A 17 β -hydroxysteroid dehydrogenase of female rabbit liver cytosol

Purification and characterization of multiple forms of the enzyme

Gamil R. ANTOUN, Irena BRGLEZ and Denis G. WILLIAMSON

Department of Biochemistry, University of Ottawa, Ottawa, Ont. K1H 8M5, Canada

(Received 11 July 1984/Accepted 10 September 1984)

Multiple forms of the soluble 17 β -hydroxysteroid dehydrogenase of female rabbit liver were identified. NAD-dependent and NADP-dependent enzyme activities were separated by affinity chromatography on agarose-immobilized Procion Red HE3B, and three forms of the NADP-dependent enzyme activity were purified by chromatofocusing. These three enzyme forms are charge isomers and have no quaternary structure. The enzymes catalysed the C-17 oxidoreduction of oestrogens and androgens; with all enzyme forms the activity towards androgens was higher than that toward oestrogens. The enzymes also exhibited 3 α -hydroxysteroid dehydrogenase activity towards androgens of the 5 β -androstane series. Comparison of the relative activities of the enzymes towards a number of oestrogen and androgen substrates revealed differences among the enzyme forms for both the oxidative and the reductive reactions. In particular, one enzyme form had a significantly lower K_m for the 3 α -hydroxysteroid substrate and a higher 3 α -/17 β -hydroxysteroid dehydrogenase activity ratio than the other two enzyme forms.

17 β -Hydroxysteroid dehydrogenase activity has been demonstrated in the liver of a variety of animals (Aoshima & Kochakian, 1963). Depending on the species, this enzyme activity may be soluble or membrane-bound and NAD- or NADP-dependent (Williamson, 1979). In general, the 17 β -hydroxysteroid dehydrogenases of liver exhibit activity towards both oestrogens and androgens, although enzyme activity relatively specific for oestrogens (Renwick *et al.*, 1981) and for androgens (Kobayashi & Kochakian, 1978) has been observed in some animals. Soluble 17 β -hydroxysteroid dehydrogenases have been purified or partially purified from the liver of guinea pig (Kobayashi & Kochakian, 1978), chicken (Renwick *et al.*, 1981) and rabbit (Thaler-Dao *et al.*, 1972). These studies have revealed that multiple forms of this enzyme activity, differing in steroid and nicotinamide nucleotide specificity, are present in the liver.

We have been examining the role of the liver 17 β -hydroxysteroid dehydrogenases in oestrogen metabolism in the female rabbit. Oestradiol-17 β is

Trivial names used: androsterone, 3 α -hydroxy-5 α -androstane-17-one; epitestosterone, 17 α -hydroxyandrost-4-en-3-one; aetiocolanolone, 3 α -hydroxy-5 β -androstane-17-one.

metabolized to its 17 α -epimer before excretion (Williams *et al.*, 1968), and both 17 α - and 17 β -hydroxysteroid dehydrogenase activities are present in rabbit liver (Breuer & Pangels, 1960). We have isolated multiple forms of a soluble 17 α -hydroxysteroid dehydrogenase from female rabbit liver with activity towards both oestrogens and androgens (Hasnain & Williamson, 1975, 1977). The purified enzymes also exhibit 3 α -hydroxysteroid dehydrogenase activity towards androgens of the 5 α -androstane series (Lau *et al.*, 1982b). The present paper describes the purification and characterization of multiple forms of the soluble 17 β -hydroxysteroid dehydrogenase of female rabbit liver. These studies were undertaken to compare the characteristics of the 17 α - and 17 β -hydroxysteroid dehydrogenases and to obtain information on the roles of the different enzyme forms in androgen and oestrogen metabolism in the rabbit.

Experimental

Materials

[1,2-³H]Androstenedione (sp. radioactivity 41 Ci/mmol), [1,2-³H]androsterone (sp. radioactivity 41 Ci/mmol), [1,2-³H]epitestosterone (sp.

radioactivity 50 Ci/mmol), [1,2-³H]aetiocholanolone (sp. radioactivity 45 Ci/mmol), [6,7-³H]oestradiol-17 β (sp. radioactivity 40 Ci/mmol), [6,7-³H]oestrone (sp. radioactivity 44 Ci/mmol) and [7-³H]testosterone (sp. radioactivity 25 Ci/mmol) were purchased from New England Nuclear, Lachine, Que., Canada. The purity of all radioactive steroids was verified by t.l.c. on Silica Gel N (Machery Nagel und Co., Düren, Germany) in the solvent system benzene/acetone (4:1, v/v). Non-radioactive steroids were purchased from Research Plus Steroid Laboratories, Bayonne, NJ, U.S.A., or Sigma Chemical Co., St. Louis, MO, U.S.A. LKB carrier ampholyte, pH 5–7 (40%, w/v), was obtained from Fisher Scientific Co., Ottawa, Ont., Canada. Polybuffer 74 and Polybuffer Exchanger PBE 94 were purchased from Pharmacia, Dorval, Que., Canada. Agarose-immobilized Procion Red HE3B (Matrex Gel Red A) was obtained from Amicon, Oakville, Ont., Canada. All chemical reagents used were of analytical grade.

Enzyme assays

Hydroxysteroid dehydrogenase activity was measured as described by Lau *et al.* (1982a,b). The specific radioactivity of the steroid substrates employed in the assays was adjusted to 4.5 μ Ci/ μ mol by the addition of non-radioactive steroid substrate, and stock solutions (0.1 mM) of the steroids were made up in methanol. The final concentration of the steroid substrates in the incubation medium was 1 μ M. Oxidation of hydroxysteroid substrates was assayed at 37°C in 67 mM-glycine/NaOH buffer, pH 9.5, with NADP⁺ (167 μ M). Reduction of oxosteroid substrates was assayed at 37°C in 67 mM-Tris/maleate buffer, pH 7.6, with NADPH (167 μ M).

Protein was measured by the method of Bradford (1976), with bovine serum albumin as the standard. Enzyme activity is expressed as units/ml or units/mg of protein. One unit of activity is defined as the amount of enzyme catalysing the oxidation of 1 μ mol of substrate/min under the specified assay conditions.

Enzyme purification

All procedures were carried out at 0–4°C. Virgin female New Zealand White rabbits (2–3 months old) were killed by cervical dislocation. The livers were removed and rinsed in ice-cold 0.15 M-KCl. The tissue (200 g) was homogenized in a Sorvall Omni-Mixer with 4 vol. of 10 mM-Tris/HCl buffer, pH 8.0, containing 0.15 M-KCl and 0.5 mM-dithiothreitol. The homogenate was centrifuged at 10000g for 30 min, and the supernatant obtained was centrifuged at 105000g for 90 min to yield the final supernatant fraction. This supernatant frac-

tion (10 g of protein) was concentrated to approx. 100 ml by ultrafiltration on a Diaflo apparatus (Amicon) with a YM-10 membrane, and insoluble material was removed from the concentrate by centrifugation at 105000g for 90 min.

The concentrated supernatant was divided into two equal portions, and each portion was applied to a column (5 cm $\times$ 95 cm) of Sephadex G-75 (superfine grade) that had been equilibrated with 10 mM-Tris/HCl buffer, pH 8.0, containing 0.5 mM-dithiothreitol. Elution of the column was carried out with this same buffer at a flow rate of 20 ml/h. Fractions (10 ml) were collected and assayed for 17 β -hydroxysteroid dehydrogenase activity. Fractions from each column having enzyme activity were pooled and concentrated to 30 ml by ultrafiltration.

The concentrated enzyme fraction (5.4 g of protein) was applied to a column (1.5 cm $\times$ 40 cm) of agarose-immobilized Procion Red HE3B that had been equilibrated with 10 mM-Tris/HCl buffer, pH 8.0, containing 0.5 mM-dithiothreitol and 1 mM-EDTA. The enzyme sample was applied at a flow rate of 6–8 ml/h. The column was eluted first with 150 ml of 10 mM-Tris/HCl buffer, pH 8.0, containing 0.5 mM-dithiothreitol, 1 mM-EDTA and 5 mM-MgCl₂, followed by a 600 ml linear gradient of NaCl from 0 to 1.0 M in this same buffer and then by a 200 ml linear gradient of NaCl from 1.0 to 2.0 M in 10 mM-Tris/HCl buffer, pH 8.0, containing 0.5 mM-dithiothreitol and 5 mM-MgCl₂. Finally the column was eluted with 400 ml of 10 mM-Tris/HCl buffer, pH 8.0, containing 0.5 mM-dithiothreitol, 10 mM-MgCl₂ and 2.5 M-NaCl. The flow rate for elution of the column was 20 ml/h, and 3.6 ml fractions were collected. The major part of the 17 β -hydroxysteroid dehydrogenase activity was present in the protein eluted with the buffer containing 2.5 M-NaCl. These fractions were pooled, glycerol was added to a final concentration of 20% (v/v) and the combined fractions were concentrated to 10 ml by ultrafiltration. The concentrated fraction was dialysed against two changes of 4 litres of 10 mM-Tris/HCl buffer, pH 8.0, containing 0.5 mM-dithiothreitol and 20% glycerol, and then centrifuged (10000g) to remove insoluble material.

Finally purification of the 17 β -hydroxysteroid dehydrogenase was achieved by chromatofocusing. The concentrated and dialysed enzyme fraction (57 mg of protein) obtained by affinity chromatography was applied to a column (1 cm $\times$ 40 cm) of Polybuffer Exchanger PBE 94 that had been equilibrated with 10 mM-imidazole/HCl buffer, pH 6.15, containing 0.5 mM-dithiothreitol. The eluting buffer was prepared by adjusting 50 ml of a solution of Polybuffer 74 to pH 5.15 with 1 M-HCl and diluting the solution to 400 ml with deaerated distilled water. Dithio-

threitol was added to a final concentration of 0.5 mM. Elution of the column with this Polybuffer 74 solution was carried out at a flow rate of 15 ml/h, and 1.5 ml fractions were collected and assayed for 17 β -hydroxysteroid dehydrogenase activity. The pH of appropriate fractions was measured at 4°C within 2 h of collection. Fractions comprising each peak of enzyme activity were pooled and concentrated by ultrafiltration. Each concentrated peak was subjected to chromatofocusing a second time under the conditions described above except that the column size was 1 cm $\times$ 10 cm and 100 ml of the diluted polybuffer solution was used for elution of the protein.

Electrophoresis in polyacrylamide gels

Polyacrylamide-gel electrophoresis was carried out as described by Davis (1964). The acrylamide monomer concentration was 10.5%. Gels were pre-run in the Tris/glycine buffer system (pH 8.3) before application of the protein sample (5–10 μ g). Electrophoresis in polyacrylamide gels containing sodium dodecyl sulphate was performed as described by Weber *et al.* (1972).

Isoelectric focusing in polyacrylamide gels was carried out by a modification of the method of Hayes & Wellner (1969). Polyacrylamide gels (0.6 cm $\times$ 9 cm) were prepared from a solution containing the following components (final concentrations): acrylamide (8%, w/v), *NN'*-methylenebisacrylamide (0.24%, w/v), carrier ampholytes, pH 5–7 (2%, w/v), ammonium persulphate (0.024%, w/v) and *NNN'N'*-tetramethylethylenediamine (0.24%, w/v). The cathode solution was 0.02 M-NaOH and the anode solution was 0.01 M-H₃PO₄. Before the application of the protein sample the gels were run at 4°C for 15 min at 200 V, then for 30 min at 300 V and finally for 30 min at 400 V. The NaOH solution was then removed from the cathode compartment,

and the gel surfaces were rinsed with distilled water. Protein samples containing glycerol (20%, w/v) were applied to the tops of the gels and overlaid with the NaOH cathode solution. Isoelectric focusing was carried out at 4°C for 30 min at 400 V followed by 3–4 h at 500 V. Gels were stained for 17 β -hydroxysteroid dehydrogenase activity with the substrate testosterone as described by Lau *et al.* (1982a).

Results

Purification of the 17 β -hydroxysteroid dehydrogenase

17-Hydroxysteroid dehydrogenase activities were measured in the 105 000g supernatant of female rabbit liver. With NADP⁺ as cofactor 17 α -dehydrogenase activity towards epitestosterone (685 μ units/mg of protein) was higher than the 17 β -dehydrogenase activity towards testosterone (560 μ units/mg of protein). 17 β -Hydroxysteroid dehydrogenase activity was also present when NAD⁺ was used as the cofactor, although the activity towards testosterone with this nicotinamide nucleotide (230 μ units/mg of protein) was less than 50% of that observed with NADP⁺.

The procedure for the purification of the 17 β -hydroxysteroid dehydrogenase is summarized in Table 1. The 17 α - and 17 β -hydroxysteroid dehydrogenase activities were co-eluted as a single peak on Sephadex G-75 gel filtration. This fraction was purified further by chromatography on agarose-immobilized Procion Red HE3B, and the enzyme elution profiles are shown in Fig. 1. Approx. 10% of the applied 17 β -dehydrogenase activity was eluted in the wash buffer. This fraction also contained 14% of the total 17 α -dehydrogenase activity. The remainder of the 17 α -dehydrogenase activity was eluted by the 0–1 M-NaCl gradient, and partial resolution of the

Table 1. Purification of the soluble NADP-dependent 17 β -hydroxysteroid dehydrogenases of female rabbit liver
Enzyme activity was measured with testosterone. For experimental details see the text.

Purification step	Protein (mg)	Total activity (munits)	Specific activity (munits/mg of protein)	Yield (%)
1. 105 000g supernatant	9980	5300	0.53	100
2. Sephadex G-75 gel filtration	5360	4300	0.80	81
3. Affinity chromatography				
Wash buffer	1720	410	0.24	71
0–1 M-NaCl	1160	220	0.19	
1–2 M-NaCl	100	150	1.50	
2.5 M-NaCl	57.0	3000	52.6	
4. Chromatofocusing				
Peak I	3.08	386	125	16
Peak II	1.31	121	92.4	
Peak III	6.27	333	53.1

Fig. 1. Affinity chromatography of the soluble 17β -hydroxysteroid dehydrogenase of female rabbit liver. The 17β -hydroxysteroid dehydrogenase fraction obtained by gel filtration on Sephadex G-75 was applied to a column of agarose-immobilized Procion Red HE3B and chromatographed as described in the text. Fractions (3.6 ml) were assayed for 17α -hydroxysteroid dehydrogenase activity with epitestosterone (▲) and for 17β -hydroxysteroid dehydrogenase activity with testosterone (●). -----, NaCl concentration.

Fig. 2. Chromatofocusing of the soluble NADP-dependent 17β -hydroxysteroid dehydrogenases of female rabbit liver. The 17β -hydroxysteroid dehydrogenase fraction eluted from the affinity column with buffer containing 2.5 M-NaCl was applied to a column of Polybuffer Exchanger PBE 94 that had been previously equilibrated with 10 mM-imidazole/HCl buffer, pH 6.15, containing 0.5 mM-dithiothreitol. Protein was eluted with a diluted Polybuffer 74 solution, pH 5.10, prepared as described in the text. The 17β -hydroxysteroid dehydrogenase activity towards testosterone (●) and the pH (-----) of the fractions (1.5 ml) collected were measured, and the fractions were pooled as indicated by the bars.

multiple forms of the enzyme was achieved. The heterogeneity of this enzyme in female rabbit liver has been described by Hasnain & Williamson (1975, 1977). Most of the 17β -hydroxysteroid dehydrogenase activity was recovered in the buffer containing 2.5 M-NaCl and 10 mM-MgCl₂ (Fig. 1 and Table 1). When this enzyme fraction was chromatofocused over the pH range 6.15–5.15 three peaks of enzyme activity were obtained (Fig. 2). Polyacrylamide-gel electrophoresis of each enzyme peak, pooled as shown in Fig. 2, showed that there was cross-contamination among the enzyme forms. Therefore each peak was chromatofocused again under the conditions employed in Fig. 2 except that the column size was 1 cm × 10 cm and the volume of Polybuffer solution used for elution was 100 ml. This procedure yielded each of the enzyme forms in a homogeneous state, as established by polyacrylamide-gel electrophoresis (Fig. 3). M_r values of 37 000, 40 000 and 39 000 were estimated for the enzyme forms I, II and III respectively by comparing the mobilities of the enzymes on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis with those of the reference proteins ribonuclease (M_r 13 000), chymotrypsinogen (M_r 25 700), fructose biphosphate aldolase (M_r 40 000), ovalbumin (M_r 45 000) and bovine serum albumin (M_r 68 000).

In order to determine whether forms of the 17β -hydroxysteroid dehydrogenase were lost or generated during purification, enzyme activity throughout this procedure was monitored by isoelectric focusing in polyacrylamide gels. Densitometer scans of gels focused over the pH range 5–7 and stained for 17β -dehydrogenase activity are shown in Fig. 4. Four major bands of 17β -dehydrogenase activity are present in the 105 000 g supernatant of female rabbit liver (Fig. 4b), three forms having similar isoelectric points and the fourth form being more acidic. This same enzyme-activity profile was obtained with gels of the Sephadex G-75 enzyme fraction (Fig. 4c). Affinity chromatography separated the acidic enzyme activity, which was eluted from the column by the wash buffer (Fig. 4d), from the three more basic enzyme forms, which were eluted from the column by buffer containing 2.5 M-NaCl (Fig. 4e). These latter enzyme forms were subsequently separated and purified by chromatofocusing (Fig. 2). The data in Fig. 4 show that enzyme forms were neither lost nor generated during the purification procedure.

The acidic 17β -dehydrogenase activity eluted in the wash buffer during affinity chromatography differs in nicotinamide nucleotide specificity from the more basic enzymes eluted from this column in buffer containing 2.5 M-NaCl (Figs. 1 and 4). The specific activity of the acidic enzyme fraction is 14-fold higher with NAD⁺ than with NADP⁺,

Fig. 3. Polyacrylamide-gel electrophoresis of the 17 β -hydroxysteroid dehydrogenases purified by affinity chromatography and chromatofocusing

The enzymes are those denoted in Fig. 2. (a) Polyacrylamide-gel electrophoresis at pH 8.3. (b) Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

whereas the more-basic enzymes are specific for NADP⁺ (results not shown). The elution sequence of the NAD⁺- and NADP⁺-dependent 17 β -dehydrogenase on affinity chromatography is consistent with the properties of agarose-immobilized Procion Red HE3B, which has been shown to have a higher affinity for NADP-dependent dehydrogenases than for NAD-dependent dehydrogenases (Watson *et al.*, 1978). The data in Fig. 4 suggest that more than one form of the 17 β -dehydrogenase is present in the acidic fraction. However, this could not be clearly established, owing to the presence of an achromatic band in the region of the acidic 17 β -dehydrogenase activity produced under the substrate-staining conditions employed. This band appeared as a trough in the densitometer scans (Figs. 4c and 4d) and was caused by a cyanide-insensitive superoxide dismutase (Weisiger & Fridovich, 1973). This enzyme can inhibit the reduction of Nitro Blue Tetrazolium and thus prevent the formation of the coloured bands normally produced with this substrate-staining technique (Beauchamp & Fridovich, 1971). Further purification of the acidic 17 β -dehydrogenase form(s), which accounted for 10% of the NADP-dependent 17 β -hydroxysteroid dehydrogenase activity of rabbit liver cytosol, was not undertaken.

Substrate specificities of the purified enzymes

The activities of the 17 β -hydroxysteroid dehydrogenases towards a number of androgen and

oestrogen substrates are shown in Table 2. The enzymes catalyse the reversible oxidation of 17 β -hydroxy C₁₈ and C₁₉ steroids and exhibit a pH optimum of 9.5 for the oxidative reaction and 7.6 for the reductive reaction. We have observed a variation in the stability of the enzyme forms during their purification and subsequent storage at 4°C. Enzyme III, which has the lowest isoelectric point, is the least stable enzyme form. Because of this variation in enzyme stability it is not possible to compare the specific activities of the enzymes directly. However, the data in Table 2 do show the relative activity of each enzyme towards the different steroid substrates, and these profiles for the three enzymes can be compared. Enzyme III is the most distinct in its 17 β -dehydrogenase activity profile. In the oxidative reaction the specific activity of this enzyme towards testosterone is 47-fold higher than towards oestradiol-17 β . This difference is 26-fold for enzyme I and 11-fold for enzyme II. In the reductive reaction the specific activity of enzyme III towards androstenedione is only 2-fold higher than towards oestrone, whereas for enzymes I and II this difference is approximately 6-fold. All forms of the 17 β -hydroxysteroid dehydrogenase exhibit 3 α -hydroxysteroid dehydrogenase activity. This activity is relatively specific for androgen substrates of the 5 β -androsterane series, the activity towards the 5 β -substrate aetiocholanolone being at least 6-fold higher than towards the 5 α -substrate androsterone (Table 2). With all enzyme forms the 3 α -hydroxysteroid

Fig. 4. Isoelectric focusing-polyacrylamide-gel electrophoresis of the soluble 17 β -hydroxysteroid dehydrogenase of female rabbit liver during purification

Electrophoresis and substrate staining of the enzyme fractions were carried out as described in the text. Each enzyme sample was dialysed against 10mM-Tris/HCl buffer, pH8.0, containing 0.5mM-dithiothreitol before electrophoresis. Approx. 2000 μ nits of enzyme activity towards testosterone were applied to each gel. Isoelectric focusing was carried out initially at 400 V for 0.5h and then at 500 V for 3.5h. Each gel was scanned from the top (cathode end) for a distance of approx. 7cm. Densitometric tracings of the gels are shown for the 105000g supernatant (b), the Sephadex G-75 gel-filtration fraction (c), the affinity-column wash fraction (d) and the affinity-column 2.5M-NaCl fraction (e). Gel (a) is a control gel of the 105000g supernatant stained in the absence of the substrate testosterone.

dehydrogenase activity is lower than the 17 β -hydroxysteroid dehydrogenase activity under the experimental conditions of the enzyme assays.

Table 2. Substrate specificities of the multiple forms of the soluble 17 β -hydroxysteroid dehydrogenase of female rabbit liver

Enzyme activity was measured at pH9.5 in the oxidative direction and at pH7.6 in the reduction direction. The experimental details are given in the text. The steroid concentration was 1 μ M and the NADP concentration was 167 μ M. For the assays with testosterone the enzyme concentration was 0.9–1.8nM. With other substrates the enzyme concentration was adjusted so that the extent of oxidation did not exceed 30%.

Substrate	Enzyme	Specific activity (munits/mg of protein)		
		I	II	III
Testosterone		125	92.4	53.1
Oestradiol-17 β		4.9	8.4	1.1
Androstenedione		96.1	76.3	12.1
Oestrone		16.2	14.0	5.6
Aetiocholanolone		32.8	36.6	35.1
Androsterone		3.9	1.5	5.3

Enzyme III exhibits the highest 3 α -dehydrogenase activity relative to its 17 β -dehydrogenase activity.

A comparison of the kinetic constants for the 17 β - and 3 α -dehydrogenase activities of each enzyme, determined with testosterone and aetiocholanolone respectively, is shown in Table 3. Preliminary studies showed that under the experimental conditions employed and at steroid concentrations ranging from 0.3 to 3 times K_m the reaction rates were linear for 30min. With testosterone as substrate enzymes I and II obeyed Michaelis-Menten kinetics, whereas enzyme III exhibited substrate inhibition at testosterone concentrations above 4 μ M. All enzymes showed substrate inhibition with aetiocholanolone. This occurred at steroid concentrations above 4 μ M with enzymes I and II and above 0.5 μ M with enzyme III. The major difference in the kinetic constants among the enzymes was observed for enzyme III. The K_m of this enzyme with aetiocholanolone is 17 times lower than the K_m with testosterone and at least 10 times lower than the K_m with aetiocholanolone for either enzyme I or II.

Discussion

Earlier studies by Thaler-Dao *et al.* (1972) had shown that female rabbit liver cytosol has 17 β -hydroxysteroid dehydrogenase activities that differ in steroid and nicotinamide nucleotide specificity. The heterogeneity of this enzyme activity has been confirmed in the present studies. We have purified, for the first time, three forms of the NADP-dependent 17 β -hydroxysteroid dehydrogenase by the use of methods based on the

Table 3. *Kinetic constants of the multiple forms of the soluble 17 β -hydroxysteroid dehydrogenase of female rabbit liver*
 The apparent K_m and V_{max} values were determined by the double-reciprocal method of Lineweaver & Burk (1934).
 The assay conditions were as described in the text except that the steroid substrate concentrations ranged from 0.3 to 3 times K_m and the incubation time was 10 min. The concentration of the fixed substrate NADP⁺ was 167 μ M.

Enzyme	Testosterone		Aetiocholanolone	
	K_m (μ M)	V_{max} (nmol/min per mg)	K_m (μ M)	V_{max} (nmol/min per mg)
I	3.2	615	1.7	217
II	3.6	505	5.0	534
III	3.0	635	0.18	107

differences in nicotinamide nucleotide specificity and isoelectric points among the different 17 β -dehydrogenase activities of rabbit liver cytosol. It is unlikely that the multiple forms of the 17 β -hydroxysteroid dehydrogenase are artifacts produced during purification. The enzyme forms could be identified in the initial liver cytosol and enzyme forms were neither lost nor generated throughout the purification procedure (Fig. 4). Furthermore, the same relative amounts of the enzyme forms were obtained from different rabbit liver preparations, and storage of the liver cytosol at 4°C for several days did not alter the enzyme pattern. Heterogeneity appears to be a feature common to several mammalian 17 β -hydroxysteroid dehydrogenases, including those of human placenta (Engel & Groman, 1974), guinea-pig liver (Kobayashi & Kochakian, 1978), guinea-pig kidney (Shen & Kochakian, 1978) and pig testis (Inano *et al.*, 1980). More recently, two forms of the enzyme have been identified in human skin (Hodgins *et al.*, 1982) and human testis (Leinonen, 1982). The characteristics of the multiple forms of the soluble 17 β -hydroxysteroid dehydrogenase of female rabbit liver purified in the present study indicate that the enzymes are charge isomers. Their M_r values, determined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, are all within 3% of the average value of 38700. This M_r value is consistent with the elution of the enzyme forms on Sephadex G-75 and reveals that the enzymes have no quaternary structure. These enzymes are similar in size to the multiple forms of the 17 α -hydroxysteroid dehydrogenase present in this tissue (Hasnain & Williamson, 1977) and in rabbit kidney (Lau *et al.*, 1982a) and to the 17 β -hydroxysteroid dehydrogenases of guinea-pig liver and kidney (Kobayashi & Kochakian, 1978; Shen & Kochakian, 1978) and pig testis (Inano *et al.*, 1977). However, they differ from the 17 β -hydroxysteroid dehydrogenase in human placenta, which is a dimer having a subunit M_r of 34000 (Burns *et al.*, 1972).

All forms of the soluble 17 β -hydroxysteroid dehydrogenase of female rabbit liver exhibit

higher activities towards androgens than towards oestrogens (Table 2). Similar specificities have been reported for the soluble 17 β -hydroxysteroid dehydrogenases of guinea-pig liver (Kobayashi & Kochakian, 1978) and kidney (Liu & Kochakian, 1972). In contrast, oestrogens are the preferred substrates for the 17 β -hydroxysteroid dehydrogenases of chicken liver (Renwick *et al.*, 1981), human placenta (Langer *et al.*, 1959), sheep ovary (Kautsky & Hagerman, 1970) and human ovary (Pittaway *et al.*, 1977).

Bifunctional enzyme activity has been reported for a number of hydroxysteroid dehydrogenases (Battais *et al.*, 1977; Shen & Kochakian, 1978; Edwards & Orr, 1978; Strickler *et al.*, 1981; Sharaf & Sweet, 1982; Lau *et al.*, 1982a,b). The present studies have demonstrated that the multiple forms of the soluble 17 β -hydroxysteroid dehydrogenase of female rabbit liver exhibit bifunctional enzyme activity. Enzymes that are homogeneous by the criterion of polyacrylamide-gel electrophoresis catalyse the oxidation of both 17 β - and 3 α -hydroxysteroids (Table 2). The 3 α -dehydrogenase activity is relatively specific for steroids of the 5 β -androstane series, the activity towards aetiocholanolone being at least 6-fold higher than towards androsterone. The multiple forms of the 17 α -hydroxysteroid dehydrogenases of female rabbit liver and kidney also have 3 α -dehydrogenase activity. However, androgens of the 5 α -androstane series are the preferred substrates for these enzymes (Lau *et al.*, 1982a,b). Furthermore, in contrast with the 17 β -hydroxysteroid dehydrogenases, the 3 α -dehydrogenase activity of the 17 α -hydroxysteroid dehydrogenases of rabbit liver exceeds their 17 α -dehydrogenase activity.

Although the general substrate specificities of the multiple forms of the female rabbit liver 17 β -hydroxysteroid dehydrogenases are similar, there are differences among the enzyme forms. Enzyme III is the most distinct in its substrate specificity. This enzyme has the highest relative 3 α -dehydrogenase activity and a distinctly low K_m with aetiocholanolone (Table 3). The molecular basis for the differences in substrate specificity of the three

forms of the 17 β -hydroxysteroid dehydrogenase, as well as the physiological significance of these differences, must await a detailed comparison of the structural and kinetic properties of these enzymes.

This work was supported by a grant from the Medical Research Council of Canada.

References

- Aoshima, Y. & Kochakian, C. D. (1963) *Endocrinology (Baltimore)* **72**, 106–114
- Battais, E., Terouanne, B., Nicolas, J. C., Descomps, B. & Crastes de Paulet, A. (1977) *Biochimie* **59**, 909–917
- Beauchamp, C. & Fridovich, I. (1971) *Anal. Biochem.* **44**, 276–287
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Breuer, H. & Pangels, G. (1960) *Acta Endocrinol. (Copenhagen)* **33**, 532–538
- Burns, D. J. W., Engel, L. L. & Bethune, J. L. (1972) *Biochemistry* **11**, 2699–2703
- Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404–427
- Edwards, C. A. F. & Orr, J. C. (1978) *Biochemistry* **17**, 4370–4376
- Engel, L. L. & Groman, E. V. (1974) *Recent Prog. Horm. Res.* **130**, 139–169
- Hasnain, S. & Williamson, D. G. (1975) *Biochem. J.* **147**, 457–461
- Hasnain, S. & Williamson, D. G. (1977) *Biochem. J.* **161**, 279–283
- Hayes, M. B. & Wellner, D. (1969) *J. Biol. Chem.* **244**, 6636–6644
- Hodgins, M. B., Hay, J. B. & Donnelly, J. B. (1982) *J. Endocrinol.* **93**, 404–413
- Inano, H., Kawakura, K. & Tamaoki, B. (1977) *J. Steroid Biochem.* **8**, 787–789
- Inano, H., Hori, Y., Tamaoki, B., Hamana, K. & Nakagawa, H. N. (1980) *J. Steroid Biochem.* **13**, 287–295
- Kautsky, M. P. & Hagerman, D. D. (1970) *J. Biol. Chem.* **245**, 1978–1984
- Kobayashi, K. & Kochakian, C. D. (1978) *J. Biol. Chem.* **253**, 3635–3642
- Langer, L. J., Alexander, J. A. & Engel, L. L. (1959) *J. Biol. Chem.* **234**, 2609–2614
- Lau, P. C. K., Layne, D. S. & Williamson, D. G. (1982a) *J. Biol. Chem.* **257**, 9444–9449
- Lau, P. C. K., Layne, D. S. & Williamson, D. G. (1982b) *J. Biol. Chem.* **257**, 9450–9456
- Leinonen, P. (1982) *J. Steroid Biochem.* **16**, 277–281
- Lineweaver, H. & Burk, D. (1934) *J. Am. Chem. Soc.* **54**, 658–666
- Liu, D. K. & Kochakian, C. D. (1972) *Steroids* **19**, 701–719
- Pittaway, D. E., Anderson, R. N. & Givens, J. R. (1977) *Acta Endocrinol. (Copenhagen)* **85**, 624–635
- Renwick, A. G. C., Soon, C. Y., Chambers, S. M. & Brown, C. R. (1981) *J. Biol. Chem.* **256**, 1881–1887
- Sharaf, M. A. & Sweet, F. (1982) *Biochemistry* **21**, 4615–4620
- Shen, C. C. & Kochakian, C. D. (1978) *Endocrinology (Baltimore)* **103**, 2040–2052
- Strickler, R. C., Tobias, B. & Covey, D. F. (1981) *J. Biol. Chem.* **256**, 316–321
- Thaler-Dao, H., Descomps, B., Saintot, M. & Crastes de Paulet, A. (1972) *Biochimie* **54**, 83–91
- Watson, D., Harvey, M. & Dean, P. (1978) *Biochem. J.* **173**, 591–596
- Weber, K., Pringle, J. R. & Osborn, M. (1972) *Methods Enzymol.* **26**, 3–27
- Weisiger, R. A. & Fridovich, I. (1973) *J. Biol. Chem.* **248**, 3582–3592
- Williams, K. I. H., Henry, D. H., Collins, D. C. & Layne, D. S. (1968) *Endocrinology (Baltimore)* **83**, 113–117
- Williamson, D. G. (1979) in *Steroid Biochemistry* (Hobkirk, R., ed.), vol. 1, pp. 83–110, CRC Press, Boca Raton