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A 3(17)β-HYDROXYSTEROID DEHYDROGENASE IN RAT ERYTHROCYTES

CONVERSION OF 5α-DIHYDROTESTOSTERONE INTO 5α-ANDROSTANE-3β,17β-DIOL AND PURIFICATION OF THE ENZYME BY AFFINITY CHROMATOGRAPHY

W. HEYNS and P. DE MOOR

Laboratorium voor Experimentele Geneeskunde, Katholieke Universiteit te Leuven, Rega Instituut, Minderbroedersstraat 10, B 3000 Leuven, (Belgium)

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SUMMARY

Rat erythrocytes contain a 3β-hydroxysteroid dehydrogenase, which converts 5α-dihydrotestosterone (17β-hydroxy-5α-androstan-3-one) reversibly into 5α-androstane-3β,17β-diol. This enzyme has a molecular weight of approx. 70 000, as estimated by Sephadex G-100 chromatography and is precipitated by $(NH_4)_2SO_4$ (25–33 g/100 ml). It is unstable under slightly alkaline conditions but stabilized by the addition of coenzyme. The K_m value for 5α-dihydrotestosterone was approx. 0.14 mM, for $NADP^+$ 9 μM and for NADPH 1.8 μM. Very similar properties were found for the 17β-hydroxysteroid dehydrogenase of rat erythrocytes. By application of affinity chromatography on Cibacron Blue F3G-A coupled to Sepharose and other techniques a 7200-fold purification of the enzyme, with respect to crude hemolysate was obtained. Since the resulting preparation still has 3β-hydroxy- and 17β-hydroxysteroid dehydrogenase activities, which coincide on polyacrylamide-gel electrophoresis, both activities are probably expressions of a single enzyme.

INTRODUCTION

In recent years particular attention has been paid to the role of 5α-dihydrotestosterone (17β-hydroxy-5α-androstan-3-one) as an intermediary in androgen action [1, 2] and to the significance of the 3α- and 3β-reduced metabolites of this steroid [3, 4]. Rat erythrocytes contain several hydroxysteroid dehydrogenase activities [5, 6] and we found extensive conversion of 5α-dihydrotestosterone into 5α-androstane-3β,17β-diol by rat blood. The properties of the 3β-hydroxysteroid dehydrogenase responsible for this conversion are studied in the present paper. The enzyme is found to interact with Blue Dextran 2000 and a similar interaction occurs with the chromophore of the latter coupled to Sepharose, thus allowing affinity chromatography of the enzyme. Since the 17β-hydroxysteroid dehydrogenase activity has this and other properties in common, we believe that both the 3β- and the 17β-hydroxysteroid dehydrogenase activities of rat erythrocytes are due to a single enzyme.

MATERIALS AND METHODS

Unlabeled steroids were obtained from Merck (Darmstadt, Germany), Sigma (St. Louis, U.S.A.) and Ikafarm (Ramat-Gan, Israel). [1,2-^{3}H]Androsterone (53 Ci/mmole), [7-^{3}H]dehydroepiandrosterone (10 Ci/mmole), 5α-[1,2-^{3}H]dihydrotestosterone (39 Ci/mmole), [6,7-^{3}H]estradiol (40 Ci/mmole) and [6,7-^{3}H]estrone (45 Ci/mmole) were purchased from New England Nuclear (Frankfurt, Germany), [1,2-^{3}H]testosterone (38 Ci/mmole) from I.R.E. (Mol., Belgium). [1,2-^{3}H]Androstane-3α,17β-diol and [1,2-^{3}H]androstane-3β,17β-diol were prepared by reduction with $NaBH_4$ of, respectively, [^{3}H]androsterone and 5α-[^{3}H]dihydrotestosterone and purified by paper chromatography.

$NADP^+$, NAD^+, NADPH, NADH, bovine serum albumin and cytochrome *c* were obtained from Sigma, primulin from Difco Laboratories (West Molesey, Great Britain), Diaflo XM-50 membranes from Amicon (Lexington, U.S.A.), Sephadex G-100, Sepharose 6B and Blue Dextran 2000 from Pharmacia (Uppsala, Sweden). Thin-layer chromatography plates and most common reagents were purchased from Merck. The phosphate buffer used in most experiments consisted of 0.1 M phosphate buffer (pH 7.1) and contained also 1 mM of EDTA and 0.2 g/L of NaN_3.

Coupling of Cibacron Blue F3G-A to Sepharose 6B

The method of Böhme et al. [7] was adapted to the use of Sepharose instead of Sephadex. To 150 ml of Sepharose-6B gel, 2.5 g of Na_2CO_3 in 50 ml of water and 1 g of Cibacron Blue F3G-A, also in 50 ml of water were added. After careful mixing, the suspension was incubated for 40 h at 45 °C with occasional stirring. Thereafter the Blue Sepharose gel was washed extensively with distilled water on a Buechner filter in order to remove the non-coupled dye. It was stored at 4 °C in the presence of 1 g/L of NaN_3. The coupled amount of dye was estimated spectrophotometrically at 610 nM after hydrolysis of the gel in 0.1 M HCl for 10 min at 90 °C. 1.16 mg of dye was coupled per ml of gel or 30 mg/g of Blue Sepharose dried with methanol, diethyl ether and application of vacuum.

Chromatography

Thin-layer chromatography of free steroids was performed on Silicagel F254 plates (Merck), which were run twice in dichloromethane–diethyl ether (85:15, by vol.) Relative mobilities to testosterone were 0.74 for 5α-androstane-3α,17β-diol, 0.84 for 5α-androstane-3β,17β-diol, 1.30 for androsterone, 1.34 for 3β-hydroxy-5α-androstane-17-one, 1.56 for 5α-dihydrotestosterone, 1.80 for androst-4-ene-3,17-dione and 2.30 for 5α-androstane-3,17-dione. A more effective separation of 3α- and 3β-hydroxysteroids was obtained by acetylation and chromatography on aluminium oxide F254, Type E plates (Merck) in the system benzene-diethyl ether (80:20, by vol.). Localization of steroids was performed by absorption of ultraviolet light for Δ_4-3-ketosteroids or by spraying with primulin [8].

Gel filtration

Gel filtration on Sephadex G-100 was performed at 4 °C with 0.1 M KCl in phosphate buffer. Blue Dextran 2000, bovine serum albumin and cytochrome *c* were used for standardisation of the column.

Preparation of purified hemolysate

Blood from adult Wistar rats was collected on heparin by section of the carotid artery under diethyl ether anesthesia. After centrifugation at 800 × *g* for 15 min the plasma and intermediary white layer were removed by aspiration. The erythrocytes were washed twice with 4 times their volume of 0.9% NaCl and hemolysed by addition of 2 vol. of distilled water and incubation for 2 h at 0 °C. The resulting crude hemolysate was centrifuged for 20 min at 40 000 × *g* at 4 °C. The supernatant was diluted with an equal volume of 0.2 M KCl in phosphate–EDTA buffer and centrifuged again under the same conditions. This procedure results in the removal of a considerable amount of protein consisting mainly of hemoglobin [9]. The supernatant, which contained 6.1 ± 1.6 (S.D.; $n = 12$)% of the protein and 61.7–72.4% ($n = 3$) of the enzymatic activity was called purified hemolysate.

Radiochemical estimation of 3-β-hydroxysteroid dehydrogenase activity

0.1 μCi of 5α-[^{3}H]dihydrotestosterone was incubated for 30 min at 37 °C in 1 ml of phosphate buffer, containing the sample at a sufficient dilution (usually 1:20) to have less than 30% conversion at the end of the incubation, and 0.5 mg of NADPH. After incubation the radioactivity was extracted with 3 ml of cyclohexane–ethyl acetate (1:1, by vol.); about 20 μg of unlabeled 5α-dihydrotestosterone and 5α-androstane-3β,17β-diol were added and thin-layer chromatography was performed. The 5α-dihydrotestosterone and 5α-androstane-3β-17β-diol zones were localized under ultraviolet light, using primulin spray, scraped off in counting vials and after addition of 10 ml of dioxane-based counting solution the radioactivity in these vials was measured by liquid-scintillation counting. The enzymatic activity was expressed in arbitrary units, corresponding to the percent conversion of 5α-dihydrotestosterone into 5α-androstane-3β,17β-diol per min, corrected for the dilution of the sample. The high K_m of the enzyme for the steroid and the low solubility of the latter precluded the use of V conditions but almost constant results were obtained over a wide range of steroid concentrations. Similar procedures were followed for the assay of other hydroxysteroid dehydrogenase activities, using various labelled steroids as substrate and $NADP^+$ or NADPH, as appropriate, as coenzyme.

Spectrophotometric estimation of hydroxysteroid dehydrogenase activity

50 μl of a 1 mg/ml solution of the steroid in ethanol and 50 μl of a 2% solution of Triton X-100 in methanol were evaporated under N_2. Then 3 ml of a 10-fold dilution of the sample in phosphate buffer and 1 ml of the same buffer containing 0.4 mg of $NADP^+$ (or NADPH) were added. After 30 min of incubation at 37 °C the tubes were placed in melting ice and their absorbance at 340 nm was compared to a control of the same composition but containing no steroids, which was run in parallel.

Polyacrylamide-gel electrophoresis

This procedure was performed in Tris–glycine buffer (pH 8.5, 3 g/L Tris, 14.4 g/L glycine) on 7.5% acrylamide gels according to the method of Barka [10]. The gels were prerun for 2 h at 1 mA/tube. Thereafter the cathodic buffer was replaced by buffer containing 0.1 mg/ml of $NADP^+$. After application of the sample (20 μl), which contained 10% of glycerol, electrophoresis was performed for 2 h at 1 mA/tube. Thereafter, the gels were cut in 2-mm slices. Each slide was divided into two equal

parts, respectively, for the determination of 17β-hydroxysteroid dehydrogenase activity (testosterone→androstenedione) and 3β-hydroxysteroid dehydrogenase activity (5α-dihydrotestosterone→5α-androstane-3β,17β-diol). This measurement was performed by incubation of the gel slices with 1 ml of phosphate buffer, containing 0.1 μCi of labelled steroid and 0.5 mg of $NADP^+$ or NADPH, as appropriate, for 1 h at 37 °C in a shaking incubator, followed by extraction and thin-layer chromatography as described. Control gels were stained with Amido Black. Total protein was measured by the method of Hartree [11], glucose 6-phosphate dehydrogenase by the method of Löhr and Waller [12].

RESULTS

Conversion of 5α-dihydrotestosterone into 5α-androstane-3β,17β-diol by rat blood

As shown in Fig. 1, incubation of 5α-dihydrotestosterone in rat blood results in the formation of a 5α-androstane-3β,17β-diol. This metabolite was identified by

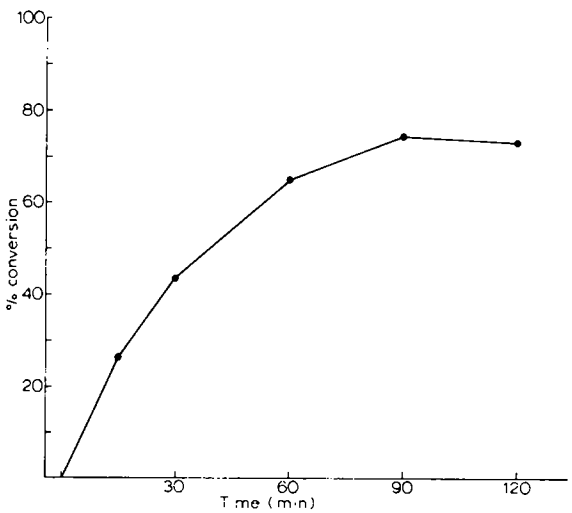

Fig. 1. Conversion of 5α-dihydrotestosterone into 5α-androstane-3β,17β-diol by rat blood as a function of time. 1 ml of blood was added to 0.2 μCi of 5α-[^{3}H]dihydrotestosterone in 0.2 ml of saline. After different periods of incubation at 37 °C 0.1 ml of this mixture was added to 2 ml of saline at 0 °C. The red blood cells were removed by centrifugation and the conversion of 5α-dihydrotestosterone into 5α-androstane-3β,17β-diol in the supernatant was measured as in the radiochemical method.

thin-layer chromatography of the free steroid and of its acetate, and by repeated crystallization in the presence of unlabelled 5α-androstane-3β,17β-diol (Table I). Since the transformation of 5α-dihydrotestosterone was observed also with washed erythrocytes but not with rat plasma, even after addition of NADPH, the 3β-hydroxysteroid dehydrogenase activity of rat blood is localized in the erythrocytes. The percent conversion of 5α-dihydrotestosterone by rat blood was almost similar for substrate concentrations between 0.6 ng and 20 μg per ml. After prolonged incubation some formation of epiandrosterone was noted.

TABLE I

IDENTIFICATION OF THE FORMED 5α-[^{3}H]ANDROSTANE-3β,17β-DIOL BY RECRYSTALLIZATION WITH UNLABELLED 5α-ANDROSTANE-3β,17β-DIOL

The radioactivity found in the 5α-androstanediol zone on thin-layer chromatography was eluted with ethanol, carrier 5α-androstane-3β,17β-diol was added and the steroid was recrystallized. On an aliquot of the crystals the weight was estimated by gas–liquid chromatography as trimethylsilyl ether on an XE-60 column, using cholestane as internal standard, the radioactivity was measured by liquid-scintillation counting.

Crystallization	Solvent	cpm/mg
1	Ethanol–water	1288
2	Ethanol–water	1289
3	Acetone–water	1319
4	Ethanol–water	1252

3β- and 17β-hydroxysteroid dehydrogenase activity in the purified hemolysate

Substrate specificity. Experiments to demonstrate hydroxysteroid dehydrogenase activity in the purified hemolysate were performed at pH 7.1. This pH is not optimal for the dehydrogenation but was chosen because of the instability of the enzyme under weakly alkaline conditions (see further).

In a first group of experiments (Table II), tritiated steroids were used as substrate and their conversion was followed radiochemically. With NADPH as cofactor a very marked reduction of the 3-oxogroup of 5α-dihydrotestosterone was observed, yielding 5α-androstane-3β,17β-diol. The 17-oxogroup (e.g. estrone, dehydroepiandrosterone, androsterone) was reduced as well. The opposite reactions were studied with NADP$^+$ as cofactor. 3β-Hydroxysteroid dehydrogenase activity occurred with

TABLE II

CONVERSION OF VARIOUS STEROIDS BY PURIFIED HEMOLYSATE (RADIOCHEMICAL ASSAY)

The enzymatic activity was measured radiochemically at a 1:20 dilution of purified hemolysate with the indicated steroids as substrate. The 3β-hydroxysteroid dehydrogenase activity, measured with 5α-dihydrotestosterone as substrate (coenzyme NADPH) was 12.6 units/ml of purified hemolysate; the 17β-hydroxysteroid dehydrogenase, measured with estradiol as substrate (coenzyme NADP$^+$) was 19.8 units/ml. For the other steroids the results are given in relation to these conversions.

Coenzyme	Substrate	Product	Relative conversion (%)
NADPH	5α-Dihydrotestosterone	5α-Androstane-3β,17β-diol	100
	Estrone	Estradiol	62
	Dehydroepiandrosterone	Androst-5-ene-3β,17β-diol	55
	Androsterone	5α-Androstane-3α,17β-diol	11
NADP$^+$	Estradiol	Estrone	100
	5α-Androstane-3β,17β-diol	3β-Hydroxy-5α-androstan-17-one	84
	Testosterone	Androstenedione	35
	5α-Dihydrotestosterone	5α-Androstane-3,17-dione	31
	3β-Hydroxy-5α-androstan-17-one	5α-Androstane-3,17-dione	3.7

3β-hydroxy-5α-androstan-17-one and 5α-androstane-3β,17β-diol as substrate. In the latter case, however, the dehydrogenation of the 17β-OH-group was much more marked, so that about 6 times more 3β-hydroxy-5α-androstan-17-one than 5α-dihydrotestosterone, and also some 5α-androstane-3,17-dione was formed. Further 17β-hydroxysteroid dehydrogenase activity was observed with 5α-dihydrotestosterone, estradiol or testosterone as substrate, but the 3α-hydroxygroup of androsterone and the 3β-hydroxygroup of dehydroepiandrosterone were not dehydrogenated to a significant extent.

In other experiments the enzymatic activity was estimated spectrophotometrically with unlabelled steroid as substrate (Table III). The results indicate that the reduction of the 3-oxogroup is limited to 5α-steroids.

TABLE III

CONVERSION OF VARIOUS NON-LABELLED STEROIDS BY PURIFIED HEMOLYSATE (SPECTROPHOTOMETRIC ASSAY)

The spectrophotometric assay of hydroxysteroid dehydrogenase activity, described in Materials and Methods was used with the indicated steroids as substrate and $NADP^+$ or NADPH as coenzyme.

Coenzyme	Substrate	$A_{340\ nm}$
NADPH	5α-Dihydrotestosterone	0.076
	5β-Dihydrotestosterone	0.000
$NADP^+$	Testosterone	0.056
	3β-Hydroxy-5α-androstan-17-one	0.013
	3α-Hydroxy-5α-androstan-17-one	0.006
	3β-Hydroxy-5β-androstan-17-one	0.003
	3α-Hydroxy-5β-androstan-17-one	0.003
	3β-Hydroxy-5α-androstane-11,17-dione	0.003
	3β,11β-Dihydroxy-5α-androstan-17-one	0.011

Inhibition of the enzymatic activity

The addition of solvents, which was necessary in some experiments, because of the low solubility of steroid in aqueous medium, produced a marked inhibition of enzymatic activity (Table IV). This was the case also for the detergents Tween-80 and Triton X-100. NaN_3 had no important inhibitory effect and $(NH_4)_2SO_4$ could be added in a high concentration with only moderate inhibition of the enzymatic activity.

Effect of pH. The enzymatic activity in the purified hemolysate was unstable under slightly alkaline conditions (Fig. 2). Similar results were obtained with Tris–HCl buffer. The inactivation of the enzyme could be prevented completely by the addition of $NADP^+$ and to some degree by the addition of glycerol which has an inhibitory effect on the reaction. Mercaptoethanol (7.5 mM) and EDTA (1 mM) gave only minor protection.

Because of this inactivation, the estimation of the optimal pH gave erroneous results when the pH of the enzyme preparation was adjusted before coenzyme was added. When the coenzyme was added first, an optimal pH of 8.2 was obtained for the dehydrogenase activity (Fig. 3). For the reduction there was a broad optimal zone between pH 6 and 7. No differences were observed between 3β- and 17β-hydroxysteroid dehydrogenase activity.

TABLE IV

INHIBITION OF THE 3β-HYDROXYSTEROID DEHYDROGENASE ACTIVITY BY VARIOUS SUBSTANCES (RADIOCHEMICAL ASSAY)

The radiochemical assay of 3β-hydroxysteroid dehydrogenase activity, described in Material and Methods, was performed in the presence of the indicated concentrations of inhibitor.

Substance	Concentration		Inhibition (%)
	% (by vol.)	g/l	
Ethanol	1.7		22
Ethanol	3.3		50
Propyleneglycol	3.3		40
Dioxane	3.3		90
Dimethylsulfoxide	3.3		43
Glycerol	5.0		26
Tween-80	3.3		92
Triton X-100	0.04		11
Triton X-100	0.1		30
Triton X-100	0.4		65
NaN_3		0.25	3
NaN_3		1.0	13
$(NH_4)_2SO_4$		18	15
$(NH_4)_2SO_4$		36	24
$(NH_4)_2SO_4$		56	30

K_m values for substrate and coenzyme. The K_m values for 5α-dihydrotestosterone and for testosterone were, respectively, 0.14 and 0.31 mM. These values were measured in the presence of a small amount (0.1%) of Triton X-100, which has an inhibitory effect on the enzymatic activity, but this addition was necessary because of the low solubility of these steroids. The K_m values for coenzyme were studied, using a

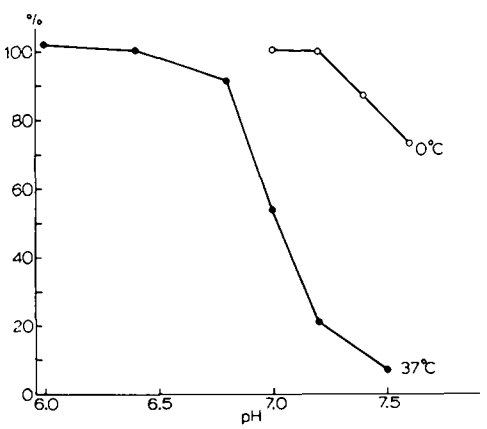

Fig. 2. Stability of the enzyme as a function of pH. To 0.15 ml of purified hemolysate 0.15 ml of phosphate buffer (I = 0.1) of various pH values were added. The samples were preincubated for 1 h at 0 or 37 °C. Thereafter 0.8 ml of phosphate buffer (pH 7.1, 0.1 M KCl) containing 0.1 μCi of [^{3}H]-testosterone and 0.4 mg of $NADP^+$ were added and after 30 min of incubation at 37 °C the conversion of testosterone into androstenedione was measured. The results are expressed as a percentage of the conversion obtained with samples of similar composition but without preincubation.

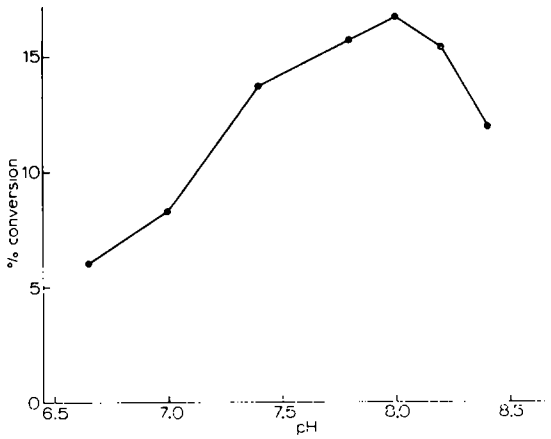

Fig. 3. 17β-Hydroxysteroid dehydrogenase activity as a function of pH. 0.1 μCi of [^{3}H]testosterone was dissolved in 0.95 ml of Tris–HCl buffers (I = 0.05) of various pH values at 37 °C. Then 0.05 ml of purified hemolysate containing 0.25 mg of NADP$^+$ was added. After 30 min of incubation at 37 °C the % conversion of testosterone into androstenedione and the pH values (at 37 °C) were measured.

constant and very low concentration of steroid and various concentrations of coenzymes [13]. For NADP$^+$ a K_m value of 9.0 μM was obtained with 3β-hydroxy-5α-androstan-17-one as substrate (3β-hydroxysteroid dehydrogenase activity) and a value of 8.2 μM with testosterone (17β-hydroxysteroid dehydrogenase activity). For NADPH the results were 1.8 and 2.1 μM with, respectively, 5α-dihydrotestosterone (3β-hydroxysteroid dehydrogenase activity) and estrone (17β-hydroxysteroid dehydrogenase activity) as substrate.

$(NH_4)_2SO_4$ precipitation. As shown in Fig. 4, both the 17β- and 3β-hydroxy-

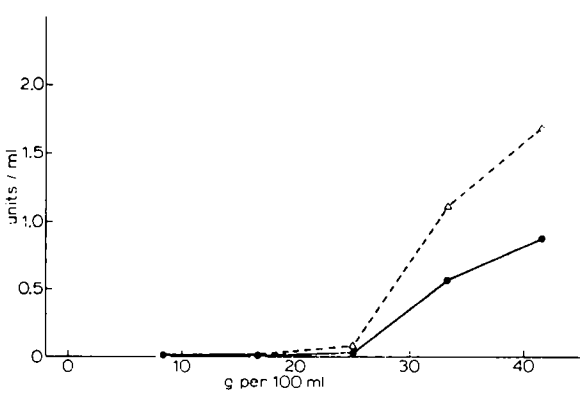

Fig. 4. Precipitation of hydroxysteroid dehydrogenase activity as a function of the concentration of $(NH_4)_2SO_4$. One ml of purified hemolysate and 5 ml of a solution of $(NH_4)_2SO_4$ (10–50 g/100 ml) were incubated for 1 h at 4 °C, then the tubes were centrifuged for 15 min at 30 000 × g. The precipitate was dissolved in 2 ml of 0.2 M Tris buffer (pH 7.2) and the radiochemical assay of hydroxysteroid dehydrogenase activity was performed with, respectively, 5α-dihydrotestosterone (●—●) and estradiol (△---△) as substrate and NADPH and NADP$^+$ as coenzyme.

steroid dehydrogenase activities could be precipitated by $(NH_4)_2SO_4$. For both activities this precipitation occurred mainly between 25 and 33 g/100 ml of $(NH_4)_2SO_4$.

Gel filtration on Sephadex G-100. In the absence of Blue Dextran 2000, the 3β- and 17β-hydroxysteroid dehydrogenase activities were eluted from a Sephadex G-100 column with a K_{av} of 0.195 corresponding to a molecular weight of approx. 70 000 (Fig. 5). The elution profile of both enzymatic activities was practically identical. When the gel filtration experiment was repeated with Blue Dextran 2000 in the sample, the hydroxysteroid dehydrogenase activity was found for the largest part in the void volume (Fig. 5). Consequently, an interaction occurs between the hydroxysteroid dehydrogenase and Blue Dextran 2000.

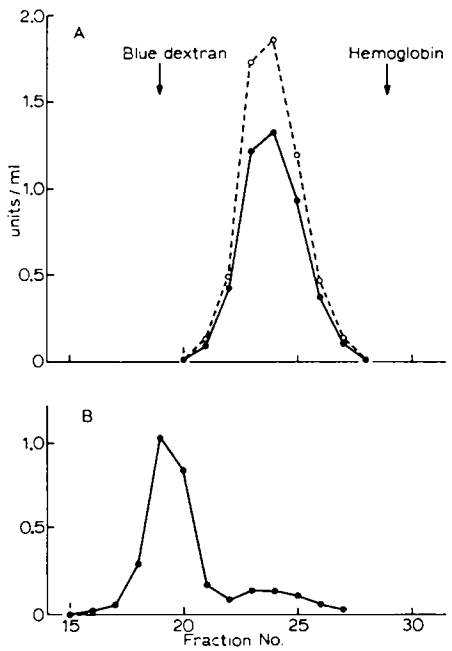

Fig. 5. Elution pattern of hydroxysteroid dehydrogenase activity from a Sephadex G-100 column. 2 ml of purified hemolysate were applied to a Sephadex G-100 column (2.4 cm × 90 cm) and eluted with 0.1 M KCl in phosphate buffer. Fractions of 10 ml were collected and evaluated radiochemically for 3β-hydroxysteroid dehydrogenase (substrate dihydrotestosterone, coenzyme NADPH, ———) and 17β-hydroxysteroid dehydrogenase (substrate estradiol, coenzyme $NADP^+$, - -). In B the sample contained also 1 mg of Blue Dextran 2000.

Affinity chromatography on Blue Sepharose. A similar interaction with the chromophore of Blue Dextran 2000 occurred also, when this dye was coupled to Sepharose. Indeed, the enzymatic activity was retained on small columns of Blue Sepharose (Fig. 6) and could be eluted from the latter by the addition of $NADP^+$ or NADPH to the eluant (Fig. 6) or by an increase of the concentration of KCl. Elution by cofactor resulted in the most efficient purification. Indeed, in the experiment shown in Fig. 6 the $NADP^+$ fractions contained 58.7% of the applied enzymatic activity and only 0.265% of the applied protein, corresponding to a purification by this step

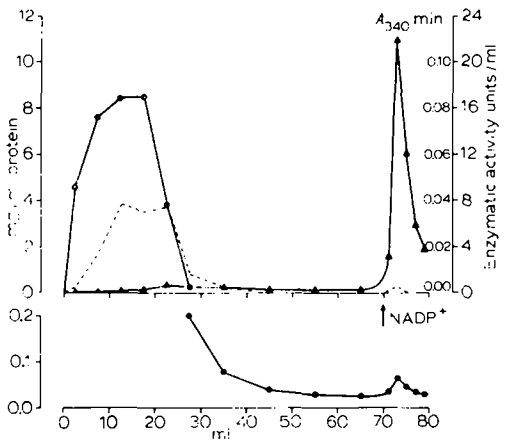

Fig. 6. Affinity chromatography with $NADP^+$ elution. To a column (internal diameter 9 mm), containing 2 ml of Blue Sepharose, 20 ml of purified hemolysate (containing 0.15 M KCl) were applied; the column was washed with 50 ml of 0.15 M KCl in phosphate buffer and eluted with 10 ml of the same buffer containing 0.2 mg/ml of $NADP^+$. All fractions were tested for protein concentration (●—●), 17β-hydroxysteroid dehydrogenase activity (units/ml; ▲—▲) and glucose-6-phosphate dehydrogenase activity ($A_{340\ nm}$/min;). Note the two scales for protein.

of 220 times. The maximal purification, obtained in the second $NADP^+$ fraction was 337 times. In a similar experiment with NADPH as eluant (Table V) both the 17β- and 3β-hydroxysteroid dehydrogenase activities were followed (Table V). The purification by the various steps and the overall purification was almost the same for both activities. Other substances with structural analogy to NADP(H), such as NAD(H), AMP, cyclic AMP and ATP did not result in elution of the enzyme. Elution was obtained by an increased concentration of KCl (e.g. 0.4 M), but in this case there was a marked elution of other proteins, resulting in a less efficient purification.

TABLE V

PURIFICATION OF THE ENZYME BY AFFINITY CHROMATOGRAPHY ON "BLUE SEPHAROSE"

Crude hemolysate was prepared from 10 ml of blood and purified as described in Materials and Methods. Affinity chromatography was executed as in Fig. 6, but NADPH (0.2 mg/ml) was used for elution of the enzyme. The 17β-hydroxysteroid dehydrogenase (I, substrate dehydroepiandrosterone) and the 3β-hydroxysteroid dehydrogenase (II, substrate 5α-dihydrotestosterone) were measured radiochemically with NADPH as coenzyme. The initial activities in the crude hemolysate were 18.2 and 41.6 units/ml for I and II, respectively.

Purification step	Volume (ml)	Protein (mg/ml)	Relative specific activity		Recovery (%)
			I	II	
Crude hemolysate	12	152	1	1	100
Purified hemolysate	21	4.3	12.3	11.6	61.0
After affinity chromatography:					
total NADPH-eluate	10	0.014	3088	2936	23.7
second NADPH-fraction	2	0.018	3567	3271	–

Polyacrylamide-gel electrophoresis. By a combination of affinity chromatography with KCl elution, gel filtration on Sephadex G-100 and affinity chromatography with $NADP^+$ elution a 7200-fold purification of the enzyme was obtained with respect to crude hemolysate. On the resulting purified enzyme preparation polyacrylamide-gel electrophoresis was performed. To stabilize the enzyme under the unfavourable alkaline conditions of the electrophoresis, $NADP^+$ was added to the sample and to the cathodic buffer. As shown in Fig. 7 the 3β- and 17β-hydroxysteroid dehydrogenase activities in the gel coincided completely. After staining a single protein band was detected, which was slightly less mobile than albumin and coincided with the steroid dehydrogenase activities. Since the observed protein band, however, was weak, the presence of other proteins cannot be excluded.

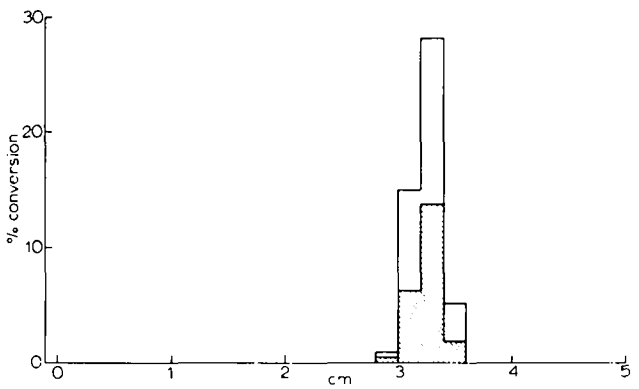

Fig. 7. Localization of 3β-hydroxysteroid dehydrogenase and 17β-hydroxysteroid dehydrogenase on polyacrylamide gel after electrophoresis of the 7200-fold purified preparation. The distance from the cathodic end of the gel is indicated. Hatched area, 3β-hydroxysteroid dehydrogenase; non-hatched area, 17β-hydroxysteroid dehydrogenase.

DISCUSSION

In the present paper it is shown that the 3β- and 17β-hydroxysteroid dehydrogenase activities of rat erythrocytes can be purified substantially by affinity chromatography on Cibacron Blue F3G-A coupled to Sepharose. This method is based on the observation that these enzymatic activities interact with Blue Dextran 2000 during gel filtration on Sephadex G-100. Indeed, Cibacron Blue F3G-A is the chromophore of Blue Dextran 2000 [7, 14]. Similar work with Cibacron Blue F3G-A coupled to Sephadex G-200 has been reported by Röschlau and Hess [14] for pyruvate kinase and by Böhme et al. [7] for phosphofructokinase. We preferred Sepharose to Sephadex G-200, because the latter is difficult to handle, due to the very pronounced swelling of the coloured Sephadex, particularly at low salt concentrations.

The elution of the enzyme from Blue Sepharose by means of its coenzyme, shows that an interaction occurs between the binding of the chromophore and the binding of the coenzyme to the enzyme. This may indicate that the enzyme has a common binding site for both components. In this case one would expect some structural relationship between coenzyme and chromophore, and such a similarity has,

indeed, been put forward by Böhme et al. [7] for the interaction of ATP, Cibacron Blue and phosphofructokinase. A similar reasoning prompted us to an unsuccessful attempt to elute the enzyme from Blue Sepharose with structural analogues to NADP(H) such as NAD(H), AMP, ATP and cyclic AMP. An alternative hypothesis is the possibility that the binding of the coenzyme changes the enzyme in such a way that it binds no longer to the chromophore. In this case the enzyme–chromophore interaction may be a rather non-specific phenomenon, as suggested by the binding of various proteins to Blue Dextran (Pharmacia Bulletin on Blue Dextran 2000 [15]). The specificity of the "affinity chromatography" might then result to a large degree from the elution by the appropriate cofactor.

According to Portius and Repke [5] the 3β-hydroxysteroid dehydrogenase activity and the better known 17β-hydroxysteroid dehydrogenase [5, 6] activity of rat erythrocytes are due to separate enzymes. Our results, however, strongly suggest that both enzymatic activities are expressions of a single enzyme. Indeed, the 3β- and the 17β-hydroxysteroid dehydrogenase activities have identical properties. This is the case for their cofactor requirement: both activities are NADP(H)-linked and their K_m values for $NADP^+$, as well as for NADPH, are practically the same. This is the case also for the effect of pH on their activity; both activities are unstable under slightly alkaline conditions and are protected by the presence of NADP. A similar protection by coenzyme has been described by Marks et al. [16] for glucose-6-phosphate dehydrogenase. The 3β- and 17β-hydroxysteroid dehydrogenase activities are precipitated in parallel by $(NH_4)_2SO_4$ and their elution patterns from Sephadex G-100 columns are identical. Furthermore, they show a marked interaction with Blue Dextran and with "Blue Sepharose", allowing affinity chromatography of the enzyme resulting in a similar degree of purification. Finally, after 7200-fold purification the enzyme preparation has still similar 3β- and 17β-hydroxysteroid dehydrogenase activities, which coincide completely on polyacrylamide-gel electrophoresis. For these reasons we believe that both activities are properties of a single enzyme which performs the interconversion of the 17-oxo and the 17β-hydroxy group of many steroids and of the 3-oxo and 3β-hydroxy group of some steroids with 5α-configuration. Examples of bi-specificity of steroid dehydrogenase have been described: e.g. the 3(17)β-hydroxysteroid dehydrogenase from *Pseudomonas testosteroni* [17], the 3α(20β)-hydroxysteroid dehydrogenase from *Streptomyces hydrogenans* [18] and the 3α(17β)-hydroxysteroid dehydrogenase from rabbit liver cytosol [19].

The 17β-hydroxysteroid dehydrogenase activity of rat erythrocytes is much higher than in human red blood cells which contain an enzyme with similar properties [20, 21]. Furthermore, rat serum contains no specific binding proteins for 17β-hydroxysteroids, such as present in human serum [22]. Consequently, the hydroxysteroid dehydrogenase is quantitatively much more important in rat blood than in human blood [6]. Since the interconversion of testosterone and androstenedione, of estradiol and estrone and of 5α-dihydrotestosterone and 5α-androstane-3β-17β-diol affects the hormonal activity of these steroids, the 3(17)β-hydroxysteroid dehydrogenase found in the erythrocytes may play a significant role in the steroid metabolism of the rat. Furthermore, the enzyme may have a function in the erythrocytes themselves, for instance in relation to the inhibition by 17-oxosteroids of glucose-6-phosphate dehydrogenase [16, 23].

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