Purification and Properties of a 3α-Hydroxysteroid Dehydrogenase from *Pseudomonas testosteroni**

Jean Boyer,† Denis N. Baron,‡ and Paul Talalay§

ABSTRACT: A procedure is described for the purification of the steroid-induced 3α-hydroxysteroid dehydrogenase of *Pseudomonas testosteroni*. The purified preparations catalyze the nicotinamide–adenine dinucleotide dependent oxidation of about 300 μmoles of androsterone/min per mg of protein at *p*H 9.0 and 25°. The purification depends upon stabilization of the enzyme in glycerol-water mixtures; removal of nucleic acids with protamine sulfate; ammonium sulfate and acetone fractionations; chromatography on carboxymethyl-cellulose; and gel filtration on Sephadex G-100. The enzyme reacts also with the thionicotinamide, the 3-pyridinealdehyde, and the acetylpyridine analogs of nicotinamide–adenine dinucleotide.

The effect of *p*H and temperature on the reaction have been measured. The application of the enzyme to the sensitive enzymatic assay of androsterone is illustrated.

Hydroxysteroid dehydrogenases catalyze nicotinamide nucleotide-dependent, stereospecific interconversions of hydroxyl and carbonyl functions located at specific positions on the steroid skeleton and side chain (Talalay, 1963). Marcus and Talalay (1956) reported on the partial purification and properties of two steroid-induced hydroxysteroid dehydrogenases of *Pseudomonas testosteroni*, a microorganism which may be grown on steroids as the only source of carbon. These authors isolated a 3α-hydroxysteroid dehydrogenase [EC 1.1.1.50], which catalyzes reaction (1),[1] and a (3 and 17)β-hydroxysteroid dehydrogenase [EC 1.1.1.51], which catalyzes reactions (2) and (3):

$$\text{androsterone} + \text{NAD}^+ \rightleftharpoons 5\alpha\text{-androstane-3,17-dione} + \text{NADH} + \text{H}^+ \quad (1)$$

$$\text{testosterone} + \text{NAD}^+ \rightleftharpoons \Delta^4\text{-androstene-3,17-dione} + \text{NADH} + \text{H}^+ \quad (2)$$

$$17\beta\text{-estradiol} + \text{NAD}^+ \rightleftharpoons \text{estrone} + \text{NADH} + \text{H}^+ \quad (3)$$

This paper describes a procedure for the preparation of highly purified α-HSD and some of the properties of this enzyme, including the effect of *p*H on the reaction equilibrium, the specificity toward nicotinamide nucleotide analogs, and the analytical applications of the enzyme to the microestimation of steroids. Delin and Porath (1963) and Delin *et al.* (1964) have recently described a purification of the α-HSD of *P. testosteroni* and have obtained a preparation of specific activity comparable to that described in this paper. Earlier studies in our laboratory (reviewed by Talalay, 1963) concerned the steroid specificity, the nucleotide specificity, the stereospecificity of hydrogen transfer, and the analytical applications of this enzyme.

Experimental Procedures

Reagents and Materials. Tap water was deionized and distilled in a glass apparatus. Dioxane and glycerol were of spectroscopic grade. Reagent grade acetone was distilled. All other chemicals were of analytical reagent quality. Ethylenediaminetetraacetate was obtained as the disodium salt. The β-mercaptoethanol was supplied by Eastman Organic Chemicals, Rochester, N.Y., and was stored at 4°. Crystalline bovine serum albumin was obtained from Armour Pharmaceutical Company, Kankakee, Ill. Ammonium sulfate (Baker Analyzed) was recrystallized twice from EDTA (Beisenherz *et al.*, 1953). All the vessels used in the purification were washed with a solution containing 2.0 g of EDTA and 1.35 g of $NaHCO_3$ per liter (*p*H 7.0).

Solutions of the following composition were used in the purification procedure: *Medium A:* 0.05 M potassium phosphate, 0.003 M EDTA, 0.05% β-mercaptoethanol, and 20% glycerol; the final *p*H was 7.0; *medium B:* 0.001 M potassium phosphate, 0.001 M EDTA, 0.05% β-mercaptoethanol, and 25% glycerol; the final *p*H was 6.4; *medium C:* 0.001 M potassium phosphate, 0.001

* From the Department of Pharmacology and Experimental Therapeutics, The Johns Hopkins University School of Medicine, Baltimore, Maryland, and the Ben May Laboratory for Cancer Research, University of Chicago, Chicago, Ill. *Received May 6, 1965.* This investigation was supported by a grant from the U.S. Public Health Service (AM 07422).

† Recipient of a Fulbright Grant. Permanent address: Clinique d'Endocrinologie de la Faculté de Médecine, Marseille, France.

‡ Rockefeller Foundation Traveling Fellow in Medicine. Permanent address: Department of Chemical Pathology, Royal Free Hospital, London, England.

§ To whom communications should be addressed.

[1] The following abbreviations are used: α-HSD for 3α-hydroxysteroid dehydrogenase; β-HSD for (3 and 17)β-hydroxysteroid dehydrogenase; NAD^+ and NADH, nicotinamide-adenine dinucleotide, oxidized and reduced forms, respectively.

M EDTA, 0.05% β-mercaptoethanol, and 10% glycerol; the final *p*H was 6.4; *medium D:* 0.03 M potassium phosphate, 0.001 M EDTA, 0.05% β-mercaptoethanol; the final *p*H was 7.25. The concentrations of all organic solvents are expressed as fractional volumes.

Nicotinamide–adenine dinucleotide and its analogs were supplied by Pabst Laboratories, Milwaukee, Wis. The following molar extinction coefficients and absorption maxima of the reduced nucleotides were assumed:[2] NAD^+ (340 mμ, a_m = 6220); thionicotinamide–adenine dinucleotide (395 mμ, a_m = 11,300); 3-acetylpyridine–adenine dinucleotide (363 mμ, a_m = 9100); 3-pyridinealdehyde–adenine dinucleotide (358 mμ, a_m = 9300); hypoxanthine–adenine dinucleotide (338 mμ, a_m = 6200). Solutions of nucleotides were either adjusted to *p*H 7 by addition of $NaHCO_3$, or not neutralized, and were stored at −20°.

17β-Estradiol was supplied by the Sigma Chemical Co., St. Louis, Mo., and androsterone (mp 182–184°) was obtained from Mann Laboratories, New York. Solutions of both steroids were prepared in dioxane (1.5 mg/ml), and were stored at 4° between assays. Progesterone used for induction purposes was donated by the Upjohn Company, Kalamazoo, Mich.

Seamless regenerated cellulose tubing was supplied by the Visking Company, Chicago. This material was soaked for several days in a solution containing 2 g of EDTA, 1.37 g of $NaHCO_3$, and 0.5 ml of β-mercaptoethanol per liter. Just before the dialysis, the tubing was washed carefully with the dialyzing medium. The soaking and washing procedures removed considerable quantities of pigment and rendered the tubing colorless.

The quantities of ammonium sulfate and of organic solvents to be added to a solution to attain a given degree of saturation were determined as previously described by Noda and Kuby (1957) and by Jarabak *et al.* (1962).

Growth of Microorganism. Pseudomonas testosteroni (ATCC 11996) (Talalay *et al.*, 1952) was grown at 30° in a liquid medium of the following composition: Difco yeast extract, 10 g; $(NH_4)_2HPO_4$, 1 g; $(NH_4)H_2PO_4$, 1 g; KH_2PO_4, 2 g; and 10 ml of trace element solution[3] per liter of deionized water. The final *p*H was adjusted to 6.65 with 1 N NaOH. The medium was sterilized at 121° for 30 minutes. Growth was carried out in 500-liter fermentors, each containing 250 liters of medium. General Electric Co. Antifoam A-60 was added at a concentration of 0.2 ml/liter. Further additions of Antifoam were made during growth when necessary. The fermentors were aerated at the rate of 125–250 liters/min and stirred at 280 rpm. Each fermentor was inoculated with 2.5 liters (1% by volume) of an 18-hour culture of the microorganism grown on the same medium. After 7.5 hours of growth, a slurry of 75 g of finely pulverized progesterone in about 600 ml of 1% Tween 60 was added to each tank (300 mg/liter), and growth continued for a total of 23 hours from the time of inoculation. The contents of the tank were cooled to 8–10° by passing tap water or cold brine through the jacket, and the cells were harvested by centrifugation at 13,500 rpm in a Sharples continuous flow supercentrifuge having a bowl of approximately 6-liter capacity. The flow rate was 2.4 to 2.6 liters/min, which resulted in sedimentation of 93–94% of the cells. The contents of each fermentation tank filled the rotor to about one-half to one-third capacity. The cell mass was removed from the rotor, transferred to 500-ml capacity plastic containers, and frozen in dry ice. The yield of packed wet cell mass was 1.9–2.2 kg for 250 liters of growth medium.

Preparation of Acetone Powders. The packed cell mass was unfrozen overnight at 4° and suspended in 1.5 liters of 0.03 M potassium phosphate buffer of *p*H 7.0 for each kilogram of wet cell mass by gentle treatment in a Waring Blendor (4-liter capacity), operated at reduced speed for a sufficient period to give a smooth, creamy suspension. Aliquots of this suspension (250 ml) were then poured in a thin stream into 2.5 liters of acetone at −20° in the 4-liter metal cup of the Waring Blendor, operated at reduced speed. The suspensions were pooled and allowed to stand at −20° overnight, at which time most of the suspension had settled, and the bulk of the supernatant could be siphoned off and discarded. The slurry was diluted with an equal volume of acetone at −20° and was poured into large stainless steel Buchner funnels (33-cm diameter, manufactured by Commercial Equipment Company, Milwaukee, Wis.) covered with two layers of Whatman No. 1 filter paper, and washed with more acetone at −20° and finally with ether at −20°. The filter cake was removed from the funnel and dried by pressing on thick filter paper in a hood until a dry, faintly beige-colored powder was obtained. The powder was transferred to large Petri dishes in a desiccator which was evacuated until no odor of organic solvents was detectable. The powder was stored in a vacuum at −20°. Under these conditions the enzyme appeared to be stable for at least one year. The yield of acetone powder by weight was approximately 25% of the weight of the centrifuged bacterial cell mass.

Chromatography. Some of the chromatographic experiments were carried out in glass columns treated with a 4% solution of dichlorodimethylsilane in toluene. Carboxymethyl-cellulose (Selectacel type 40) was supplied by the Carl Schleicher and Schuell Co., Keene, N.H. The exchange capacity was rated by the manufacturer as 0.78 meq/g. The CM-cellulose (30 g) was suspended in about 1000 ml of water and treated for 1 minute in a Waring Blendor. The ion exchanger was packed in large columns (3.5-cm diameter) and was washed with 2 liters of 1.0 M potassium phosphate, *p*H 6.4, followed by 10 liters of a solution containing 0.001 M potassium phosphate, 0.001 M EDTA, and 0.05% β-mercaptoethanol, at a final *p*H of 6.4. For chromatography, the ion exchanger was packed by gravity in appropriate size glass columns provided with

[2] Stein *et al.* (1963) give a_m = 11,900 at 398 mμ for reduced thionicotinamide–adenine dinucleotide. The other extinction values are those given by Siegel *et al.* (1959).

[3] The composition of the trace mineral solution is: $MgSO_4 \cdot 7H_2O$, 20 g; NaCl, 1 g; $ZnSO_4 \cdot 7H_2O$, 0.5 g; $MnSO_4 \cdot 3H_2O$, 0.5 g; $CuSO_4 \cdot 5H_2O$, 0.05 g; 0.1 N H_2SO_4, 10 ml; in 1 liter of distilled water.

a glass wool support. The top of the cellulose was overlaid with glass beads to a depth of 5–10 mm to avoid disturbance of the surface when the samples were applied. The columns were connected to a gradient device, giving a linear concentration gradient. This consisted of two interconnected lucite cylinders of equal cross section, one of which was provided with a stirrer.

Sephadex Gel Filtration. Sephadex G-100 was supplied by Pharmacia, Uppsala, Sweden. The dry powder was suspended in a sufficient quantity of medium D and stored at room temperature for at least 24 hours to assure complete swelling. The "fines" were removed by several decantations and the gel was packed into a column (95 × 2 cm). The surface of the gel was covered with a fine nylon mesh and the column was transferred to a cold room maintained at 4°. This column had a total bed volume of 370 ml. The void volume (110 ml) was determined by applying a small quantity of a crude mixture of proteins and monitoring the effluent by light absorbance measurements at 280 mμ. It was assumed that the earliest rise in absorbance was caused by proteins which were completely excluded from the gel.

Protein Concentrations. These were determined by one of the following methods. (1) Biuret method (Gornall *et al.*, 1949): An appropriate dilution of the protein in a final volume of 1.5 ml was mixed with 1.5 ml of biuret reagent, stirred, and incubated at 30° for 30 minutes. Absorbance measurements were made in cuvets of 1.0-cm light path at 540 mμ against a reagent blank. Several dilutions of crystalline bovine serum albumin (10 mg/ml) served as standards. (2) Measurement of the absorbances at 280 and 260 mμ were made according to Warburg and Christian (1936). The protein concentration was calculated by the formula: $1.50 \cdot A_{280} - 0.75A_{260}$ = protein concentration in mg/ml (Kalckar, 1947). These optical measurements were carried out in quartz cuvets of 1.0-cm light path. The measurements were made against blanks of composition similar to the protein-containing solutions examined.

Measurement of Dehydrogenase Activity. Spectrophotometric assays were carried out in Pyrex cuvets of 1.0-cm light path, in a Beckman Model DU spectrophotometer or a Gilford automatic absorbance recorder (Model 2000, Gilford Instrument Company, Oberlin, Ohio) coupled to a Beckman monochromator. The temperature of the cell compartments was regulated to 25 ± 0.5° (unless otherwise indicated) by circulating water through thermospacers. The reaction systems contained in a final volume of 3.0 ml: 200 μmoles of sodium pyrophosphate buffer (*p*H 8.9), 0.5 μmole of NAD^+, 30 μg of androsterone for α-HSD or 30 μg of 17β-estradiol for β-HSD, each in 0.02 ml of dioxane, and enzyme. The final *p*H was 9.0 (assay system A). The reaction was initiated by addition of enzyme (0.01–0.10 ml), and absorbance readings were taken at 340 mμ against a blank containing all ingredients except the steroid solution, which was replaced by 0.02 ml of dioxane. The reaction velocities were determined by graphic means, and calculated from the slopes of the initial linear portions of plots of absorbance against time. Dilutions of the enzyme were prepared when necessary with 1% crystalline bovine serum albumin.

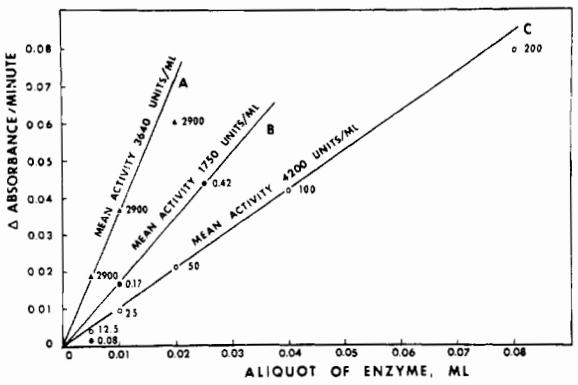

FIGURE 1: Effect of protein concentration in the assay system on the measured activity of 3α-hydroxysteroid dehydrogenase. All assays were carried out on a partially purified enzyme preparation which was obtained by chromatography on CM-cellulose, and contained 0.05 mg of protein/ml (specific activity = 84,000 units/mg of protein). (A) Aliquots of 5–20 μl were assayed in reaction systems containing 2.9 mg of bovine serum albumin/ml. (B) Aliquots of 5–25 μl were assayed directly without additional protein. (C) Aliquots of 5–80 μl were assayed after the enzyme had been diluted by addition of 3 volumes of bovine serum albumin (10 mg/ml). The assays were carried out without introducing additional protein into the reaction system. The figures adjacent to each experimental point give the protein concentration in each cuvet (μg/ml). The apparent mean activity of the original enzyme solution is calculated from each curve.

Difficulties were experienced in obtaining consistent measurements when dilute solutions of highly purified enzymes were assayed. When the protein concentration in the reaction cuvet was less than about 0.5 μg/ml, the measured velocities were low, although in most instances a linear relation appeared to exist between the measured velocity and the quantity of enzyme added (Figure 1). When the protein concentration in the assay system was raised by the addition of crystalline bovine serum albumin, maximum velocities were obtained when the total protein concentration was greater than 25–50 μg/ml. If the concentration exceeded 1 mg/ml, some decline in reaction velocity was observed. The albumin preparations were devoid of measurable hydroxysteroid dehydrogenase activity. No differences in reaction velocity were observed when the additional albumin was added with the enzyme, or if it was present in the assay system when the dilute enzyme was added. These observations prompted a revision of the assay by the addition of 500 μg of crystalline bovine serum albumin to each cuvet (assay system B).

One unit of enzyme activity represents a change of absorbance of 0.001/min at 340 mμ under the

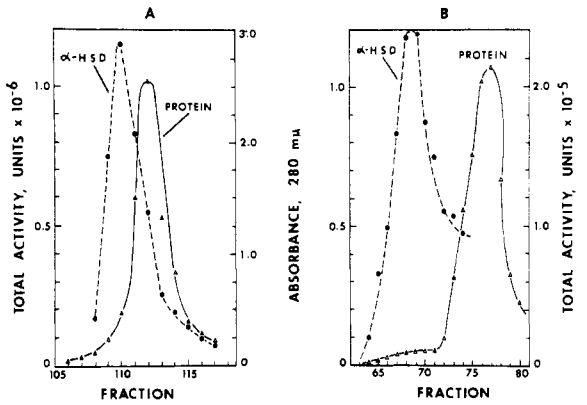

FIGURE 2: Chromatography on CM-cellulose. (A) First chromatography (step 5). The column (46 × 2.2 cm) was equilibrated against medium B by a short washing just prior to chromatography. The enzyme (32 ml) was applied at a rate of approximately 20 ml/hour. Fractions (8.5 ml) were collected into tubes containing a mixture of 1.0 ml of 1.0 M potassium phosphate, *p*H 7.0, and 1.0 ml of glycerol. The fractions are numbered from the point of loading of the sample. The column was then washed with medium B until fraction 22 was collected and then with medium C until fraction 50 was collected. Fractions 20–30 represented unadsorbed enzyme and contained 9.45 × 10^5 units (13.6%) of α-HSD and 5.14 × 10^5 units (58.2%) of β-HSD. These fractions were pigmented. Elution was carried out with a linear gradient of increasing phosphate and decreasing glycerol concentrations at a constant *p*H of 6.4. The mixing chamber initially contained 290.4 ml of medium C, and was connected to a reservoir which contained 300 ml of 0.06 M potassium phosphate, 0.001 M EDTA, 0.05% β-mercaptoethanol, at a final *p*H of 6.4. The dead space between the gradient device and the column surface had a volume of 50 ml; the hold-up volume of the packed column was 170 ml. The phosphate and glycerol gradients reached the bottom of the column coincidental with collection of fraction 75. The entire chromatography required 22 hours and was carried out at 4°. Protein concentrations were determined by light absorbances at 280 mμ. Fractions 108–117 contained 4.21 × 10^6 units of α-HSD (60.7% of the quantity applied). (B) Second chromatography (step 5). The column (22 × 2.2 cm) was prepared as described above. The enzyme (38 ml; 152 mg of protein) contained 2.37 × 10^6 units of α-HSD of specific activity 15,600 units/mg protein and 3.06 × 10^5 units of β-HSD of specific activity 2025 units/mg protein. The enzyme was applied at a rate of approximately 20 ml/hour. Fractions (5.5 ml) were collected in tubes containing 1 ml of a mixture of equal volumes of glycerol and 1.0 M potassium phosphate, *p*H 7.0. The numbering of the fractions began with the loading of the sample. The column was washed with medium B until fraction 7 was collected and then with medium C until fraction 25. Fractions 15–22 contained 3.57 × 10^5 units (15.1%) of unadsorbed α-HSD. Elution was carried out under conditions similar to those for (A). The gradient consisted of 145.8 ml of medium C and 150 ml of solution containing 0.06 M potassium phosphate, 0.001 M EDTA, and 0.05% β-mercaptoethanol, at a *p*H of 6.4. The dead space above the column was 40 ml and the hold-up volume was 72 ml. The gradient reached the bottom of the column coincidental with collection of fraction 46. The entire chromatography was carried out at 4° and was completed in 15 hours.

stated conditions and is equivalent to the reduction of 0.48 mμmole of NAD^+/min. Since the 17β-estradiol is oxidized at a maximal rate of 13.3% of that of testosterone (Marcus and Talalay, 1955), the values obtained for the activity of the 17β-HSD with 17β-estradiol as substrate were multiplied by 7.5 in order to make them comparable to the testosterone units previously defined (Marcus and Talalay, 1956).

Results and Discussion

Stability of 3α-Hydroxysteroid Dehydrogenase. Instability of the α-HSD presented a serious problem, especially when dealing with dilute solutions of highly purified preparations. The sensitivity of this enzyme and other hydroxysteroid dehydrogenases to sulfhydryl reagents and heavy metal cations has been previously noted (Talalay and Marcus, 1956). However, the use of glass-distilled water and of ammonium sulfate recrystallized from EDTA, and the inclusion of β-mercaptoethanol and of EDTA in all solutions, did not prevent entirely losses of activity. The addition of glycerol to solutions of α-HSD exercised a considerable stabilizing effect, and advantage was taken of this finding in carrying out the purification. Although the stabilizing effect of glycerol on α-HSD was not studied systematically, some of the findings may be summarized. The enzyme does not tolerate rapid freezing and thawing at a *p*H below 6.6. Thus, at *p*H 6.0 or 6.25, 70–75% of the activity is lost when partially purified preparations are treated in this way, whereas the addition of 20% glycerol provides complete protection against the deleterious effects of freezing and thawing. In other experiments dilute solutions of α-HSD (0.11 mg/ml of protein) were stored at 25° in the presence or absence of 25% glycerol. The glycerol-containing solutions retained full activity for at least 7 days, whereas in the absence of the stabilizer the entire enzyme activity was lost during this period.

Chromatography on Ion-Exchange Celluloses. Both α- and β-HSD were adsorbed to a major extent on CM-cellulose equilibrated with 0.001 M potassium phosphate at *p*H 6.4, either in the presence or absence of 20–25% glycerol. However, under a variety of conditions, the two enzymes were eluted simultaneously and no significant separation of the enzymes could be obtained by chromatography on CM-cellulose, although substantial purification of both enzymes was always achieved. In several trials with this system it was noted that a higher proportion of the β-HSD activity loaded onto the CM-cellulose column always remained un-

TABLE I: Purification of 3α-Hydroxysteroid Dehydrogenase.[a]

Step	Volume (ml)	Protein Concentration (mg/ml)	Total Activity α-HSD (units × 10^{-6})	Specific Activity α-HSD (units/mg protein)	Total Activity β-HSD (units)	Ratio β-HSD/α-HSD (%)	Total Purification	Total Yield (%)
1. Crude extract	325	14.3[b]	16.2	3,500	1.09×10^7	67.3		(100)
2. Protamine sulfate supernatant	350	12[b]	16.6[c]	3,950	1.05×10^7	63.2	1.13	102
3. 50–75% ammonium sulfate precipitate	54	29.2	10.7	6,790	2.63×10^6	24.6	1.94	66.0
4. 40–60% acetone precipitate	26	18.7[b]	8.32	17,000	1.56×10^6	18.7	4.86	51.4
Dialysis and centrifugations	32	12.8[b]	6.94	17,000	8.83×10^5	12.7	4.86	42.8
5A. First CM-cellulose effluent	34	0.24	2.07	249,000	...	...	88.0	20.6
5B. Second CM-cellulose effluent	62	0.04	1.26	508,000	...	...		
Pooled 0–75% ammonium sulfate precipitate	1.7	6.07	2.08	202,000	5.35×10^4	2.6	...	12.8
6. Sephadex effluent	30	0.098	1.96	667,000	...	...	191	12.1
7. 0–75% ammonium sulfate precipitate	38.8	0.078	2.16	713,000	2.40×10^4	1.11	204	13.3

[a] Activity measurements for steps 1 through 4 were carried out in assay system A. Assay system B was used in steps 5 through 7. [b] These protein concentrations were determined by the biuret method. All other protein measurements were made by the light absorbances at 280 and 260 mμ. [c] In all experiments, the measured total activity in step 2 was 2–15% higher than in the crude extract (step 1).

adsorbed (10–38%), while the adsorption of α-HSD was consistently more efficient (80–99%).

Glycerol was found to influence the behavior of the enzymes on CM-cellulose. In the presence of 25% glycerol, a much larger fraction of the β-HSD than of the α-HSD applied to the column remained unadsorbed. Thus, when a solution containing 228,000 units of α-HSD and 111,000 units of β-HSD was applied to a CM-cellulose column equilibrated with 0.001 M potassium phosphate in 25% glycerol at *p*H 6.4, about 80% of the β-HSD activity was recovered in the washings and only about 10% of the α-HSD.

The inclusion of glycerol also profoundly affected the elution of the enzymes. In the presence of 20% glycerol and an increasing phosphate gradient at *p*H 6.4, α- and β-HSD were eluted almost immediately upon application of the gradient (0.005 M). In the absence of glycerol, when other conditions remained the same, the phosphate concentration required for elution was approximately 0.03–0.04 M. It was therefore possible to obtain a substantial separation of the enzymes by carrying out the adsorption in 25% glycerol. The use of a linear gradient of rising phosphate and decreasing glycerol concentrations resulted in a satisfactory elution of the enzyme, and at the same time the low concentrations of glycerol present in the enzyme fractions contributed to the stability.

The *p*H at which the CM-cellulose chromatography is carried out is very critical. At *p*H 6.7 in 0.001 M phosphate the CM-cellulose loses 70% of its capacity for adsorbing the α-HSD. At *p*H 6.0, this cellulose exchanger adsorbed α-HSD very well, but the enzyme is somewhat unstable at this *p*H.

Gel Filtration on Sephadex. Delin and Porath (1963) have reported an apparently complete separation of α- and β-HSD in relatively crude extracts by filtration on Sephadex G-100. In repetition of their experiments, it was found that with crude enzyme preparations (approximately 5000 units of α-HSD/mg of protein) the peaks of α-HSD and β-HSD activities were eluted at 1.3 and 1.5 void volumes, respectively. However, when more highly purified enzyme preparations were used (81,000 and 200,000 units of α-HSD/ mg of protein), no separation of the two enzymes was achieved, although in all cases a substantial purification of the enzymes was obtained.

The extracts of testosterone- or progesterone-induced cells also contain Δ^5-3-ketosteroid isomerase (Kawahara *et al.*, 1962), but this enzyme is retarded to a much greater extent than the hydroxysteroid dehydrogenases in Sephadex G-100 filtration. Delin *et al.* (1964) have reported that the isomerase emerges at 1.95 void volumes.

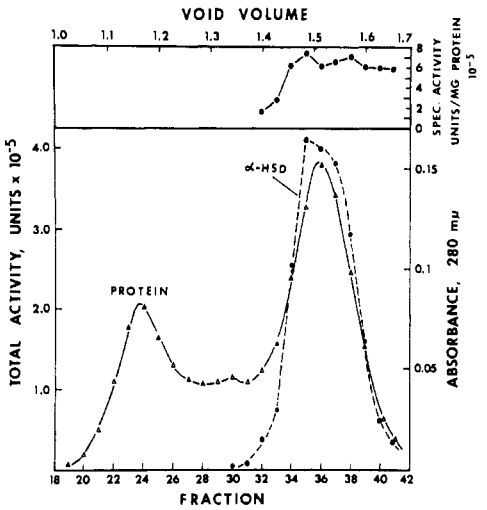

FIGURE 3: Sephadex G-100 gel filtration of 3α-hydroxysteroid dehydrogenase. The details of column size and manner of sample application are given in the text. Fractions of 3.2 ml were collected in test tubes containing 1.0 ml of glycerol. The flow rate was approximately 20 ml/hour. Nearly the entire activity was recovered in 10 fractions (32–41), indicating a 19-fold dilution of the enzyme on gel filtration.

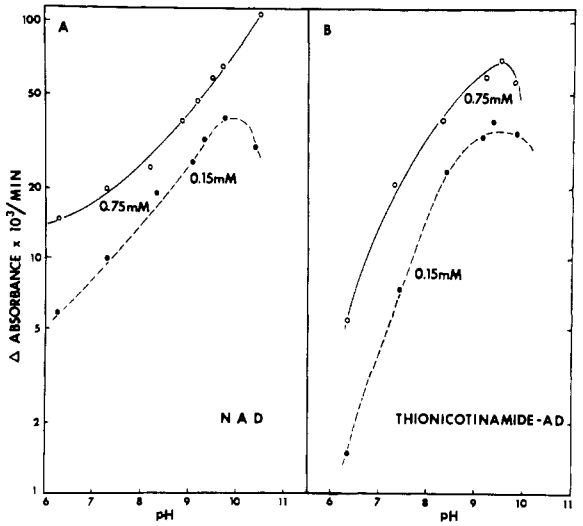

FIGURE 4: Initial velocities of oxidation of androsterone by 3α-hydroxysteroid dehydrogenase as a function of *p*H. (A) With NAD and (B) with thionicotinamide–adenine dinucleotide as hydrogen acceptors. Initial velocities were measured at 25° in reaction systems of 3.0-ml volume containing: 100 μmoles of buffer (sodium pyrophosphate for *p*H 6.3 to 9.85 and sodium carbonate–bicarbonate for *p*H 10.4), 0.131 μmole of androsterone in 0.1 ml of methanol, 0.45 or 2.25 μmoles of NAD or thionicotinamide–adenine dinucleotide, and 0.24 μg of α-HSD (specific activity = 148,000 units/mg of protein). Absorbance was measured against a blank containing all ingredients except steroid at 340 or 395 mμ.

Purification Procedure

All operations were carried out at 2–5°, unless otherwise specified. The results of the procedure are summarized in Table I.

Step 1: Extraction of Enzyme. The acetone powder (30 g) was added slowly to 270 ml of medium A, and the mixture was stirred with a magnetic bar for 2 hours. The suspension was centrifuged at 20,000 × *g* for 1 hour, and the residue was extracted with 150 ml of the same medium for 1 hour and again centrifuged. The straw-yellow, slightly turbid first extract (210 ml) was combined with the clearer second extract (115 ml).

Step 2: Protamine Sulfate Precipitation. A solution of protamine sulfate (46.5 ml; 20 mg/ml) was then added slowly with stirring. The quantity of protamine sulfate (930 mg) was one-fifth of the total amount of protein (4650 mg) contained in the extract. The copious, sticky precipitate was permitted to accumulate for 1 hour and then centrifuged for 10 minutes at 7000 × *g*. The clear yellow supernatant was decanted and subjected without delay to step 3.

Step 3: Ammonium Sulfate Fractionation. Ammonium sulfate was added to 35% of saturation, and the concentration was then raised in 5% increments to 60% of saturation and then in a single step to 75% of saturation while the *p*H was maintained between 6.8 and 7.0 by the addition of dilute ammonium hydroxide. Each precipitate was permitted to accumulate for 8 hours, centrifuged for 20 minutes at 20,000 × *g*, and then dissolved in a small volume of medium A. The fractions were stored at 4°.

Step 4: Acetone Fractionation. The 50–75% ammonium sulfate fractions were combined and subjected to fractional precipitation with acetone, in 10% increments from 30 to 70% of acetone, while the temperature was gradually lowered to −20°. The mixtures were stirred for 5 minutes after each addition of acetone and centrifuged for 10 minutes at 10,000 × *g* at the same temperature as that at which each precipitation was carried out. Each precipitate was dissolved in medium B, and insoluble material (which was especially abundant in the 30–50% acetone fractions) was removed by centrifugation and washed. The 40–60% acetone fractions were combined and dialyzed for 19 hours against four 1-liter changes of medium B. A slight turbidity which usually developed in the solution was removed by centrifugation, and the precipitate was discarded.

Step 5: Chromatography on CM-Cellulose. The dialyzed solution was applied to a CM-cellulose column which had been equilibrated against medium B just prior to chromatography. After loading the enzyme, the column was thoroughly washed with medium B, and then with medium C. The enzyme was then eluted by a linear gradient of increasing phosphate concentration and decreasing glycerol concentration at *p*H 6.4. Full details are given with Figure 2A, which

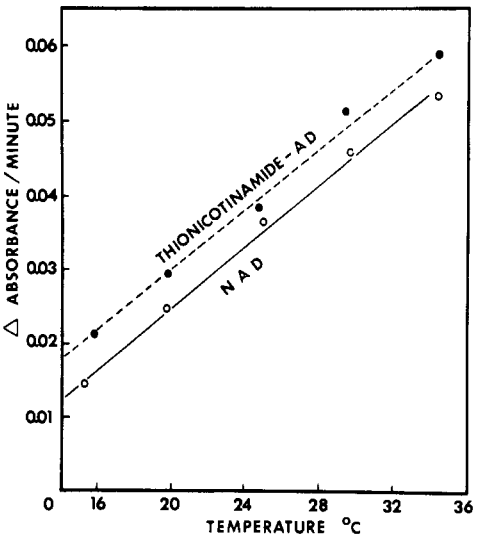

FIGURE 5: Effect of temperature on the reaction rate of 3α-hydroxysteroid dehydrogenase. Initial velocities were measured (at 340 or 395 mμ) in 3.0-ml systems containing: 100 μmoles of sodium pyrophosphate buffer (the final *p*H for NAD was 9.9 and for thionicotinamide–adenine dinucleotide was 9.5), 0.42 μmole of NAD or thionicotinamide–adenine dinucleotide, 0.131 μmole of androsterone in 0.1 ml of methanol, and 30 units of α-HSD (specific activity = 148,000 units/mg of protein). Controls contained all ingredients except steroid. The temperature was varied by adjusting the thermostat of the circulator supplying the thermospacers, and the actual cuvet temperature was measured immediately after running the reaction.

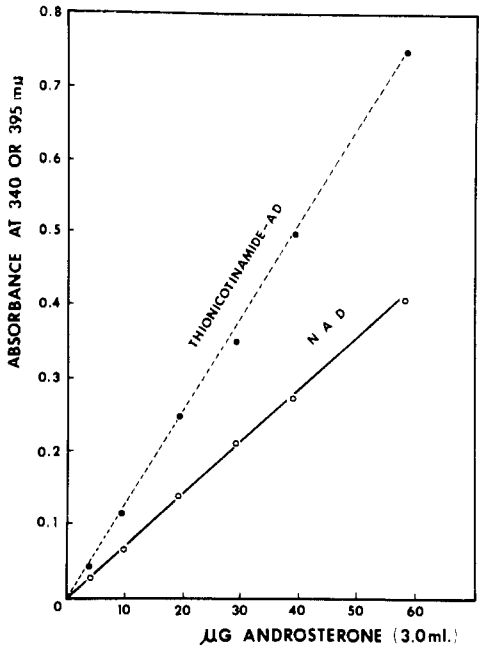

FIGURE 6: Enzymatic assay of androsterone showing linear relation between total change in absorbance and quantity of steroid. Reactions were followed to completion in systems of 3.0 ml final volume containing: 100 μmoles of sodium pyrophosphate buffer (*p*H 9.9 for NAD and *p*H 9.4 for thionicotinamide–adenine dinucleotide), 3.9 to 58 μg of androsterone in 0.1 ml of methanol, 0.45 μmole of NAD or of thionicotinamide–adenine dinucleotide, and an excess of α-HSD. Controls contained all ingredients except steroid. Total change in absorbance at 340 or 395 mμ is recorded at the time the reaction was complete. Temperature, 25°.

shows the elution of the α-HSD and its specific activity. The major portion of the enzyme activity was eluted just ahead of a pink pigment which also coincided with the main protein peak (fraction 112). Fractions 108, 109, and 110 contained a total of 2.07×10^6 units of α-HSD activity (29.8% of step 4) and had specific activities of 163,000, 310,000, and 237,000 units/mg of protein, respectively. The fractions were pooled, ammonium sulfate was added to 75% saturation, and the slurry was stored at 4° (precipitate A). Fractions 111 to 117 contained 2.14×10^6 units of α-HSD (30.8% of step 4) with lower specific activities. They were combined and ammonium sulfate was added to 75% saturation. The precipitate was collected by centrifugation at 20,000 × *g* for 30 minutes and dissolved in fractions 22, 23, and 24, which represented unadsorbed enzyme (5.35×10^5 units of α-HSD, or 7.72% of step 4). This solution was dialyzed for 18 hours against medium B (four 1-liter changes), and was applied to a smaller CM-cellulose column (22 × 2.2 cm). This column was operated in a manner similar to the larger CM-cellulose column used for the first chromatography (Figure 2B).

Fractions 64–72 contained 1.26×10^6 units of α-HSD activity with a mean specific activity of 508,000 units/mg of protein (33-fold purification). These fractions were combined and precipitated by addition of ammonium sulfate to 75% saturation (precipitate B).

Step 6: Gel Filtration on Sephadex. Precipitates A and B were collected by centrifugation at 20,000 × *g* for 60 minutes and dissolved in the smallest possible volume of medium D. A small quantity of insoluble material was removed by centrifugation. The solution (1.7 ml; 2.08×10^6 units of α-HSD), which was clear and pale yellow, was applied to a Sephadex G-100 column (95 × 2 cm; total bed volume = 370 ml; void volume = 110 ml) and filtered by addition of medium D (Figure 3). The fractions of the highest specific activity had a ratio of the light absorbance at 280 mμ to the absorbance at 260 mμ in the range of 2.04 to 2.18. The enzyme activity peak appeared at 1.5 void volumes and was preceded by a protein peak at 1.1 to 1.2 void volumes. The total activity recovered was 1.96×10^6 units of α-HSD (94% of step 5). These fractions were combined and solid ammonium sulfate was added to 75% saturation. The precipitate was stored in a slurry at 4°. The specific activity of α-HSD in fractions 34–40 was nearly constant between 600,000 and 700,000 units/mg of protein. This corresponds to an activity

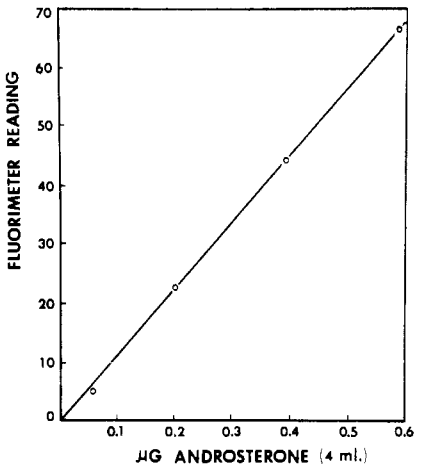

FIGURE 7: Fluorescence measurement of the oxidation of androsterone. Reactions were followed to completion at room temperature (22°) in a Turner fluorimeter using incident light filter at 360 mμ and emergent light filter at 415 mμ. The reaction vessels contained in a final volume at 4.0 ml: 200 μmoles of sodium pyrophosphate buffer, *p*H 9.4, 0.058–0.58 μg of androsterone in 0.15 ml of methanol, 0.41 μmole of NAD, and an excess of α-HSD. Controls contained all ingredients except steroid, and the final measurements are compensated for the fluorescence of controls.

of 290 to 340 μmoles of androsterone oxidized/min per mg of protein. Delin *et al.* (1964) have recently described the purification of this enzyme to a point of homogeneity by gel filtration on Sephadex G-100, followed by column electrophoresis. The product was homogeneous according to a number of criteria (Squire *et al.*, 1964), and for the oxidation of androsterone had a specific activity of 198 μmoles/min per mg of protein.

Properties of the 3α-Hydroxysteroid Dehydrogenase

Reaction Rates with Different Nucleotides. The initial rates of reduction of a number of nicotinamide nucleotide analogs by androsterone were examined with purified preparations of α-HSD. Initial velocities were measured at 25° in 3.0-ml systems containing: 100 μmoles of sodium pyrophosphate buffer (to give a final *p*H of 9.4), 4 μmoles of each nucleotide, 0.24 μg of α-HSD (specific activity of 146,000 units/mg of protein) for NAD and thionicotinamide–adenine dinucleotide or 1.20 μg of enzyme protein for the other nucleotides, and 0.131 μmole of androsterone in 0.05 ml of methanol. Measurements were made at the absorption maximum for each nucleotide and are corrected for difference in extinction of the reduced nucleotides. Under these conditions the following relative initial rates of reaction were obtained: for NAD, 100; for thionicotinamide–adenine dinucleotide, 73; for 3-pyridinealdehyde–adenine dinucleotide, 5.2; for 3-acetylpyridine–adenine dinucleotide, 3.6; and for hypoxanthine–adenine dinucleotide, 0. These results are similar to those reported by Talalay and Williams-Ashman (1960) with a much less pure enzyme preparation and under somewhat different experimental conditions. The Michaelis constants and relative maximum velocities for NAD at *p*H 9.9 and thionicotinamide–adenine dinucleotide at *p*H 9.4 were measured by the double reciprocal plots of Lineweaver and Burk in the presence of saturating concentrations of androsterone. The K_m for NAD was 1.97×10^{-4} M and for thionicotinamide–adenine dinucleotide it was 1.70×10^{-4} M. The maximum velocity (V_m) for thionicotinamide–adenine dinucleotide was 48% of that for NAD.

Effect of pH on Rate of Oxidation with NAD and Thionicotinamide–Adenine Dinucleotide. The hydrogen ion concentration of the reaction medium affects the reaction velocity and the position of the equilibrium of the oxidation (Talalay, 1963). Figure 4 shows the effect of *p*H on the initial rates of oxidation of saturating concentrations of androsterone by α-HSD in the presence of two concentrations of NAD and of thionicotinamide–adenine dinucleotide. The rates rise markedly with increasing *p*H.

Effect of Temperature on Reaction Rate. Figure 5 shows the effect of temperature in the range of about 15 to 34° on the initial rates of oxidation of androsterone using NAD and thionicotinamide–adenine dinucleotide as hydrogen acceptors. The rates rise essentially linearly with temperature. The Q_{10} (20–30°) is 1.8 for NAD and 1.7 for thionicotinamide–adenine dinucleotide.

Quantitative Assay of Androsterone. The application of hydroxysteroid dehydrogenases to the sensitive and specific microestimation of steroids has already been described (Hurlock and Talalay, 1957; Talalay, 1960). Since thionicotinamide–adenine dinucleotide has a higher oxidation-reduction potential than NAD and also a higher extinction coefficient in the reduced form than NADH (Anderson and Kaplan, 1959; Stein *et al.*, 1963), it presents advantages in the enzymatic assay of androsterone and other 3α-hydroxysteroids.

Figure 6 compares the enzymatic assay of varying amounts of androsterone using NAD and thionicotinamide–adenine dinucleotide as hydrogen acceptors. A linear relation is obtained with both coenzymes, and the sensitivity obtainable with thionicotinamide–adenine dinucleotide is about 1.75 times greater than with NAD. Figure 7 demonstrates the application of fluorimetry to the assay of androsterone, using the method of Lowry *et al.* (1957). In a Turner fluorimeter it was possible to measure accurately 0.1 μg of androsterone in a 4.0-ml volume. The sensitivity could therefore be increased by at least a factor of 10, using smaller volumes and a more sensitive instrument.

Effect of pH on Equilibrium of Oxidation of Androsterone. The *p*H profoundly affects the position of the equilibrium of oxidation of androsterone. With purified preparations of α-HSD, the equilibrium constants (Talalay, 1963) were measured at various *p*H values. For both NAD and thionicotinamide–adenine dinucleotide, a linear relation of unit slope was obtained for a plot of log *K* against *p*H. From the data of these experiments, the mean K_H with NAD and thionicotinamide–

TABLE II: Equilibrium Constants and Oxidation-Reduction Potentials of 3α-Hydroxysteroid Dehydrogenase with NAD and Thionicotinamide–Adenine Dinucleotide (TNAD).[a]

Reaction	K_H (M)	E_0 (v)
Androsterone + $NAD^+ \rightleftarrows$ androstane-3,17-dione + NADH + H^+	9.2×10^{-9}	−0.241
Androsterone + $TNAD^+ \rightleftarrows$ androstane-3,17-dione + TNADH + H^+	6.0×10^{-8}	−0.217

[a] K_H is the equilibrium constant from the equation ([NADH or TNADH][steroid ketone][H^+])/([NAD^+ or $TNAD^+$]·[steroid alcohol]). The values given are the means obtained under the experimental conditions described with Figure 4. E_0', the oxidation-reduction potential of the reaction as a whole, has been calculated as $E_0' = RT \ln K_H/nf$.

adenine dinucleotide was calculated. Previous measurements of K_H(NAD) have given the value of 7.4×10^{-9} M (Talalay, 1963), which may be compared with the present finding of 9.2×10^{-9} M (Table II). The results demonstrate the much more favorable equilibrium for the oxidation of the steroid obtained with the thionicotinamide analog ($K_H = 6.0 \times 10^{-8}$ M). The oxidation-reduction potential (E_0') for NAD is −0.320 v (Burton and Wilson, 1953) at *p*H 7.0. The potential E_0' for thionicotinamide–adenine dinucleotide can be computed from these results and is found to be −0.297 v, which may be compared with the value of Anderson and Kaplan (1959) of −0.285 v, obtained for a different enzymatic system.

Acknowledgments

The authors are indebted to Drs. H. J. Koepsell and D. H. Peterson of the Upjohn Company, Kalamazoo, Michigan, for making available the facilities for the large scale growth of the microorganism. It is a pleasure also to acknowledge generous gifts of steroids from the Upjohn Company and from Syntex S.A., Mexico, D.F.

References

Anderson, B. M., and Kaplan, N. O. (1959), *J. Biol. Chem. 234*, 1226.

Beisenherz, G., Boltze, H. J., Bücher, T., Czok, R., Garbade, K. H., Meyer-Arendt, E., and Pfleiderer, G. (1953), *Z. Naturforsch. 8b*, 555.

Burton, K., and Wilson, T. H. (1953), *Biochem. J. 54*, 86.

Delin, S., and Porath, J. (1963), *Biochim. Biophys. Acta 67*, 197.

Delin, S., Squire, P. G., and Porath, J. (1964), *Biochim. Biophys. Acta 89*, 398.

Gornall, A. G., Bardawill, C. J., and David, M. M. (1949), *J. Biol. Chem. 177*, 751.

Hurlock, B., and Talalay, P. (1957), *J. Biol. Chem. 227*, 37.

Jarabak, J., Adams, J. A., Williams-Ashman, H. G., and Talalay, P. (1962), *J. Biol. Chem. 237*, 345.

Kalckar, H. M. (1947), *J. Biol. Chem. 167*, 461.

Kawahara, F. S., Wang, S.-F., and Talalay, P. (1962), *J. Biol. Chem. 237*, 1500.

Lowry, O. H., Roberts, N. R., and Kapphahn, J. I. (1957), *J. Biol. Chem. 224*, 1047.

Marcus, P. I., and Talalay, P. (1955), *Proc. Roy. Soc. (London), Ser. B: 144*, 116.

Marcus, P. I., and Talalay, P. (1956), *J. Biol. Chem. 218*, 661.

Noda, L., and Kuby, S. A. (1957), *J. Biol. Chem. 226*, 541.

Siegel, J. M., Montgomery, G. A., and Bock, R. M. (1959), *Arch. Biochem. Biophys. 82*, 288.

Squire, P. G., Delin, S., and Porath, J. (1964), *Biochim. Biophys. Acta 89*, 409.

Stein, A. M., Lee, J. K., Anderson, C. D., and Anderson, B. M. (1963), *Biochemistry 2*, 1015.

Talalay, P. (1960), *Methods Biochem. Analy. 8*, 119.

Talalay, P. (1963), *Enzymes 7*, 177.

Talalay, P., Dobson, M. M., and Tapley, D. F. (1952), *Nature 170*, 620.

Talalay, P., and Marcus, P. I. (1956), *J. Biol. Chem. 218*, 675.

Talalay, P., and Williams-Ashman, H. G. (1960), *Recent Progr. Hormone Res. 16*, 1.

Warburg, O., and Christian, W. (1936), *Biochem. Z. 287*, 291.