Purification and Properties of a New Testosterone 17 β -Dehydrogenase (NADP⁺) from Guinea-Pig Liver

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As a result of studies of guinea-pig liver testosterone 17 β -dehydrogenase (NADP⁺) (EC 1.1.1.64), a new testosterone 17 β -dehydrogenase was discovered. The new enzyme was purified to a single homogeneous protein from the 105000g-supernatant fraction of guinea-pig liver by (NH₄)₂SO₄ fractional precipitation and two gel-filtration stages, DEAE-cellulose column chromatography and hydroxyapatite column chromatography. It was characterized by many properties. The enzyme has almost the same properties as the classical testosterone 17 β -dehydrogenase (NADP⁺) (EC 1.1.1.64), with respect to cofactor requirement, pH optima for dehydrogenation, effect of phosphate ion on the NAD⁺-dependent reaction and molecular weight, but characteristic differences were observed in substrate-specificity between the two dehydrogenases. With various androstane derivatives, the configuration of the A/B-ring junction was closely connected with enzyme activity. 5 α -Androstanes, such as 5 α -androstane-3 α ,17 β -diol, 5 α -androstane-3 β ,17 β -diol and 17 β -hydroxy-5 α -androstane-3-one, and 5 β -congeners, such as 5 β -androstane-3 α ,17 β -diol, 5 β -androstane-3 β ,17 β -diol and 17 β -hydroxy-5 β -androstane-3-one, served as substrates for both the EC 1.1.1.64 enzyme and the new enzyme. The EC 1.1.1.64 enzyme oxidized testosterone more rapidly than did the new enzyme. These comparisons were based on the relative activities, apparent K_m values and apparent V_{max} values.

In a previous paper we demonstrated that testosterone 17 β -dehydrogenase (NADP⁺) (EC 1.1.1.64), obtained from guinea-pig liver cytosol, participates in the reversible oxidation of 3-hydroxyhexobarbital [5-(3'-hydroxy-1'-cyclohexen-1'-yl)-1,5-dimethylbarbituric acid], the microsomal oxygenation product of hexobarbital (Kageura & Toki, 1971). This enzyme was subsequently purified to homogeneity as 3-hydroxyhexobarbital dehydrogenase (Kageura & Toki, 1975). During investigation of 3-hydroxyhexobarbital dehydrogenase as a testosterone 17 β -dehydrogenase (NADP⁺) (EC 1.1.1.64), another testosterone 17 β -dehydrogenase was isolated from the same source (Kageura & Toki, 1971, 1974).

The present paper describes the purification and properties of the new testosterone 17 β -dehydrogenase (referred to as the 'new enzyme' throughout). The role of this enzyme in the metabolism of 17 β -hydroxy steroids has been discussed in comparison with that of classical testosterone 17 β -dehydrogenase (NADP⁺) (referred to as the 'EC 1.1.1.64 enzyme' throughout).

Materials and Methods

Materials

Testosterone was supplied from Teikoku Hormone Manufacturing Co., Tokyo, Japan. 17 β -Hydroxy-5 β -androstane-3-one was purchased from Ikapharm,

Ramat-Gan, Israel; 5 β -androstane-3 β ,17 β -diol, 17 α -hydroxyandrost-4-en-3-one and androst-5-ene-3 β ,17 β -diol were from Steraloids, Pawling, NY, U.S.A.; 17 β -hydroxy-5 α -androstane-3-one and other steroids were from Sigma Chemical Co., St. Louis, MO, U.S.A.; the oxidized and reduced forms of NAD and NADP were from Oriental Yeast Co., Tokyo, Japan; cytochrome *c* (horse heart), myoglobin (sperm-whale), chymotrypsinogen A (ox pancreas), ovalbumin and albumin (bovine serum) were from Mann Research Laboratories, Orangeburg, NY, U.S.A.; alcohol dehydrogenase (horse liver and yeast) was from Boehringer, Mannheim, Germany; Sephadex G-25, Sephadex G-75 (superfine grade) and Sephadex G-100 were from Pharmacia Fine Chemicals AB, Uppsala, Sweden; DEAE-cellulose (DE-32) was from W. and R. Balston, Maidstone, Kent, U.K.; hydroxyapatite was from Seikagaku Kogyo Co., Tokyo, Japan; Ampholine carrier ampholites were from LKB Produkter AB, Bromma, Sweden.

Methods

Enzyme assay. Testosterone 17 β -dehydrogenase activity was measured by the change in A_{340} (1 cm light-path) in a Shimadzu (Kyoto, Japan) Double-40 spectrophotometer. The reaction mixture was 1.5 ml containing 0.1 M-Na₂HPO₄/0.01 M-NaOH buffer, pH 10.7, 0.15 μ mol of testosterone (in methanol),

0.5 μ mol of NADP⁺ and enzyme solution (0.02–0.05 ml). The reaction was carried out at 25°C. A unit of activity was defined as the amount of enzyme that forms 1 μ mol of NADPH/min at 25°C. Specific activity is expressed in terms of units/mg of protein. Protein concentration was determined by the method of Lowry *et al.* (1951) after all the protein was precipitated by the method of Folin & Wu (1919); bovine serum albumin was the standard.

Purification of testosterone 17 β -dehydrogenase. The new testosterone 17 β -dehydrogenase was purified by a modification of the procedure for 3-hydroxyhexobarbital dehydrogenase (Kageura & Toki, 1975). All steps were carried out at 0–4°C and each enzyme fraction was concentrated in a Diaflo PM-10 filter (Amicon Corp., Lexington, MA, U.S.A.). Standard sodium phosphate buffer (0.005 M-, 0.05 M- or 0.1 M-NaH₂PO₄/Na₂HPO₄), pH 8.0, containing 0.05% 2-mercaptoethanol was used.

Step 1: Tissue extraction. The livers obtained from Hartley male guinea pigs (about 700 g body wt.) were homogenized with 2 vol. of 0.1 M-NaH₂PO₄/Na₂HPO₄ buffer, pH 7.4, and centrifuged successively at 9000 g for 20 min in a Kubota (Tokyo, Japan) KR-200B refrigerated centrifuge, and at 105 000 g for 60 min in a Martin Christ (Osterode am Hartz, Germany) Omega I ultracentrifuge.

Step 2: (NH₄)₂SO₄ fractionation. The 105 000 g supernatant was treated with saturated (NH₄)₂SO₄ solution adjusted to pH 7.4 with aq. NH₃, and the precipitate between 45 and 80% saturation was collected by centrifugation. The precipitate was dissolved in 47.5 ml of standard 0.1 M-sodium phosphate buffer, and the solution was subdivided into 12 ml portions, which were stored at –20°C.

Step 3: Sephadex G-100 gel filtration. The (NH₄)₂SO₄ fraction (12 ml) was applied to a Sephadex G-100 column (2.5 cm $\times$ 90 cm), and eluted with standard 0.05 M-sodium phosphate buffer at a flow rate of 15 ml/h. The active fractions were combined and concentrated to about 2 ml.

Step 4: Sephadex G-75 gel filtration. The Sephadex G-100 fraction was applied to a Sephadex G-75 (superfine) column (2.5 cm $\times$ 90 cm). Separation was carried out with the same buffer as in step 3, at a flow rate of 3 ml/h. The active fractions (about 96 ml) were combined and concentrated to 2 ml. To the concentrated solution, 10 ml of standard 0.005 M-sodium phosphate buffer was added and concentrated again. This process was repeated three times.

Step 5: DEAE-cellulose DE-32 column chromatography. The concentrated Sephadex G-75 fraction was applied to the DEAE-cellulose column (1.5 cm $\times$ 75 cm), and eluted with a linear gradient formed from 300 ml each of standard 0.005 M- and 0.05 M-sodium phosphate buffer. Fractions (10 ml) were collected at a flow rate of 5 ml/h. During this step, the enzyme activity catalysing the dehydrogenation

of testosterone was separated into two peaks, fraction A (tubes 9–14) and fraction B (tubes 20–30). Fraction A (30 ml) was combined and concentrated to 4 ml. It was confirmed that fraction B corresponds to classical testosterone 17 β -dehydrogenase (NADP⁺) (EC 1.1.1.64), which also possesses 3-hydroxyhexobarbital dehydrogenase activity.

Step 6: Hydroxyapatite column chromatography. The concentrated fraction A was applied to a hydroxyapatite column (1.5 cm $\times$ 13 cm). Protein was eluted from the column with a linear gradient formed from 100 ml each of standard 0.05 M-sodium phosphate buffer and the same buffer containing 0.5 M-NaCl. Fractions (10 ml) were collected at a flow rate of 15 ml/h. The active fractions (tubes 14–19) were collected and used for the study of the new enzyme. The concentrated fraction B (5.4 ml) was also applied to a hydroxyapatite column (1.5 cm $\times$ 10 cm) and eluted with a linear gradient formed from 100 ml each of standard 0.05 M- and 0.1 M-sodium phosphate buffer. The active fractions (tubes 13–18) were collected and used for the study of the EC 1.1.1.64 enzyme.

Electrophoresis. Polyacrylamide-gel disc electrophoresis and sodium dodecyl sulphate/polyacrylamide-gel disc electrophoresis were performed by the methods of Davis (1964) and of Weber & Osborn (1969) respectively. Density-gradient isoelectric focusing was performed as described by Haglund (1971). In each case, the purified enzyme solution was desalted by passage through a Sephadex G-25 column (1.5 cm $\times$ 40 cm) with 0.05 M-NaH₂PO₄/Na₂HPO₄ buffer, pH 8.0, or 1% glycine solution. All other procedures were carried out as reported previously (Kageura & Toki, 1975).

Results

Enzyme purification

In the present purification procedure an effort was made to eliminate contaminating protein which persistently accompanied the new enzyme. A summary of the purification procedure of the guinea-pig liver testosterone 17 β -dehydrogenases is given in Table 1. The two enzymes were separated from each other by DEAE-cellulose DE-32 column chromatography. At the final step, the new enzyme was purified 48-fold from the supernatant fraction with a yield of 11%, and the EC 1.1.1.64 enzyme was purified 171-fold with a yield of 33%. A quarter of the total testosterone dehydrogenating activity was due to the new enzyme.

Purity of enzyme

The purified preparation of the new enzyme was examined by polyacrylamide-gel disc electrophoresis. At pH 9.4, the enzyme migrated as a single zone

Table 1. *Summary of purification of the new testosterone 17 β -dehydrogenase and the EC 1.1.1.64 enzyme*

The enzymes were assayed with testosterone as described under 'Methods'. The supernatant was obtained from 123.5g of guinea-pig liver.

Fraction	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification	Yield (%)
Supernatant	7831	106	0.014	1	100
(NH ₄) ₂ SO ₄	3306	89	0.027	2	84
Sephadex G-100	430	77	0.178	13	72
Concentrated	427	77	0.180	13	73
Sephadex G-75	143	63	0.439	33	59
Concentrated	141	60	0.428	32	57
DEAE-cellulose DE-32					
New enzyme	66	12	0.189	14	12
EC 1.1.1.64	37	39	1.049	78	37
Concentrated					
New enzyme	65	12	0.186	14	11
EC 1.1.1.64	37	37	0.992	74	35
Hydroxyapatite					
New enzyme	18	11	0.647	48	11
EC 1.1.1.64	15	35	2.316	172	33

and did not contain any minor contaminating protein. The enzyme also appeared as a single sharp band by polyacrylamide-gel disc electrophoresis in sodium dodecyl sulphate.

Substrate specificity

The relative rates of oxidation, apparent K_m values and apparent V_{max} values for various types of 17 β -hydroxy steroids are listed in Table 2. In contrast with 5 α -androstane derivatives, all of the 5 β -isomers showed higher activity than testosterone even at lower substrate concentration. Among these compounds, 5 β -androstane-3 α ,17 β -diol exhibited extremely high activity, 12 times that of testosterone at three-fifths of the substrate concentration. On the contrary, 5 α -isomers showed lower activity than testosterone. The relative rate for 19-nortestosterone was about 90% of that for testosterone. Several 3-hydroxy steroids having a 17-oxo group were examined; however, the rate of oxidation of these compounds was less than 10% of that of testosterone or zero. Oestradiol-17 β and 17 α -hydroxy steroids (e.g. 17 α -hydroxyandrost-4-ene, 17 α -hydroxy-5 β -androst-3-one and oestradiol-17 α) did not act as substrates.

The apparent K_m value and apparent V_{max} value were calculated by the method of Lineweaver & Burk (1934). Most of the K_m values obtained from various steroids ranged from 1 to 10 μ M. 17 β -Hydroxy-5 β -androst-3-one showed the lowest K_m value and 17 β -hydroxy-5 α -androst-3-one the highest. The V_{max} values almost parallel the relative rates of oxidation of the steroids. The K_m/V_{max} values

indicate that the new enzyme utilizes 5 β -androstane-3 α ,17 β -diol and 17 β -hydroxy-5 β -androst-3-one most efficiently as substrates; 17 β -hydroxy-5 α -androst-3-one and 19-nortestosterone are not good substrates for the enzyme.

Isoelectric density-gradient electrofocusing

Fig. 1 shows the results of the isoelectric-focusing study. In this experiment, three substrates, testosterone, 17 β -hydroxy-5 β -androst-3-one and 17 β -hydroxy-5 α -androst-3-one, all gave the same isoelectric point of pH 8.94. The homogeneity of the enzyme preparation was also confirmed, because only the third peak monitored by the A_{280} contained protein, and this coincided with enzyme activity.

Test with combined substrates

To confirm that the new enzyme dehydrogenates different types of 17 β -hydroxysteroids, testosterone, 17 β -hydroxy-5 β -androst-3-one and 17 β -hydroxy-5 α -androst-3-one were selected as substrates and the mixed-substrate test was performed by the method of Adams (1949). As shown in Table 3, the total rate of the reaction was less than the sum of the rates of the reactions measured separately. These results indicate that a single enzyme is responsible for the oxidation of the 17 β -hydroxy steroids.

Detection and identification of the reaction product

The reaction product was extracted with ethyl acetate and identified by t.l.c. on silica gel HF₂₅₄

Table 2. *Substrate specificity of new testosterone 17 β -dehydrogenase*

Relative activities were measured with 0.1 mM of substrate at 15s intervals. Details are described under 'Methods'. The K_m and V_{max} values were calculated from double-reciprocal plots. The maximal substrate concentration used for kinetic studies was 0.06 mM (substrates 3 and 7) and 0.1 mM (other steroids listed). The following compounds showed less than 10% of the relative activity observed with testosterone: 3 α -hydroxy-5 α -androstan-17-one, 3 β -hydroxy-5 α -androstan-17-one, 3 α -hydroxy-5 β -androstan-17-one, 3 β -hydroxy-5 β -androstan-17-one, androst-5-ene-3 β ,17 β -diol and 3 β -hydroxyandrost-5-en-17-one. The following compounds showed no detectable activity: 17 α -hydroxyandrost-4-en-3-one, 17 α -hydroxy-5 β -androstan-3-one, oestradiol-17 α and oestradiol-17 β .

Substrate	Relative activity (%)	K_m (μ M)	V_{max} (μ mol/min per mg of protein)	K_m/V_{max}
1. Testosterone	100	27	0.75	36
2. 19-Nortestosterone	93	53	0.79	67
3. 5 α -Androstane-3 α ,17 β -diol*	63	23	0.61	37
4. 5 α -Androstane-3 β ,17 β -diol*	25	—	—	—
5. 17 β -Hydroxy-5 α -androstan-3-one	30	77	0.42	183
6. 5 α -Androstan-17 β -ol*	3	—	—	—
7. 5 β -Androstane-3 α ,17 β -diol*	1180	11	8.9	1
8. 5 β -Androstane-3 β ,17 β -diol	134	29	0.96	30
9. 17 β -Hydroxy-5 β -androstan-3-one	330	6.7	3.0	2
10. 5 β -Androstan-17 β -ol*	117	—	—	—

* Because of the low solubility of these compounds, the reaction was performed at 37°C. Substrate concentrations used for the reaction mixture were 0.06 mM for 5 α -androstan-3 α ,17 β -diol and 5 β -androstan-3 α ,17 β -diol, and 0.02 mM for 5 α -androstan-3 β ,17 β -diol, 5 α -androstan-17 β -ol and 5 β -androstan-17 β -ol. By comparison with the activity of testosterone assayed at 37°C and 25°C, the results obtained for these substrates were converted into values at 25°C.

Fig. 1. *Isoelectric density-gradient electrophoresis of new testosterone 17 β -dehydrogenase*

The purified enzyme solution was desalted by passing through a Sephadex G-25 column with 1% glycine solution. The column was run in a pH3–10 ampholyte preparation for 41 h. ●, A_{280} ; ▲, pH; ○, enzyme activity with testosterone; △, enzyme activity with 17 β -hydroxy-5 α -androstan-3-one; □, enzyme activity with 17 β -hydroxy-5 β -androstan-3-one. All fractions (3 ml) were assayed as described under 'Methods'.

(E. Merck A.-G., Darmstadt, Germany) with benzene/acetone (4:1, v/v) as solvent. The products from testosterone, 17 β -hydroxy-5 β -androstan-3-one

Table 3. *Test with mixed substrates*

The enzyme was assayed as described under 'Methods'. The reaction was measured at 15s intervals.

Substrate	Concentration (mM)	NADPH produced (nmol/min per ml)
Testosterone	0.1	5.78
17 β -hydroxy-5 α -androstan-3-one	0.1	0.97
Combined	—	2.53
Testosterone	0.25	7.23
17 β -Hydroxy-5 β -androstan-3-one	0.01	23.14
Combined	—	9.64

and 17 β -hydroxy-5 α -androstan-3-one gave the same R_F values as authentic androst-4-ene-3,17-dione (R_F 0.61), 5 β -androstan-3,17-dione (R_F 0.49) and 5 α -androstan-3,17-dione (R_F 0.64) respectively. Each spot was detected under a u.v. lamp, and confirmed by a colour reaction after spraying with H_2SO_4 /methanol (1:1, v/v).

Enzyme stability

Although the enzyme activity without 2-mercaptoethanol decreased significantly within 3 days, the purified enzyme was stable for at least 10 days when kept in the presence of 0.05% 2-mercaptoethanol; it then decreased to less than 30% of the activity within 1 month at 0–4°C. The enzyme activity from

each purification step was lost by freezing, except for the $(\text{NH}_4)_2\text{SO}_4$ fraction, which retained activity for at least 2 weeks.

pH optima

The pH optima were 10.6 with NADP^+ and 6.1 with NADPH , and the forward reaction proceeded at a rate more than 15 times that of the backward reaction. The optimum pH value with NADP^+ was essentially identical with that of the EC 1.1.1.64 enzyme (pH 10.7) (Kageura & Toki, 1975), but the value with NADPH was different from the pH 7.9 for the EC 1.1.1.64 enzyme. At the optimal pH, the apparent K_m values were 27 μM for testosterone and 47 μM for NADP^+ .

Effect of phosphate ion

Although the activity with NADP^+ was relatively independent of the buffer composition, the activity with NAD^+ was influenced by the presence of phosphate ion and increased with the molar concentration of the phosphate buffer. Very similar phenomena were also observed with the EC 1.1.1.64 enzyme (Kageura & Toki, 1974, 1975).

The kinetic study was made with 17 β -hydroxy-5 β -androstane-3-one as substrate in 0.009 M- Na_2HPO_4 /0.091 M- $\text{Na}_4\text{P}_2\text{O}_7$ buffer, pH 10, and in 0.06 M-glycine/0.6 M- NaCl /0.04 M- NaOH buffer, pH 10. The K_m values for 17 β -hydroxy-5 β -androstane-3-one in the two different buffer components were identical (43 μM), and the $V_{\max}$ value in phosphate buffer was 5 times that in glycine buffer. On the other hand, the K_m values for NAD^+ were different in the two buffers: 0.68 mM in phosphate buffer and 3.5 mM in glycine buffer. NAD^+ gave the same $V_{\max}$ values of 17.8 nmol/min per ml in the two buffers. The results indicated that phosphate ion appears to play an effective role in the affinity of NAD^+ for the enzyme.

In phosphate buffer, the $V_{\max}$ value for 17 β -hydroxy-5 β -androstane-3-one was about 80% of that with NADP^+ . The optimum pH with NAD^+ was 9.9.

Molecular weight

The molecular weight as measured by Sephadex G-100 chromatography and sodium dodecyl sulphate/polyacrylamide-gel disc electrophoresis was 39 000 and 31 000 respectively. Bovine serum albumin (mol.wt. 68 000), ovalbumin (mol.wt. 45 000), horse

Fig. 2. Elution profile of the enzyme activities for 17 β -hydroxy-5 α -androstan-3-one and 17 β -hydroxy-5 β -androstan-3-one on a Sephadex G-100 column (a) and a DEAE-cellulose DE-32 column (b)

Details of the chromatographic procedures are given in the text. Each fraction was assayed for enzyme activities at 0.1 mM substrate concentration as described under 'Methods'. $\bullet$, A_{280} ; Δ , enzyme activity with 17 β -hydroxy-5 α -androstan-3-one; $\square$, enzyme activity with 17 β -hydroxy-5 β -androstan-3-one.

liver alcohol dehydrogenase (mol.wt. 41000), yeast alcohol dehydrogenase (mol.wt. 37000), chymotrypsinogen (mol.wt. 25000), myoglobin (mol.wt. 17800) and cytochrome *c* (mol.wt. 12400) were used as markers. However, alcohol dehydrogenases were omitted when gel filtration was performed.

Influence of temperature

The activation energy for the new enzyme, which catalyses the dehydrogenation of 17 β -hydroxy-5 β -androstane-3-one, was estimated to be 22.0 kJ (5270 cal) by Arrhenius plots over the range 25–45°C,

and 21.9 kJ (5250 cal) for the dehydrogenation of testosterone. However, it was elevated to 71.9 kJ (17200 cal) and 71.3 kJ (17060 cal) for the dehydrogenation of 17 β -hydroxy-5 β -androstane-3-one and testosterone respectively, provided that the new enzyme was free from 2-mercaptoethanol. On the other hand, the activation energy for the EC 1.1.1.64 enzyme was 27.2 kJ (6500 cal) for the dehydrogenation of 17 β -hydroxy-5 α -androstane-3-one, a typical substrate, and 28.0 kJ (6700 cal) for testosterone, irrespective of the presence of 2-mercaptoethanol. The maximal rate was achieved at 50°C for the new enzyme and at 47°C for the EC 1.1.1.64 enzyme.

*Because of the low solubility of 5 α -androstane-3 β ,17 β -diol, the specific activities obtained at 0.02 mM substrate concentration are listed instead of V_{max} values.

Scheme 1. Pathway of testosterone metabolism, and V_{max} values of new testosterone 17 β -dehydrogenase and the EC 1.1.1.64 enzyme

The V_{max} values for the new enzyme/the EC 1.1.1.64 enzyme are given above the arrows. Names of the compounds: I, testosterone; I-A, 17 β -hydroxy-5 α -androstane-3-one; I-A-1, 5 α -androstane-3 α ,17 β -diol; I-A-2, 5 α -androstane-3 β ,17 β -diol; I-B, 17 β -hydroxy-5 β -androstane-3-one; I-B-1, 5 β -androstane-3 α ,17 β -diol; I-B-2, 5 β -androstane-3 β ,17 β -diol; II, androst-4-ene-3,17-dione; II-A, 5 α -androstane-3,17-dione; II-A-1, 3 α -hydroxy-5 α -androstane-17-one; II-A-2, 3 β -hydroxy-5 α -androstane-17-one; II-B, 5 β -androstane-3,17-dione; II-B-1, 3 α -hydroxy-5 β -androstane-17-one; II-B-2, 3 β -hydroxy-5 β -androstane-17-one,

Comparison of the new enzyme and the EC 1.1.1.64 enzyme activities for 17 β -hydroxy-5 α -androstan-3-one and 17 β -hydroxy-5 β -androstan-3-one

The total activities of the two enzymes in the supernatant fraction were 96.3 units for 17 β -hydroxy-5 α -androstan-3-one and 128.4 units for 17 β -hydroxy-5 β -androstan-3-one. In the next (NH₄)₂SO₄ fraction, 85% of the activity for 17 β -hydroxy-5 α -androstan-3-one was recovered and 82% for the 5 β -isomer. The two enzymes were eluted simultaneously in approximately similar yields (72–74%) on Sephadex G-100 gel filtration (Fig. 2a), and were also eluted similarly to each other from Sephadex G-75. At the DEAE-cellulose DE-32 column-chromatography stage, the elution profile of each enzyme activity was separated into two peaks, although they ran closely parallel with each other (Fig. 2b). The yields of the two enzymes at the hydroxyapatite fraction were very different for the two substrates: for 17 β -hydroxy-5 α -androstan-3-one, 3.2% (new enzyme) and 37.7% (EC 1.1.1.64 enzyme); for 17 β -hydroxy-5 β -androstan-3-one, 30.6% (new enzyme) and 4.2% (EC 1.1.1.64 enzyme).

All the enzyme activities for 17 β -hydroxy-5 α -androstan-3-one and 17 β -hydroxy-5 β -androstan-3-one obtained from the supernatant fraction of guinea-pig liver correspond to either the new enzyme or the EC 1.1.1.64 enzyme, and each enzyme has selectivity for the configuration at the A/B-ring junction. When 17 β -hydroxy-5 β -androstan-3-one was used as substrate, the final purification of the new enzyme was 130-fold from the supernatant fraction.

Discussion

The new enzyme showed similarity to the classical testosterone 17 β -dehydrogenase (NADP⁺) (EC 1.1.1.64) from the same source; however, the most characteristic difference was in substrate-specificity. Only the established EC 1.1.1.64 enzyme is able to metabolize 3-hydroxyhexobarbital. Although both enzymes participated in the dehydrogenation of various androgenic 17 β -hydroxy steroids and, in

general, the new enzyme gave lower K_m values than the EC 1.1.1.64 enzyme, the reactivity of each enzyme was different in relation to steroidal A/B-ring configuration.

Scheme 1 shows the metabolic pathway of testosterone. The V_{max} values of the two enzymes for 17 β -hydroxy steroids are also cited. Testosterone is converted *in vivo* through two main pathways, the 17 β -hydroxyl pathway and the 17-ketonic pathway (Baulieu & Pierre, 1964a,b). Both testosterone 17 β -dehydrogenases are concerned with the several reactions connected directly with these two pathways. The V_{max} values indicated that the 17 β -hydroxy steroids with the 5 β -configuration were more suitable substrates than 5 α -analogues for the new enzyme, and vice versa for the EC 1.1.1.64 enzyme, except for 5 β -androstan-3 α ,17 β -diol. The results also showed that, when the V_{max} values of the two enzymes for the same substrate were compared, the new enzyme gave higher activity than the EC 1.1.1.64 enzyme for the dehydrogenation of 5 β -androgens, and vice versa for 5 α -androgens. It is thus characteristic that the two enzymes possess different activity for the metabolism of 5 α - and 5 β -congeners of 17 β -hydroxy steroids.

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