowry *et al.*[8] with BSA as standard.

RESULTS

Enzyme purification. The purification scheme of the endometrial 17β-HSD is presented in Table 1. Since secretory endometrium contains considerably more enzyme activity than proliferative endometrium [9–15] only cytosol from secretory endometrium was used. Most of the enzyme activity precipitated between 35 and 50% saturation of ammonium sulphate.

Passage of the redissolved 35–50% ammonium sulphate precipitate through a Sephadex G-200 column resulted in a 4·3-fold purification and a recovery of 26% (Table 1).

Figure 1 shows that most of the 17β-HSD activity was eluted from Sephadex G-200 in one peak (fractions no. 75–90). These fractions were applied to a DEAE-Sephadex column as described in the method section. The elution profile of protein and enzyme activity is shown in Fig. 2. The bulk of the 17β-HSD

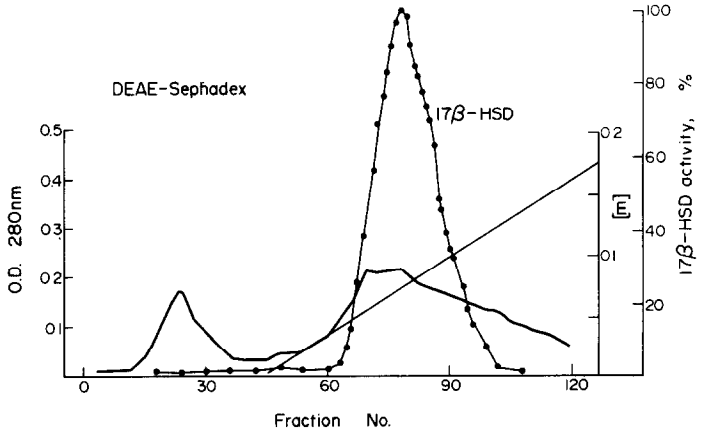

Fig. 2. Ion exchange chromatography of pooled fractions no. 75–90 obtained from Sephadex G-200 gel filtration. A column (1·5 × 30 cm.) of DEAE-Sephadex was equilibrated with 0·005 M potassium phosphate buffer (pH 7·0) containing 12 mM mercaptoethanol, 1 mM EDTA and 20% (v/v) glycerol. After application of samples the column was washed with the above buffer and eluted with a linear phosphate gradient (0·005–0·2 M) in the same buffer. Fractions no. 70–90 were collected for the next purification step.

Straight line: phosphate gradient.
●—● 17β-HSD activity (nmol/min/fraction)
—— protein at 280 nm.

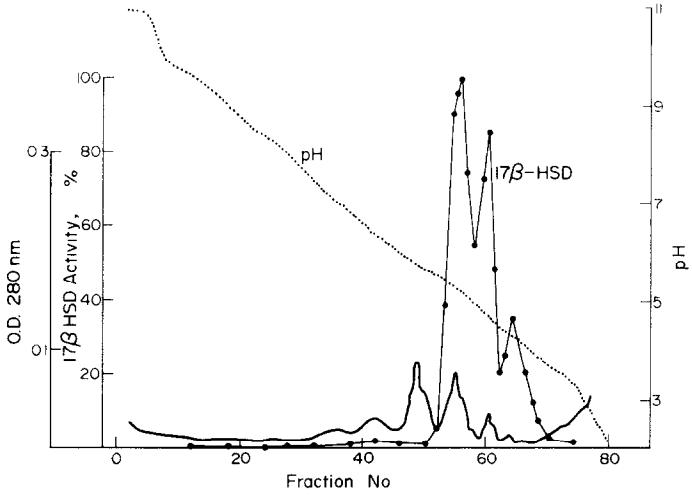

Fig. 3. Isoelectric focusing in a sucrose gradient (pH 3–10) of pooled fractions no. 70–90 from DEAE-Sephadex chromatography. Experimental details are given under "Materials and Methods".
●—● 17β-HSD activity (nmol/min/fraction)
—— O.D. at 280 nm (protein content)
····· pH gradient

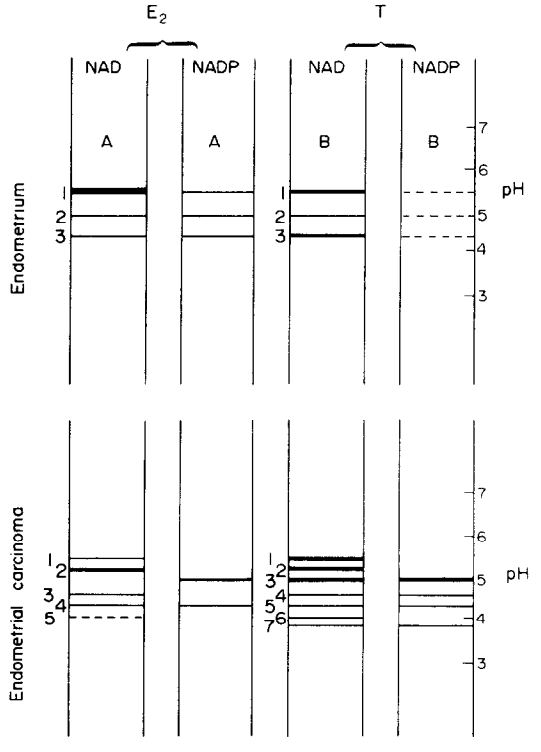

Fig. 4. Diagram of activity patterns of the soluble 17β-HSD from secretory (or proliferative)* endometrium and endometrial carcinoma as obtained by isoelectric focusing in acrylamide gels (pH 3–10). Staining for 17β-HSD activity was carried out as described under "Materials and Methods". Gels A were incubated with estradiol (E_2) and gels B with testosterone (T). Cofactors were NAD or NADP. Arabic numerals to the left of the "gels" serve to identify the densitometric peaks shown in Figs. 5 and 6.

* Secretory and proliferative endometrium yielded the same patterns, but bands from secretory endometrium were considerably stronger.

was eluted in a single peak at a phosphate concentration between 50 and 150 mM. This step resulted in an overall 7·5-fold purification and a total recovery of 19%. The enzyme recovered from ion-exchange chromatography was applied to an LKB column for isoelectric focusing in a sucrose gradient. Three enzymatically active peaks could be separated, focused around pH 5 (Fig. 3). The overall purification achieved in these peaks ranged from 13- to 39-fold (Table 1).

Isoelectric focusing in polyacrylamide gels. Figure 4 shows a diagram of the different activity patterns

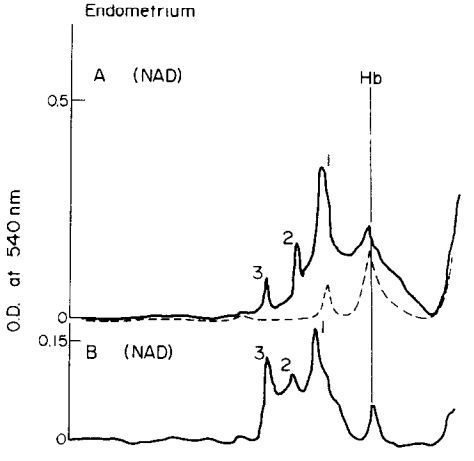

Fig. 5. Densitometric scans of two of the gels from the upper half of Fig. 4. Gels A and B (normal endometrium, cofactor NAD) were scanned at 540 nm with a Gilford spectrophotometer. A rough quantification of the relative amount of 17β-HSD activity in each band is provided by the height of the densitometric peaks. Arabic numerals on the peaks serve to identify corresponding bands of enzyme activity of the gels shown in Fig. 4. Dotted lines indicate scans of gels without enzyme activity (blanks). Hb = absorption caused by haemoglobin.

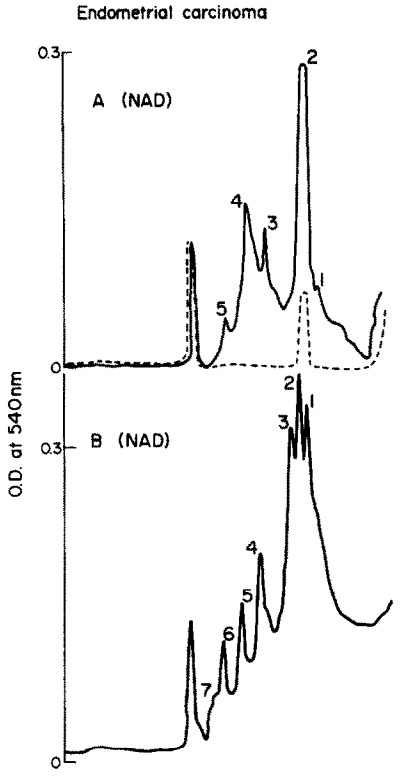

Fig. 6. Densitometric scans of two of the gels from the lower half of Fig. 4. Gels A and B (endometrial carcinoma, cofactor NAD) were scanned at 540 nm as described under Fig. 5.

obtained. Normal endometrium (proliferative and secretory) yielded three enzymatically active bands with both estradiol and testosterone as substrates. In contrast, the activity patterns from endometrial carcinoma consisted of 2, 4 or 7 bands, depending on the substrate (E_2 or T) and the cofactor (NAD or NADP). The densitometric scans (seen in Figs. 5 and 6) provide a rough quantification of the relative amounts of the various 17β-HSD activities.

The patterns were well reproducible. With the normal (7 cases) and pathological endometrial tissues (5 cases) there were moderate quantitative variations from patient to patient.

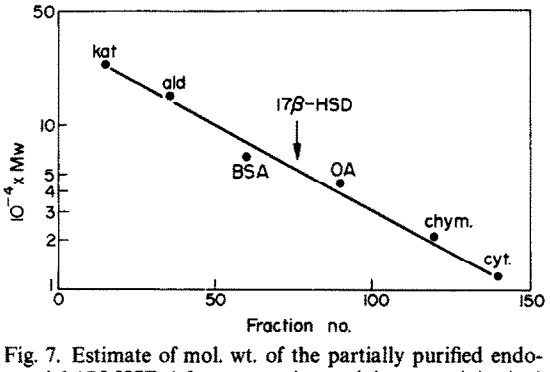

Fig. 7. Estimate of mol. wt. of the partially purified endometrial 17β-HSD (after ammonium sulphate precipitation) by gel chromatography on Sephadex G-200. Marker proteins were cytochrome c (cyt.), chymotrypsinogen A (chym.), ovalbumin (OA), bovine serum albumin (BSA), aldolase (ald.) and catalase (kat.).

Determination of molecular weight. A molecular weight between 50,000 and 54,000 was computed from the graph seen in Fig. 7.

DISCUSSION

This paper describes the partial purification of the soluble NAD(P)-dependent 17β-HSD from secretory endometrium and demonstrates by isoelectric focusing that the activities found in normal endometrium and endometrial carcinoma differed considerably in P_I-values as well as substrate and coenzyme specificities. On polyacrylamide gels (pH 3–10) normal endometrium yielded a pattern of three bands with both estradiol and testosterone as substrates while activity patterns from endometrial carcinoma consisted of 2, 4 or 7 bands depending on which of the above substrates or which cofactor was used.

The partial purification and separation of the heterogeneous forms of the enzyme from human endometrium has been achieved in four steps, namely ammonium sulphate precipitation, gel filtration, ion exchange chromatography and isoelectric focusing.

No definite answer can yet be given as to whether the observed heterogeneity of the 17β-HSD is due to procedural artifacts or to the existence of truly different enzymes ("isoenzymes"). The existence of isoenzymes has been postulated, e.g. by Engel and co-workers for the soluble 17β-HSD of human placenta [4, 16].

In this study procedural artifacts have been rendered unlikely in as far as the activity patterns (and respective P_I-values) were very similar in sucrose gradients and in gels (compare Figs. 3 and 5) and because the activity patterns were largely independent of the duration of focusing and the amount of protein or ampholytes used. Other causes of "artificial" heterogeneity, such as *in vitro* degradation, have not been eliminated. In view, however, of the results of others who have presented evidence for the existence of 17β-HSD-isoenzymes in other tissues [1, 4] it may well be possible that also the 17β-HSD from normal and neoplastic endometrium exists in multiple molecular forms. This heterogeneity could be caused by the combination of different polypeptides ("subunits") [17] or of identical polypeptides but modified for example by covalently bound phosphate or carbohydrate.

The molecular weight of the soluble 17β-HSD from secretory endometrium (50,000 to 54,000) is lower than the value found for the human placental enzyme (62,000 to 65,000) [4].

It should be emphasized, however, that calculation of molecular weights by the use of gel filtration are only approximative 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.

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