Isolation and Characterization of 17 β -Hydroxy Steroid Dehydrogenase from Human Erythrocytes

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(Received 10 November 1971)

1. The 17 β -hydroxy steroid dehydrogenase was solubilized during haemolysis of erythrocytes and was isolated from the membrane-free haemolysate. Membrane preparations isolated in different ways did not contain 17 β -hydroxy steroid dehydrogenase activity. The 17 β -hydroxy steroid dehydrogenase activity in the haemolysate was concentrated by repeated ammonium sulphate precipitation and gel filtration on Sephadex G-150. The 17 β -hydroxy steroid dehydrogenase activity of the purified preparation per unit weight of protein was 350–3000 times higher than the activity of the crude erythrocyte haemolysate. The 20 α -hydroxy steroid dehydrogenase activity was lost during this purification procedure. 2. The 17 β -hydroxy steroid dehydrogenase was NADP-dependent and had a pH optimum for conversion of testosterone between 8.5 and 10. For the molecular weight of the enzyme a value of 64000 was calculated from Sephadex chromatography results. 3. *p*-Chloromercuribenzoate inhibited the enzymic activity. The oxidative activity of the enzyme for the 17 β -hydroxyl group was only partly inhibited when a large excess of 17-oxo steroids was added. The catalysing activity of the enzyme was influenced by the NADP⁺/NADPH ratio. The oxidation of the 17 β -hydroxyl group in the presence of NADP⁺ proceeded faster than the reduction of the 17-oxo group with NADPH. When both reduced and oxidized cofactors were present the oxidation of the 17 β -hydroxyl group was inhibited to a considerable extent. 4. The enzyme had a broad substrate specificity and not only catalysed the conversion of androstanes with a 17 β -hydroxyl group, or 17-oxo group, but also the conversion oestradiol $\rightleftharpoons$ oestrone. In addition the steroid conjugates dehydroepiandrosterone sulphate and oestrone sulphate were also converted. There were no indications that more than one 17 β -hydroxy steroid dehydrogenase was present in the partially purified preparation.

Human erythrocytes contain dehydrogenase activities capable of oxidizing the 17 β -hydroxyl group of oestradiol and oestradiol 3-sulphate (Portius & Repke, 1960; Migeon *et al.*, 1962; Jacobsohn & Hochberg, 1968), testosterone (Jacobsohn & Hochberg, 1968; Lindner, 1961; Van der Molen & Groen, 1968) and androst-5-ene-3 β ,17 β -diol (Nicol *et al.*, 1969). It has also been shown that human erythrocytes can catalyse the conversion of progesterone into 20 α -hydroxypregn-4-en-3-one (Van der Molen & Groen, 1968). The significance of the presence of these steroid dehydrogenases in erythrocytes is not known. These enzymes may have a function *in vivo* in maintaining the balance between oxidized and reduced steroids in blood. In addition the dehydrogenase may fulfil a physiological role in the erythrocyte itself, because some 17-oxo steroids can inhibit the erythrocyte glucose 6-phosphate dehydrogenase, whereas the corresponding 17 β -hydroxy steroids are inactive in this respect (Marks & Banks, 1960; McKerns & Kaleita, 1960).

This investigation was carried out to determine the

cellular localization in the erythrocyte of the 17 β -hydroxy steroid dehydrogenase catalysing the reaction of testosterone into androstenedione. In addition the isolation and partial purification of the enzyme were undertaken and the characterization of the enzyme in terms of molecular weight and substrate specificity was studied. Finally, the localization and the relationship between glucose 6-phosphate dehydrogenase activity and reduction of 17-oxo steroids in erythrocytes was investigated.

Materials and Methods

Materials

The following ³H- and ¹⁴C-labelled steroids were obtained from New England Nuclear Corp., Frankfurt, Germany or The Radiochemical Centre, Amersham, Bucks., U.K.: 4-¹⁴C-labelled androst-4-ene-3,17-dione (56 mCi/mmol), dehydroepiandrosterone (56 mCi/mmol), dehydroepiandrosterone sulphate (56 mCi/mmol), progesterone (60 mCi/mmol),

testosterone (60 mCi/mmol); 1,2- $^3\text{H}_2$ -labelled androst-4-ene-3,17-dione (50 Ci/mmol), testosterone (37 Ci/mmol), progesterone (35 Ci/mmol), 20 α -hydroxypregn-4-en-3-one (32.7 Ci/mmol); 7 α - ^3H -labelled dehydroepiandrosterone (16 Ci/mmol) and dehydroepiandrosterone sulphate (10 Ci/mmol); 6,7- $^3\text{H}_2$ -labelled oestrone (46 Ci/mmol), oestradiol (56 Ci/mmol) and oestrone 3-sulphate (40 Ci/mmol). When necessary, these steroids were purified by paper chromatography or t.l.c. to a radiochemical purity of over 98%.

[7- ^3H]Androstenediol (androst-5-ene-3 β ,17 β -diol) was prepared by reduction of [7- ^3H]dehydroepiandrosterone with the 17 β -hydroxy steroid dehydrogenase preparation from erythrocytes. The incubation and isolation procedures used are described under the section dealing with determination of steroid dehydrogenase activity by incubation with radioactive substrates. The amounts of enzyme and NADP used were, however, five times higher than those used for the measurement of the enzyme activity.

NAD $^+$, NADH, NADP $^+$ and NADPH, glucose 6-phosphate, glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and lactate dehydrogenase (EC 1.1.1.27) were obtained from Boehringer, Mannheim, Germany. Unlabelled steroids were obtained from Steraloids, Pawling, N.Y., U.S.A.

Partial purification of 17 β -hydroxy steroid dehydrogenase from erythrocytes

Erythrocytes were isolated from fresh heparinized blood or from out-dated blood-bank blood. The upper layer and leucocyte-containing layer were separated from the erythrocytes by centrifugation and carefully removed. The cells were washed three times with 0.9% NaCl solution. The washed cells were haemolysed by mixing with 2.5 vol. of water followed by stirring for 1 h at 4°C. The haemolysate was centrifuged for 20 min at 30000 g_{av} for separation of the clear supernatant from the layer containing cell debris. The 17 β -hydroxy steroid dehydrogenase was isolated from this supernatant (the 'membrane-free' haemolysate) by a modification of the fractionating procedure used by Jacobsohn & Hochberg (1968). Equal amounts of supernatant and saturated $(\text{NH}_4)_2\text{SO}_4$ (pH 7) (700 g/litre) were mixed and stirred overnight at 4°C. The precipitate was collected after centrifuging at 30000 g_{av} and dissolved in a solution with a final concentration of $(\text{NH}_4)_2\text{SO}_4$ of 20% of saturation [140 g of $(\text{NH}_4)_2\text{SO}_4$ /litre]. This solution was again centrifuged and any residual precipitate discarded. The supernatant was mixed with $(\text{NH}_4)_2\text{SO}_4$ to a concentration of 50% of saturation (350 g/litre) and the $(\text{NH}_4)_2\text{SO}_4$ precipitation procedure was repeated.

Fig. 1. Comparison of steroid dehydrogenase activity, glucose 6-phosphate dehydrogenase activity and protein content of fractions collected from a Sephadex G-150 column

Gel filtration on Sephadex of the 17 β -hydroxy steroid dehydrogenase-containing preparations, obtained after $(\text{NH}_4)_2\text{SO}_4$ precipitation of membrane-free haemolysates of erythrocytes, was performed as described in the Materials and Methods section. Steroid dehydrogenase activity (■) was measured as the percentage of [^{14}C]testosterone formed from [^{14}C]androstenedione, and glucose 6-phosphate dehydrogenase activity (○) was measured spectrophotometrically (Bishop, 1966). The protein contents of the different fractions are given on a relative basis as percentages of transmission at 254 nm (—).

The enzyme solution obtained after $(\text{NH}_4)_2\text{SO}_4$ precipitation was further purified on a Sephadex G-150 column. The column (25 mm $\times$ 1000 mm) was equilibrated at 4°C with 0.05 M-phosphate buffer, pH 7.0, containing 0.1 M-KCl, 1 mM-EDTA, 1 mM-mercaptoethanol and 0.02 mM-NADP.

The column was run by reverse flow. Portions of the solution containing the enzymic activity associated with 100–150 mg of protein were applied to the bottom of the column in 4 ml of buffer and the column was eluted overnight. Fractions of 3 ml each were collected. The fractions with highest dehydrogenase activity (Fig. 1) were pooled. Equal amounts of this enzyme solution and saturated $(\text{NH}_4)_2\text{SO}_4$ solution were mixed, stirred for 30 min at 4°C and centrifuged at 30000 $g_{av.}$. The precipitate was collected, dissolved in $(\text{NH}_4)_2\text{SO}_4$ solution (140 g/litre) and stored at –15°C. The loss of activity was only a few per cent over a period of several months. Desalting of $(\text{NH}_4)_2\text{SO}_4$ -containing solutions was carried out by chromatography on Sephadex G-25.

Enzyme determinations

Enzymic activity of steroid dehydrogenase-containing preparations was determined either spectrophotometrically or by incubation with radioactive steroids.

Spectrophotometric determination of 17 β -hydroxy steroid dehydrogenase activity. The activity of the enzyme was determined in a Gilford model 2400 recording spectrophotometer at 37°C by measuring E_{340} of the reduced cofactor formed. In a cuvette (10 mm $\times$ 4 mm) 0.1 ml of a 1.65 mM steroid solution (or other amounts as indicated in the legends to tables and figures) in ethanol–benzene (1:9, v/v) was mixed with 0.1 ml of propylene glycol–methanol (1:4, v/v) and the volatile solvents were evaporated in a stream of N_2 at 37°C. During this procedure the steroid was concentrated in a small drop of propylene glycol and could subsequently be readily dissolved in buffer solution. This amount of propylene glycol does not influence the enzymic reactions. To the steroid in the cuvette 0.1 ml of 0.4 M-tris–HCl buffer, pH 8.5, 0.1 ml of 1.5 M-NaCl and 0.1–0.4 ml of enzyme solution were then added, and water to make a total volume of 1 ml. After preincubation for 5 min the reaction was started by the addition of 0.1 ml of 3 mM-NADP $^+$. The reaction rate was almost constant for periods up to 60 min. The extinction was recorded at 2 min intervals and the enzymic activity was defined as the average reaction rate during the first 30 min after the initiation of the reaction. The specific activity was calculated from these reaction rates and the protein content of the preparations, determined by the method of Lowry *et al.* (1951).

Determination of steroid dehydrogenase activity by

incubation with radioactive substrates. In an incubation flask a small amount of labelled steroid (e.g. 0.1 μCi of ^{14}C -labelled steroid, specific radioactivity 50 mCi/mmol) dissolved in ethanol–benzene (1:9, v/v) was concentrated with carrier steroid (16.5 nmol if not specified in the legend to tables and figures) and propylene glycol as described for the spectrophotometric assay. Steroids with a 17 β - or 20 α -hydroxyl group were incubated in a medium containing 0.1 ml of 3.0 mM-NADP $^+$, 0.1 ml of 0.4 M-tris–HCl buffer, pH 8.5, 0.1 ml of 1.5 M-NaCl, 0.1–0.5 ml of the solution containing the enzymic activity and water to make a total volume of 1.0 ml. Steroids with a 17- or 20-oxo group were incubated in a medium containing 0.1 ml of 3.0 mM-NADPH, 0.1 ml of 0.4 M-tris–HCl buffer, pH 7.5, 0.1 ml of 1.5 M-NaCl, 0.1 ml of 10 mM-glucose 6-phosphate, 0.1–0.5 ml of the solution containing the enzymic activity and water to make a total volume of 1.0 ml. All enzyme preparations contained small amounts of glucose 6-phosphate dehydrogenase. The incubation was carried out for 3 h at 37°C in a shaking water bath as a routine.

At the end of the incubation period known amounts of the ^3H -labelled steroid substrate and steroid product were added to correct for losses during isolation and the steroids were extracted immediately. Non-conjugated steroids were extracted either with ether (Van der Molen & Groen, 1968) or with ethyl acetate (Brinkmann *et al.*, 1970).

For isolation of the conjugated steroids 4 ml of methanol was added very slowly to the incubation mixture under constant shaking. After centrifugation the clear supernatant was separated and the protein precipitate was twice extracted with 4 ml of 80% (v/v) ethanol. The supernatant and the two ethanol extracts were combined. The recovery of radioactive steroid by this type of extraction was better than 95%. The steroid sulphates were then solvolysed with ethyl acetate at pH 1 (Burstin & Lieberman, 1958). The ethyl acetate layer was concentrated and subjected to t.l.c. or paper chromatography.

The oxo steroids were separated from the hydroxy steroids in a Bush A2 or a Bush B1 system or on silica-gel thin-layer plates in the solvent system benzene–ethyl acetate (3:2, v/v) (Bush, 1961; Van der Molen & Groen, 1968). The areas containing radioactivity were localized after chromatography by scanning with a radiochromatogram or thin-layer plate scanner and were eluted with methanol. The concentrated eluate was dissolved in a modified Bray's (1960) solution. Radioactivity was counted in a Packard model 3375 liquid-scintillation spectrometer. Quench corrections were calculated from external standard ratios. Corrections for loss of steroid during isolation were calculated from $^3\text{H}/^{14}\text{C}$ ratios as described previously (Van der Molen & Groen, 1968).

Results

Localization of 17 β -hydroxy steroid dehydrogenase in the erythrocyte

The 17 β -hydroxy steroid dehydrogenase could be solubilized by haemolysis (Table 1). The rates of conversion of androstenedione into testosterone in intact cells and in membrane-free haemolysates prepared from the same amount of cells were almost equal. The membranes prepared by haemolysis with water saturated with CO₂ (Parpart, 1942) retained only a small fraction of the dehydrogenase activity of the intact cell. Membranes from which most of the haemoglobin was removed by washing with phosphate buffers ('white ghosts' prepared by the

method of Dodge *et al.*, 1963) did not show activities significantly different from the results obtained in control incubations.

The enzymic activity catalysing the conversion of progesterone into 20 α -hydroxypregn-4-en-3-one was also localized in the erythrocyte cytoplasm.

Partial purification of 17 β -hydroxy steroid dehydrogenase

After haemolysis of erythrocytes and removal of membranes the enzyme was concentrated by a twice-repeated (NH₄)₂SO₄ precipitation procedure. The enzymic activity catalysing the conversion of androstenedione into testosterone was localized mainly

Table 1. 17 β -Hydroxy steroid dehydrogenase activity of human erythrocyte fractions

Incubation conditions: 20 μ g of androstenedione (0.065 μ mol) was mixed with [¹⁴C]androstenedione (less than 0.01 μ mol) and dissolved as described in the Materials and Methods section. Krebs-Ringer bicarbonate buffer, pH 7.4 (1.2 ml), supplemented with 50 μ mol of glucose, 2 μ mol of NADH, 2 μ mol of NADPH and 15 μ mol of glucose 6-phosphate was used as incubation medium. The erythrocyte preparations added were, successively: (a) 2 ml of washed erythrocytes; (b) 2 ml of a total haemolysate of erythrocytes [prepared by sonication of 2 ml of washed erythrocytes (2 $\times$ 5 s, 21 kHz at 4°C)]; (c) 2 ml of a 'membrane-free' haemolysate prepared as described in the Materials and Methods section; (d) 2 ml of an erythrocyte membrane suspension prepared by haemolysis of erythrocytes with water saturated with CO₂ (Parpart, 1942); (e) 2 ml of an erythrocyte membrane suspension prepared by the method of Dodge *et al.* (1963). The erythrocyte fractions used in Expts. (b)–(e) were obtained from the same amounts of erythrocytes as used in Expt. (a). Expt. (f) was a control incubation, with an incubation medium containing 2 ml of buffer instead of an erythrocyte preparation. The range of the observed values for testosterone formation is given.

Expt.	Preparation	Testosterone formed (μ mol/3 h)
(a)	Whole erythrocyte	0.0120–0.0160
(b)	Total haemolysate	0.0115–0.0120
(c)	'Membrane-free' haemolysate	0.0110–0.0175
(d)	Membranes (CO ₂ –water)	0.0027–0.0030
(e)	Membranes (Dodge method)	0.0008–0.0009
(f)	Control	0.0004–0.0008

Table 2. Partial purification of 17 β -hydroxy steroid dehydrogenase from human erythrocytes

The specific activity was measured as the percentage of [¹⁴C]testosterone formed from [¹⁴C]androstenedione under standard conditions as described in the Materials and Methods section, divided by the amounts of protein used. Specific activities are compared in column 4 relative to the activity of an erythrocyte haemolysate.

Step of purification	Total volume (ml)	Total protein (g)	Relative specific activity	Recovery of total activity (%)
Haemolysate	900	310	1	100
Second precipitation with ammonium sulphate and resuspension	20	13	100–250	50–100
Suspension in ammonium sulphate after Sephadex G-150 column chromatography	20	0.112	350–1300	20–40

Table 3. 17 β -Hydroxy steroid dehydrogenase, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities of human erythrocyte fractions

Enzymic activities were determined spectrophotometrically by following the change in E_{340} . 17 β -Hydroxy steroid dehydrogenase was measured as described in the Materials and Methods section. Glucose 6-phosphate and 6-phosphogluconate dehydrogenase were measured by the method of Bishop (1966).

Fraction	NADPH produced (nmol/min per mg of protein)		
	17 β -Hydroxy steroid dehydrogenase	Glucose 6-phosphate dehydrogenase	6-Phosphogluconate dehydrogenase
Total haemolysate	± 0.001	6.6	3.1
Second $(\text{NH}_4)_2\text{SO}_4$ precipitate	0.1–0.2	280	0.38
Sephadex G-150 fraction	0.8–1.3	650	0.22
DEAE-Sephadex A-50 fraction	0.3	1200	

in the fraction precipitating between 20% and 50% of saturation of the enzyme preparation with $(\text{NH}_4)_2\text{SO}_4$. The enzyme was purified further by chromatography on Sephadex G-150. Part of the enzymic activity was lost in this step (Table 2). Initial attempts to purify the enzyme on a DEAE-Sephadex A-50 column were not successful. Although a threefold purification and removal of residual traces of haemoglobin could be obtained, the recovery of the enzymic activity was less than 10%. During the purification procedure of the steroid dehydrogenase the glucose 6-phosphate dehydrogenase activity of the different fractions also increased considerably (Table 3). The glucose 6-phosphate dehydrogenase activity of these fractions was much higher than the steroid dehydrogenase activity.

In contrast the 6-phosphogluconate dehydrogenase activity was lost during the $(\text{NH}_4)_2\text{SO}_4$ precipitation procedure.

The analysis of the different fractions collected from the Sephadex G-150 column revealed that glucose 6-phosphate dehydrogenase and steroid dehydrogenase activity were clearly separated (Fig. 1), although small amounts of glucose 6-phosphate dehydrogenase were always present in fractions containing steroid dehydrogenase activities. The fraction nos. 95–120 (Fig. 1) were collected, concentrated by $(\text{NH}_4)_2\text{SO}_4$ precipitation as described in the Materials and Methods section and used as purified enzyme preparation.

Properties of the enzyme

An approximate value for the molecular weight of the enzyme was estimated on Sephadex G-150, according to the method of Andrews (1965) as described by Determan (1968). By using this method the molecular weight of a partially purified enzyme

Fig. 2. Determination of the pH optimum for the reaction testosterone $\rightarrow$ androstenedione catalysed by the purified 17 β -hydroxy steroid dehydrogenase preparation from human erythrocytes

NADPH produced (nmol/30min) at 37°C was measured spectrophotometrically as described in the Materials and Methods section. The buffer solutions used were: ●, 0.04M-phosphate buffer; Δ, 0.04M-tris-HCl buffer; ■, 0.04M-glycine buffer.

can be calculated from a plot of the logarithm of the molecular weight of various reference proteins versus the elution volume of these proteins. A value of 64000 was calculated for the steroid dehydrogenase. This value for the molecular weight can only be considered to represent an approximation of the true molecular weight if no dissociation into subunits

or association of enzyme monomers occurs on the Sephadex column.

The pH-dependence for the enzymic conversion of testosterone into androstenedione is shown in Fig. 2. The reaction rate did not vary much in the range pH 8.5–10 and was somewhat dependent on the buffer solution used. For the standard spectrophotometric assay a tris-HCl buffer, pH 8.5, was used. The buffer solution contained NaCl in physiological concentration. Although omission of NaCl at pH 8.5 had little effect on the conversion rate of testosterone, an effect of ionic strength on the conversion of oestradiol and oestradiol sulphate by an erythrocyte steroid dehydrogenase was reported by Jacobsohn & Hochberg (1968). The conversion of androstenedione into testosterone was expected to have an optimum value at acidic pH. The enzyme preparations became turbid at pH values below 7, and the enzymic activity was almost completely lost. Incubations to study the reduction of the oxo steroids were therefore carried out at pH 7.5. These reactions could not be studied by the spectrophotometric assay, because the oxidation velocity of NADPH was too slow for accurate measurements and could not be accelerated by greater amounts of substrate as a result of the limited solubility of the steroids. The cofactor

dependence of the hydroxy steroid dehydrogenase is given in Table 4. The enzyme has an absolute requirement for NADP⁺ or NADPH for its action. No measurable conversion could be detected in the presence of NAD⁺ or NADH.

Michaelis-Menten constants for the conversion of testosterone into androstenedione were estimated by the spectrophotometric assay. The K_m values for testosterone oxidation were obtained as shown in Fig. 3. The K_m value with testosterone as variable substrate was 1.7×10^{-4} M and with NADP⁺ as variable substrate 2.4×10^{-4} M. Although the values found in duplicate experiments were always in agreement, these results should be interpreted with reservation because they were obtained with only partially purified preparations, which exhibit comparatively little enzyme activity. Owing to limited substrate solubility and low reaction velocities, wider ranges of substrates could not be used.

Substrate specificity

The results of experiments concerning the substrate specificity of the enzyme are summarized in Table 5. A comparison was made of the conversion rates of different steroids by enzyme fractions obtained during

Table 4. Cofactor requirement of human erythrocyte 17 β -hydroxy steroid dehydrogenase

Enzymic activities were determined as described in the Materials and Methods section, with the exception of the cofactor solution used. The incubations with [¹⁴C]testosterone (A) contained 16 μ M steroid; with [¹⁴C]-androstenedione (C) 0.3 μ M steroid was used and incubations were for 3 h. In the spectrophotometric assay (B), NAD(P)H production was measured for 30 min in a cuvette containing 160 μ M steroid, 0.3 mM-NAD⁺, 0.3 mM-NADP⁺, 0.3 mM-NADH, 0.3 mM-NADPH. NADH-generating system: 0.3 mM-NADH, 0.01 ml of ethanol and 0.6 unit of alcohol dehydrogenase (EC 1.1.1.1)/ml. NADPH-generating system: 0.3 mM-NADPH, 1 mM-glucose 6-phosphate and glucose 6-phosphate dehydrogenase (EC 1.1.1.49) (0.15 unit/ml). The range of the observed values is given.

(A) Testosterone oxidation: [¹⁴ C]testosterone → [¹⁴ C]androstenedione		
Cofactor	Androstenedione formed (nmol)	
NADP ⁺	2.6–3.1	
NAD ⁺	0.10–0.45	
None	0.10–0.35	
(B) Testosterone oxidation (<i>E</i> ₃₄₀)		
Cofactor	NAD(P)H formed (nmol)	
NADP ⁺	6.4	
NAD ⁺	<0.15	
None	<0.15	
(C) Androstenedione reduction: [¹⁴ C]androstenedione → [¹⁴ C]testosterone		
Cofactor	Testosterone formed (nmol)	
NADPH	0.054–0.058	
NADPH-generating system	0.051–0.065	
NADH	0.004–0.005	
NADH-generating system	0.004–0.005	
None	0.003–0.004

Fig. 3. Double-reciprocal plots of testosterone oxidation in the presence of various amounts of NADP⁺ and of NADP⁺ reduction in the presence of various amounts of testosterone

(a) Testosterone oxidation by 17β-hydroxy steroid dehydrogenase from human erythrocytes with NADP⁺. Concentration of NADP⁺ used: ●, 0.15 mM; □, 0.30 mM; ▲, 0.60 mM; ○, 0.90 mM. (b) NADP⁺ reduction by 17β-hydroxy steroid dehydrogenase from human erythrocytes with testosterone. Concentrations of testosterone used: ●, 43 μM; □, 86 μM; ▲, 172 μM; ○, 258 μM. The enzymic activity was determined spectrophotometrically as described in the Materials and Methods section. *v*, nmol of NADPH produced/min by 1 mg of enzyme preparation in 1 ml of buffer solution.

Table 5. Conversion of different steroids by human erythrocyte 17-βhydroxy steroid dehydrogenase

The amounts of steroids converted are expressed relative to the amount of testosterone oxidized to androstenedione in the haemolysate. The enzyme preparations used were an erythrocyte haemolysate obtained by repeated freezing and thawing of erythrocytes in Krebs–Ringer buffer (final concn. 150mg of protein/ml of incubation medium), the enzyme preparation obtained after the second precipitation with (NH₄)₂SO₄ as described in the Materials and Methods section, and the preparation obtained after Sephadex G-150 chromatography. Incubations with labelled substrates (16 nmol) were carried out as described in the Materials and Methods section.

Enzyme preparation	Amount of steroid converted		
	Haemolysate	(NH ₄) ₂ SO ₄ precipitate	Sephadex G-150 fraction
Cofactor NADP ⁺ :			
Testosterone → androstenedione	1	1120	6500
Androstenediol → dehydroepiandrosterone	4	2750	13000
Oestradiol → oestrone	1.3	2500	9500
20α-Hydroxypregn-4-en-3-one → progesterone	0.7	<10	<50
Cofactor NADPH:			
Androstenedione → testosterone	1.7	125	600
Dehydroepiandrosterone → androstenediol	3.5	500	2800
Oestrone → oestradiol	1.5	225	1300
Progesterone → 20α-hydroxypregn-4-en-3-one	2	<5	<25
Dehydroepiandrosterone sulphate → androstenediol sulphate	2	350	1500
Oestrone sulphate → oestradiol sulphate	2	500	2500

the purification procedure. All rates are expressed relative to the reaction rate for the conversion of testosterone into androstenedione in a haemolysate. The

upper part of Table 5 gives the oxidation rates of the different steroids. Androstenediol was converted by all fractions more rapidly than testosterone. The

enzyme preparation can also oxidize 17β-hydroxy-5α-androstan-3-one (dihydrotestosterone). The conversion rates of testosterone, androstenediol and oestradiol were much higher for the more purified fractions.

Fig. 4. Inhibition of the conversion oestradiol → oestrone by androstenediol

▲, Double-reciprocal plot of the uninhibited reaction; ■, double-reciprocal plot of the reaction inhibited by the addition of 86 nmol of androstenediol. The enzymic activity was determined by incubation as described in the Materials and Methods section; the concentration of NADP⁺ was 0.4mM. Each point on the graph was calculated from three incubations of respectively 10, 20 and 30min duration. *v*, nmol of oestrone formed/min in 1ml of incubation medium.

However, the reaction rate of 20α-hydroxypregn-4-en-3-one with the fractions obtained after (NH₄)₂SO₄ precipitation was comparatively small. The same pattern can be observed in the lower part of the table showing the rates of the reactions in the opposite direction. The speed of the reduction reactions with NADPH were relatively small, compared with the oxidation reactions. As well as the free dehydroepiandrosterone and oestrone, the steroid conjugates dehydroepiandrosterone sulphate and oestrone sulphate were also converted by the purified enzyme preparations.

These results lead to the conclusion that the erythrocyte contains two dehydrogenases for steroids. The enzymic activity attacking the 20-oxo group of progesterone is lost during the purification procedure.

The ratio of the amounts of 17-oxo steroid or 17β-hydroxy steroid converted by the different enzyme preparations remains more or less constant during purification. To obtain additional evidence for the hypothesis that only one enzyme in the partially purified preparation reacts with the 17β-hydroxyl group of different steroids, incubations were carried out in which two steroids were incubated together. The results of one of these experiments is given in Fig. 4. The effect of androstenediol on the conversion rate of labelled oestradiol into oestrone was measured at different oestradiol concentrations and the double-reciprocal plot of reaction rate versus oestradiol concentration was plotted. Oestradiol conversion was competitively inhibited by the presence of androstenediol. This effect is not due to limited availability of cofactor, because NADP⁺ was added in large excess. Results similar to those in Fig. 4 could be obtained if androstenediol was the labelled steroid and oestradiol was added to the medium. The conversion rate of androstenediol and oestradiol incubated together was considerably lower than the sum of the individual conversion rates of these steroids incubated alone.

Table 6. Effect of partial heat-denaturation of 17β-hydroxy steroid dehydrogenase on conversion rate of androstenediol into dehydroepiandrosterone and of oestradiol into oestrone

The partially heat-denatured enzyme preparation was obtained by heating 0.15ml of the purified enzyme preparation diluted with 2.5ml of Krebs-Ringer solution, pH7.35, for 15min at 43°C. Incubation with 16nmol of labelled substrate was carried out for 30min at 37°C as described in the Materials and Methods section. Expts. A and B are duplicate experiments with different enzyme preparations.

Expt.	Substrate	Product formed (nmol)		Residual activity after heat-denaturation (%)
		Original enzyme preparation	Heat-denatured enzyme preparation	
A	Androstenediol	5.5	3.3	63
A	Oestradiol	2.7	1.5	57
B	Androstenediol	3.60	1.95	55
B	Oestradiol	1.93	0.87	45

Table 7. *Effect of various agents on the conversion of different steroids by human erythrocyte 17 β -hydroxy steroid dehydrogenase*

Conversion rates of steroid substrates were determined as described in the Materials and Methods section. Reaction rates with testosterone and androstenediol were measured spectrophotometrically; the conversion of androstenedione was determined by incubation. The reaction rates in the presence of the added compounds are given as percentages of the reaction rate of a control experiment with the same concentrations of substrate and cofactor, but without addition. In the experiment with albumin the reaction medium contained 4% (w/v) human serum albumin.

Substrate (nmol)	Cofactor (nmol)	Substance added (nmol)	Relative conversion rate (without addition = 100)
Testosterone	NADP ⁺ 400	Androstenedione 320	88
		Androstenedione 320	71
		NADPH 200	40
		NADPH 400	30
		Dehydroepiandrosterone 250	75
		sulphate	
		Dehydroepiandrosterone 250	75
		sulphate	
		<i>p</i> -Chloromercuribenzoate 10	80
		<i>p</i> -Chloromercuribenzoate 100	32
		Chloromercuribenzenes- <i>p</i> -sulphonate 10	82
		Chloromercuribenzenes- <i>p</i> -sulphonate 100	38
		Mg ²⁺ 1000	100
		Mn ²⁺ 100	100
		Zn ²⁺ 10	90
		Albumin	21
Androstenediol	NADP ⁺ 200	NADPH 100	71
		NADPH 200	56
		NADPH 400	35
		Dehydroepiandrosterone 250	75
		sulphate	
160	400	Dehydroepiandrosterone 250	80
		sulphate	
Androstenedione 16	NADPH 200	NADP ⁺ 200	83
		NADP ⁺ 400	72

Heat-denaturation of the enzyme preparation affects both the capacity of this preparation to catalyse the oxidation of the 17 β -hydroxyl group of oestradiol and of androstenediol in a comparable way (Table 6). Although the residual activity after heat-treatment for the conversion of oestradiol was somewhat lower, this difference cannot be considered significant.

Effect of reaction products and various other agents on enzymic activity

The effect of addition of an excess of NADPH during the oxidation of both testosterone and androstenediol in the presence of NADP⁺ was comparatively

large (Table 7). If twice as much NADPH was added as NADP⁺ the oxidation rate was only in the order of 30% of the uninhibited reaction. Addition of NADP⁺ during the reduction of androstenedione had much less effect. Also, after addition of the 17-oxo steroids androstenedione or dehydroepiandrosterone sulphate only small inhibitory effects were observed under the conditions of the experiments described in Table 7. The thiol-group-blocking agents *p*-chloromercuribenzoate and chloromercuribenzenes-*p*-sulphonic acid inhibited the conversion of testosterone into androstenedione. Addition of the cations Mg²⁺, Mn²⁺ and Zn²⁺ to the assay medium had little effect. The addition of albumin, known to bind steroids in a non-specific manner (Sandberg *et al.*, 1957), caused a considerable decrease in reaction velocity.

Discussion

The enzyme 17 β -hydroxy steroid dehydrogenase was isolated from the membrane-free haemolysate of human erythrocytes. Green *et al.* (1965) and Wins & Schoffeniels (1969) have suggested that most glycolytic enzymes and oxidoreductases are associated with the membrane of erythrocytes. It is not very likely that this conclusion also applies to the 17 β -hydroxy steroid dehydrogenase of erythrocytes, because in our experiments the enzyme was solubilized under mild conditions of haemolysis. It is not possible, however, to distinguish between an enzyme really free in the intracellular fluid and an enzyme associated with the membrane with very weak forces, which may be solubilized during haemolysis.

The steroid dehydrogenase was purified by $(\text{NH}_4)_2\text{SO}_4$ precipitation and gel filtration. The erythrocyte contains relatively small amounts of this enzyme and considerable activity was lost during chromatography on Sephadex or on ion-exchange columns. It was therefore only possible to obtain a partially purified preparation. The enzyme can catalyse the oxidation of 17 β -hydroxy steroids, as well as the reduction of 17-oxo steroids. The activity per amount of protein of the purified preparation for the reduction of androstenedione to testosterone was at least 350 times higher than that of the crude erythrocyte haemolysate. For the oxidation of testosterone to androstenedione and of androstenediol to dehydroepiandrosterone the difference between specific activities of haemolysates and purified preparations was still higher (3000–6000 times) (Table 5). It is unlikely that the different purification factors obtained for the different steroid substrates in the assay is due to the presence of two different enzymes for the oxidation and the reduction at the 17-oxygen position of the steroid molecule. Therefore a difference in binding of the substrates testosterone and androstenedione to proteins in the haemolysate may be considered. Other studies from our laboratory have shown that the proteins in an erythrocyte haemolysate can bind considerable amounts of steroids and that testosterone is bound more firmly than androstenedione (A. O. Brinkmann & H. J. Van der Molen, unpublished work). Therefore the concentration of free testosterone in the haemolysate will be lower than the concentration of free androstenedione, resulting in relatively lower conversion rates for testosterone by a haemolysate. During the assay of the enzymic activity of the purified enzyme preparation, the incubation medium contains only small amounts of protein and binding of steroid to proteins will be of minor importance. The difference between conversion rates of steroid substrates by haemolysates and purified enzyme fractions might therefore be greater for testosterone than for androstenedione.

The availability of cofactors in the haemolysate

might also affect the conversion rates of the different steroids. The enzyme showed an almost absolute requirement of NADP^+ for oxidation of 17 β -hydroxy steroids and of NADPH for reduction of 17-oxo steroids. If traces of endogenous NADPH are present in the haemolysate in addition to the amount of NADP^+ added, the conversion of testosterone into androstenedione might be partly inhibited. This inhibitory effect of NADPH on the oxidation of 17 β -hydroxy steroids could be demonstrated by the addition of NADPH during oxidation of testosterone with the purified enzyme preparation in the presence of NADP^+ (Table 7).

Traces of glucose 6-phosphate in the haemolysate together with glucose 6-phosphate dehydrogenase might favour the availability of the cofactor in the reduced state. Van der Molen & Groen (1968) demonstrated that erythrocytes can catalyse the inter-conversion between progesterone and 20 α -hydroxy-pregn-4-en-3-one. During purification of the 17 β -hydroxy steroid dehydrogenase activity the enzymic activity catalysing the conversion of 20 α -hydroxy-pregn-4-en-3-one was lost.

The results of our study of the substrate specificity of 17 β -hydroxy steroid dehydrogenase preparations suggest that a single enzyme with a broad substrate specificity catalyses the conversion of the 17 β -hydroxyl or 17-oxo group of androstanes as well as the conversion between oestradiol and oestrone. Both the conjugated steroids dehydroepiandrosterone sulphate and oestrone sulphate were readily converted.

Conclusive evidence for the presence of only one 17 β -hydroxy steroid dehydrogenase in erythrocytes cannot be obtained as long as the enzyme is not completely pure. Many of the criteria (Dixon & Webb, 1966) that are normally accepted as proof that a single enzyme in a partially purified preparation acts on several substrates are fulfilled, however, for the partially purified preparation used for the present studies. The ratio of the enzymic activities for conversion of the various steroid substrates with a 17 β -hydroxyl or 17-oxo group was constant for the enzyme preparations obtained in the different purification steps. In addition the reaction rate for conversion of two of the substrates, androstenediol and oestradiol, added simultaneously to the incubation was smaller than the sum of the individual reaction rates and each substrate competitively inhibited the conversion of the other substrate. Also partial heat-denaturation of the enzyme preparation resulted in a comparable decrease in enzyme activity for these two substrates.

The biological significance of the 17 β -hydroxy steroid dehydrogenase in human erythrocytes is still not clear. The enzyme might have some effect on the ratio oxidized steroid/reduced steroid under conditions *in vivo* (e.g. of androstenedione and testos-

sterone) as has been discussed previously (Van der Molen & Groen, 1968). Patt *et al.* (1970) observed small differences in reduction rate of androstenedione incubated in Krebs-Ringer buffer with washed erythrocytes of non-pregnant, pregnant or hirsute women. The amount of enzyme in the erythrocytes is comparatively small and when erythrocytes are suspended in plasma only small amounts of steroid (e.g. 10% of the testosterone in plasma) are bound to the erythrocytes (Sandberg *et al.*, 1966).

The enzyme 17 β -hydroxy steroid dehydrogenase could also have a function in the regulation of the ratio 17-oxo steroids/17 β -hydroxy steroids in the erythrocyte itself. In this respect the inhibition of glucose 6-phosphate dehydrogenase from mammalian cells by certain oxo steroids is noteworthy (Marks & Banks, 1960; McKerns & Kaleita, 1960; Douville & Warren, 1968). Dehydroepiandrosterone and androstenedione demonstrate a particularly strong inhibitory effect on glucose 6-phosphate dehydrogenase. It is known from the work of Theorell *et al.* (1966) that certain alcohol dehydrogenases from liver can convert steroids. Therefore the possibility was considered that glucose 6-phosphate dehydrogenase is not only inhibited by steroid, but that glucose 6-phosphate dehydrogenase also has the capability to reduce the oxo steroid. This hypothesis was not supported by the results of Sephadex column chromatography (Fig. 1), which clearly showed that steroid dehydrogenase and glucose 6-phosphate dehydrogenase could be separated.

If glucose is available to the erythrocytes, as under conditions *in vivo*, then the large amounts of glucose 6-phosphate dehydrogenase compared with 17 β -hydroxy steroid dehydrogenase will favour the formation of 17 β -hydroxy steroids from 17-oxo steroids. Inhibition of glucose 6-phosphate dehydrogenase by steroids in the erythrocyte might therefore be of minor importance, whereas the 17 β -hydroxy steroids do not inhibit glucose 6-phosphate dehydrogenase (Marks & Banks, 1960; McKerns & Kaleita, 1960; Douville & Warren, 1968).

The authors are grateful for the supply of out-dated blood obtained from the Blood Bank of the Netherlands Red Cross at Rotterdam.

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