Comparison of the 3α- and 20β-Hydroxysteroid Dehydrogenase Activities of the Cortisone Reductase of *Streptomyces hydrogenans*†

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ABSTRACT: The 3α- and 20β-hydroxysteroid dehydrogenase (HSD) activities of cortisone reductase in *Streptomyces hydrogenans* have been examined to determine whether both activities are due to one enzyme. This question was raised when changes in the commercial preparations of the enzyme reduced the 3α-HSD activity to 5% of its original level while retaining full 20β-HSD activity. In our experiments, the enzyme was purified to crystallinity and partially characterized. The 3α- and 20β-HSD activities were both coinduced and copurified. The 3α- and 20β-HSD activities were compared using the crystalline enzyme for studies of substrate competition, thermal inactivation at 52 °C, loss of activity with three haloacetoxysteroids, and the effects of Me_2SO and temperature on the reaction rate. These studies support the conclusion that the 3α- and 20β-HSD activities are due to the same enzyme molecule. In addition, it appears that the binding sites for the two activities do not act independently.

The 20β-hydroxysteroid dehydrogenase (HSD[1]) of *S. hydrogenans*, also called "cortisone reductase", was discovered by Hübener and co-workers in 1958 (Lindner et al., 1958). A decade later, a 3α-HSD activity was identified with cortisone reductase on the basis of constant ratios of 3α-HSD activity to 20β-HSD activity observed in three successive enzyme preparations (Pocklington and Jeffery, 1968). The 3α,20β-HSD is an inducible, NADH-requiring, tetrameric enzyme of apparently identical subunits (Hübener et al., 1959; Hübener, 1963; Blomquist, 1972, 1973); its molecular weight is approximately 100 000 (Blomquist, 1972, 1973). It is commercially available.

Evidence that the 3α- and 20β-HSD activities were due to one enzyme was not particularly strong. Kinetic competition experiments using mixtures of substrates gave inconclusive results (Pocklington and Jeffery, 1968, 1969; Gibb and Jeffery, 1971). Perhaps the best evidence came from the comigration of the 3α- and 20β-HSD activities on polyacrylamide disc gels (Blomquist, 1973; Rapaport and Orr, unpublished). In addition, the two activities showed identical rates of inactivation in acid (pH 4.5; Blomquist, 1973).

Preliminary experiments in our laboratory (Rapaport and Orr, 1973a,b) had shown intriguing differences between the 3α- and 20β-HSD activities: (1) Me_2SO in the assay mixture apparently increased 3α-HSD activity while decreasing 20β-HSD activity; (2) an inhibitor of steroid dehydrogenases, 17β-hydroxy-2-hydroxymethylene-17α-methyl-5α-androstan-3-one, inhibited only the 3α-HSD activity; and (3) the 20β-HSD activity demonstrated an unremarkable primary isotope effect with (4*S*)-[4-^{2}H]NADH, whereas the 3α-HSD activity had an unusually high isotope effect.

We set out to extend these observations and to study the effects of various haloacetoxysteroids, reported to inactivate the 20β-HSD activity, upon both the 3α- and 20β-HSD activities, using the commercially supplied enzyme. Batches of the enzyme bought during 1973–1974, however, showed markedly different properties from those used in the earlier studies; the 3α-HSD activity had decreased to approximately 5% of its former value, while the 20β-HSD activity remained as high as before. The commercially prepared enzyme, no longer a well-defined entity, was therefore unsuitable for comparative studies of the 3α- and 20β-HSD activities, and the identification of both activities with one enzyme was open to question. During previous studies of induction and preparations of the enzyme, only the 20β-HSD activity had been monitored. It was therefore decided to reexamine the relationship between the two dehydrogenase activities.

Experimental Procedure

Materials. Streptomyces hydrogenans, type strain no. 19631, was obtained from the American Type Culture Collection. Oatmeal agar and constituents of media were purchased from Difco Labs (Detroit, Mich.).

Steroids were obtained from commercial sources, except 17β-hydroxyandrostan-2-one, which was the gift of Dr. R. L. Clarke (Sterling-Winthrop). Before use, steroids were purified if necessary.

Growth of S. hydrogenans. The liquid medium used was that found optimal for cortisone reductase induction and very good for *S. hydrogenans* growth in fermentor cultures by Nesemann and co-workers (1960, page 93). Bacteria were also grown on oatmeal agar.

Assays of Enzyme Activity. (a) With Steroid Ketone Substrates and NADH. Assay mixtures contained potassium phosphate buffer (pH 6.4; 10 mM final concentration), NADH (0.025 mM), and steroid dissolved in methanol. Control assay mixtures contained all components except steroid. Enzyme was added and the decrease in absorbance (A) at 340 nm was recorded on a Gilford 2000 spectrophotometer.

(b) With Steroid Alcohols and NAD^+. Assay mixtures contained potassium phosphate buffer (pH 8.9; 10 mM final

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[1] Abbreviations used are: HSD, hydroxysteroid dehydrogenase; NAD, nicotinamide adenine dinucleotide; NADH, reduced NAD; DEAE, diethylaminoethyl; EDTA, (ethylenedinitrilo)tetraacetic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

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concentration), NAD^+ (0.77 mM), and steroid dissolved in methanol. The control assay mixture contained all components except steroid. Enzyme was added to each cuvette and increases in A with time were recorded.

Protein Assay. Protein was assayed by the method of Lowry et al. (1951).

Purification of 3α,20β-HSD. A Fermacell Fermentor (New Brunswick Scientific Co., N.J.) was used to grow a 30-L culture of *S. hydrogenans*. A 1% inoculum was used. The culture was stirred and aerated vigorously and kept between 26 and 27 °C. After 15 h, the cell density had reached 485 Klett units and the inducer [11β,21-dihydroxypregna-4,17(20)-dien-3-one (10 g) in methanol (250 mL)] was added. After 24 h of fermentation in the presence of the inducer, the culture was harvested using a refrigerated Sharples Super Centrifuge. The packed cells (519 g) were resuspended in 5 volumes of washing buffer I (Träger, 1973) and centrifuged for 15 min at 18 000*g*. After this step, all purification procedures were done at 4 °C.

The washed cells were resuspended in 5 volumes of washing buffer and disrupted by one pass through a Manton-Gaulin submicron disperser at 9000 psi. Cell debris was removed by centrifugation for 1 h at 18 000*g*. An aqueous streptomycin sulfate solution (18 g in 100 mL, pH 7.0) was added to the crude enzyme extract. The mixture was stirred for 2 h and then centrifuged for 1 h at 20 000*g*. The precipitate was discarded.

The resulting proteins were fractionated by the addition of saturated $(NH_4)_2SO_4$ (1 mM in EDTA, pH 7.2). The protein fraction that precipitated between 28 and 40% saturation with $(NH_4)_2SO_4$ was collected by centrifugation, dissolved in 0.1 M potassium phosphate (pH 7.2) containing 20% glycerol, and dialyzed overnight against the same buffer.

The enzyme was further purified by chromatography on DEAE-cellulose and Sephadex G-100, as described in the legend in Figure 1. The enzyme was then crystallized by the method of Hübener and Sahrholz (1960); crystallization occurred at approximately 26% saturation with $(NH_4)_2SO_4$. The crystals were dissolved in 0.1 M potassium phosphate (pH 8.0) containing 20% glycerol. This solution, designated crystal crop 1, was divided and frozen. A second crystal crop was obtained from the crystal crop 1 supernatant solution by adding saturated $(NH_4)_2SO_4$ solution to approximately 35% saturation. These crystals were designated crystal crop 2.

A sample of crystal crop 1 was recrystallized. Crop 1 enzyme (10 mg, 0.8 mL) was diluted to 4.0 mL with buffer and recrystallized by the addition of $(NH_4)_2SO_4$ solution to approximately 30% saturation. The crystals were dissolved and the crystallization was repeated.

Disc Gel Electrophoresis. Analytical polyacrylamide disc gel electrophoresis was performed in gels containing 7.5% acrylamide and 20% glycerol (gel system 1, Maurer, 1971). Protein was stained in the gels with 0.2% Coomassie brilliant blue in 12.5% trichloroacetic acid. HSD activity was detected by incubating gels in the dark at room temperature with hydroxysteroid (5 μmol in 2.5 mL of methanol), NAD^+ (15 μmol), Nitro Blue Tetrazolium (3.1 μmol), phenazine methosulfate (0.8 μmol), and potassium phosphate (0.1 μmol, pH 7.3) in a total volume of 10 mL (Gabriel, 1971). Control incubations, performed without steroid, confirmed the specificity of the activity stain. Substrates were 17α,20β,21-trihydroxypregn-4-en-3-one for 20β-HSD activity, 3α-hydroxyandrostan-17-one for 3α-HSD activity, and 3β-hydroxyandrostan-17-one for 3β-HSD activity.

Substrate Utilization Incubations. When steroid ketones were used as substrates, the incubation mixture consisted of NADH (25 μmol), enzyme (25 μg), and steroid (5 μmol in 1 mL of methanol) in 30 mL of buffer (0.03 M potassium phosphate, 1 mM EDTA, and 20% glycerol, pH 5.5). When steroid alcohols were used as substrates, the incubation mixture contained NAD^+ (25 μmol), enzyme (25 μg), and steroid (5 μmol in 1 mL of methanol) in 30 mL of buffer (0.03 M potassium phosphate, 1 mM EDTA, and 20% glycerol, pH 8.5). After incubation overnight at room temperature, each solution was extracted twice with CH_2Cl_2 (2 × 50 mL). The organic layer was filtered and dried; steroids were identified by TLC and combined gas chromatography-mass spectrometry.

Gas Chromatography-Mass Spectrometry. Both combined gas chromatography-mass spectrometry (GC-MS) and direct probe mass spectrometry was performed using an LKB 9000 gas chromatograph-mass spectrometer.

Amino Acid Analysis. Samples of enzyme from crystal crop 1 were subjected to acid hydrolysis. A Beckman Model 121 automatic amino acid analyzer was used to perform amino acid analysis. Magnetic circular dichroism (Holmquist and Vallee, 1973) was used to quantitate the tryptophan residues; *N*-acetyl-L-tryptophan was the standard.

Synthesis of Haloacetoxysteroids. Cortisone 21-iodoacetate was synthesized after the method of Ganguly and Warren (1971): mp 193 °C (dec), lit. 180 °C (dec). The NMR and mass spectra of the synthetic compound were consistent with the assigned structure.

16α-Hydroxyprogesterone was synthesized by chromous acetate reduction of 16α,17α-epoxyprogesterone; it was converted to the bromoacetate by a modification of the method of Sweet et al. (1972), using 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfate. The product crystallized readily and was recrystallized: mp 120–122.5 °C, lit. mp 120–122 °C. The identity of the product was confirmed by mass spectrometry.

17β-Bromoacetoxyandrostan-3-one, a new compound, was synthesized from the alcohol using the same general method as for the synthesis of 16α-bromoacetoxyprogesterone. The product crystallized in flat plates from ethanol: yield 44%; mp 120–122 °C; homogeneous by TLC in ether/benzene (2:1). Mass spectrometry supported the assigned structure.

Incubation of Enzyme with Haloacetoxysteroids. Incubation mixtures contained buffer, enzyme, and steroid. The mixture was kept in the dark at room temperature and periodically assayed with 17β-hydroxyandrostan-3-one and progesterone.

The effects of cortisone 21-iodoacetate (0.3 μmol in 0.3 mL of methanol) were tested using potassium phosphate (0.1 M, pH 7.0, 4.7 mL) and enzyme (37 μg). In one experiment, NADH (0.18 μmol) was added to the reaction mixture along with cortisone 21-iodoacetate.

16α-Bromoacetoxyprogesterone (150 nmol in 400 μL of ethanol) was added to potassium phosphate (0.05 M, pH 7.0, 9.6 mL) and enzyme (0.15 mg). For control incubations, (a) steroid was omitted, (b) bromoacetate anion (300 nmol) was substituted for the steroid, or (c) 16α-hydroxyprogesterone (150 nmol) was substituted for the steroid. In protection experiments, either 17β-hydroxyandrostan-3-one or progesterone was added to the complete reaction mixture at twice the concentration of the haloacetoxysteroid, or NADH was added to the reaction mixture at four times the concentration of the haloacetoxysteroid.

17β-Bromoacetoxyandrostan-3-one (150 nmol in 400 μL of ethanol) was tested using Tris buffer (0.05 M, pH 9.0, 9.6 mL) and enzyme (0.15 mg). Control incubations contained the same components, except (a) steroid was omitted or (b) bromoacetic acid (300 nmol) was substituted for the steroid.

TABLE I: Purification: 3α,20β-Hydroxysteroid Dehydrogenase.

step	protein (g)	total act. (μmol/min) 3-HSD	total act. (μmol/min) 20-HSD	sp. act (μmol/min·mg) 3-HSD	sp. act (μmol/min·mg) 20-HSD	yield (%) 3-HSD	yield (%) 20-HSD	act. ratio (3-HSD/20-HSD)
(1) crude homogenate	18.7	3380	5460	0.20	0.29	100	100	0.70
(2) streptomycin sulfate		3500	5250			91	96	0.67
(3) ammonium sulfate (28–40%)	1.30	1580	2460	1.22	1.90	41	45	0.64
(4) DEAE-cellulose	0.200	940	1750	4.7	8.7	25	32	0.54
(5) Sephadex G-100	0.095	740	1480	7.8	15.5	19	27	0.50
(6) crystals, crop 1	0.062	375	920	6.1	15.0	9.8	17	0.41

TABLE II: Kinetic Constants.

substrate	saturating substr	V/E (μmol/min·mg)	K_m (μM)	R^2
	(A) For Substrates of 3α,20β-HSD			
progesterone	NADH	29	2.8	0.93
17β-hydroxyandrostan-3-one	NADH	20.0	140	0.99
cortisone	NADH	17.9	24	0.99
17β-hydroxyandrostan-2-one	NADH	1.29	460	0.99
NADH	cortisone	29	9.9	0.99
NADH	17β-hydroxyandrostan-3-one	10.2	9.0	0.99

substrate	saturating substr	V/E (μmol/min·mg)	K_m (μM)	reference
	(B) From the Literature			
progesterone	NADH	20	2.2	Pocklington and Jeffery (1968)
		6.1	1.1	White and Jeffery (1972)
		11.58	3.95	Sweet et al. (1972)
17β-hydroxyandrostan-3-one	NADH	19.6	289	Pocklington and Jeffery (1968)
		14	112	Gibb and Jeffery (1973)
cortisone	NADH		51	Hübener (1963)
		10.9	29.3	Gibb and Jeffery (1971)
NADH	cortisone		7.2	Hübener (1963)

Results

Purification of 3α,20β-HSD. Both the 3α- and 20β-HSD activities of the cortisone reductase were followed during a complete purification of the enzyme to crystallinity to detect if the two activities were separable. A culture was prepared, induced with 11β,21-dihydroxypregna-4,17(20)-dien-3-one, harvested, and treated as described under Experimental Procedure.

DEAE-cellulose chromatography (Figure 1, top) gave good separation of the bulk of the protein from the 3α- and 20β-HSD activities. Over the peak of enzyme activity, no separation of 3α-HSD from 20β-HSD was seen. Sephadex G-100 chromatography (Figure 1, bottom) gave some additional separation of active from inactive protein, but no separation of 3α- and 20β-HSD activities was observed. The ratio of the two activities remained relatively constant over the peak.

Crystals from crop 1 averaged 10-μm in length. Gel electrophoresis showed crystal crop 1 to contain no inactive protein; crystal crop 2 contained about 3% contaminating protein.

The purification summary is given in Table I. During the purification procedure, the ratio of total 3-HSD to 20β-HSD activity decreased slightly. The decreases seemed to be due to dialysis and/or freezing of the enzyme between purification steps (unpublished data) as well as to the purification steps themselves, but no separation of 3α- from 20β-HSD activities was observed.

The progress of the purification was followed by polyacrylamide disc gel electrophoresis. From step to step, staining of gels for protein indicated increasing dominance of the triplet of bands corresponding to the 3α- and 20β-HSD activities, and staining for activity showed coincidence of the two activities. After ammonium sulfate fractionation, 3β-HSD activity was no longer detectable. Gels after electrophoresis with crystal crop 1 enzyme had three bands staining for activity and protein; inactive protein bands were not found. The enzyme was, therefore, not homogeneous but appeared to consist only of 3α,20β-HSD activity. The two minor bands each contained approximately 10% of the protein and activity.

Although crystal crop 1 appeared to contain pure 3α,20β-HSD, a sample was recrystallized twice. The results indicated constant specific activities and activity ratios. The remainder of crystal crop 1 was therefore not recrystallized before use in further experiments.

Characterization

Activities of Crystalline 3α,20β-HSD. Crystal crop 1 was examined for activity with a variety of steroids. The 3α- and 20β-HSD activities had been demonstrated with steroid ketones during purification and with steroid alcohols during staining for activity after electrophoresis. Other steroids were tested using the incubation technique described under Experimental Procedure. Reversible 2β- and 16β-HSD activities were observed, whereas no 17β-HSD was found.

Kinetic Studies. Kinetic constants were determined for 2-, 3- and 20-ketosteroids (Table II). The 16β-HSD reaction was too slow for spectrophotometric experiments. Generally, the kinetic constants determined here are comparable to those in the literature.

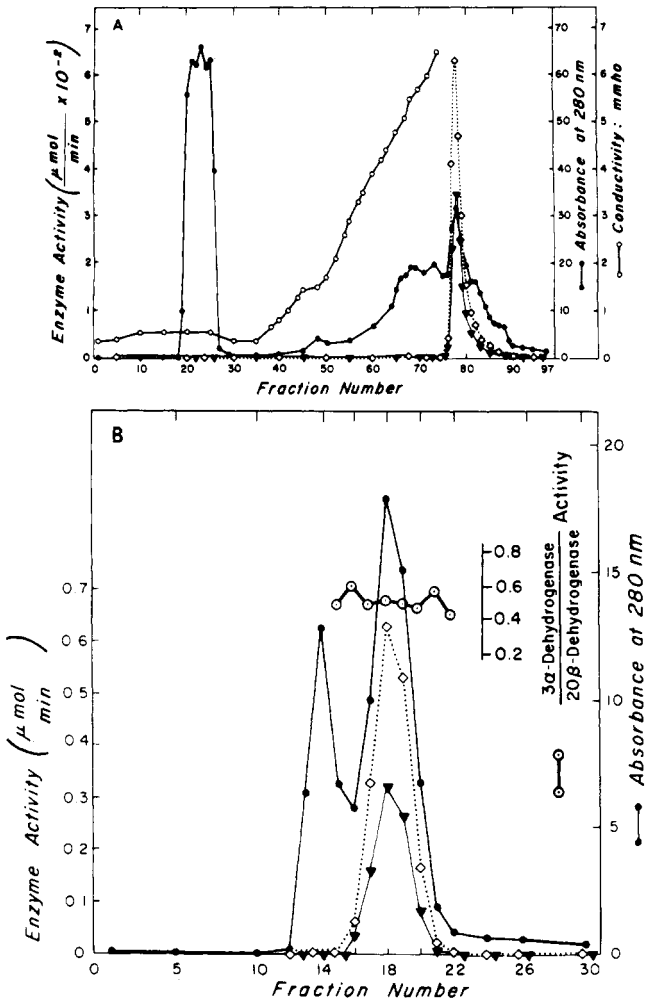

FIGURE 1: DEAE-cellulose chromatography and gel filtration on Sephadex G-100. (A) DEAE-cellulose chromatography. A Whatman DE-52 column (5 × 40 cm) was equilibrated with 0.025 M Tris buffer (pH 8.7) in the cold, containing 20% glycerol. The enzyme fraction that precipitated between 28 and 40% saturation with $(NH_4)_2SO_4$ was applied to the column, and fractions of 20 mL were collected. Active enzyme was eluted with a gradient of 0.05 M Tris (pH 8.7) containing 20% glycerol and the same buffer plus 0.25 M NaCl. (B) Gel filtration on Sephadex G-100. A column (1.6 × 100 cm) of Sephadex G-100 (Pharmacia) was equilibrated with 0.1 M potassium phosphate (pH 7.4) containing 20% glycerol. The pooled active fractions from the DEAE-cellulose column (no. 77–80) were concentrated to 6.0 mL by ultrafiltration with a UM-10 membrane (Amicon), applied to the column, and eluted with the equilibration buffer. Fractions of 3.5 mL were collected: (●-●) absorbance at 280 nm; (◇- -◇) 20β-HSD activity, assayed with progesterone; (▼-▼) 3α-HSD activity, assayed with 17β-hydroxyandrostan-3-one; (○-○) conductivity, mmho (top figure only); (⊙—⊙) ratio of 3α-HSD/20β-HSD activity (bottom figure only).

Amino Acid Analysis. The amino acid analysis (Table III) is expressed as residues per tetramer, since it has not been firmly established that the four subunits of 3α,20β-HSD are identical. The molecular weight based on amino acid composition is 92 000, excluding Cys residues. The influence of the minor component enzymes of 3α,20β revealed by gel electrophoresis on the amino acid analysis is not know. Tryptophan was determined by magnetic circular dichroism. The only amino terminal is alanine (Ford, unpublished results).

Carbohydrate Analysis. In a 200-μg sample of dry enzyme, no sialic acid was found, and only 1.5 μg of glucose (probably due to contamination) was detected. Galactose could have been detected at a level of 0.2 μg but none was found.

Competition Studies. Competition experiments were done with equimolar concentrations of 17β-hydroxyandrostan-3-one and cortisone. The total reaction rate for the steroid mixture was determined from the rate of disappearance of NADH. From the kinetic constants given in Table II, theoretical curves were calculated for additive, mutually competitive, and mutually obstructive reactions using the equations given by Gibb and Jeffery (1971). The results (Figure 2) indicated that the reaction velocities were not additive; there may be interaction between the site of the 3α-HSD activity and that for the 20β-HSD activity, although other explanations cannot be excluded. The differences between the three models are greatest at high steroid concentrations, where the experimental results indicated mutual competition.

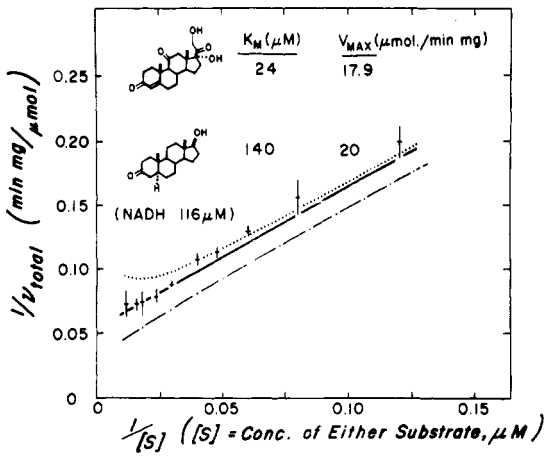

FIGURE 2: Competition between 17β-hydroxyandrostan-3-one and cortisone at equal concentration: (|) data points ±2 standard deviations. Theoretical lines: (- - -) additive reactions; (—) mutually competitive inhibition; (- · -) mutually obstructive inhibition.

TABLE III: Amino Acid Content of 3α,20β-HSD.

amino acid	residues/ tetramer
Lys	19.5
His	17.0
Arg	38.9
Asp	47.0
Thr	56.0
Ser	33.4
Glu	65.9
Pro	17.6
Gly	112.6
Ala	113.3
Cys	*a*
Val	73.1
Met	≥25.9
Ile	31.5
Leu	58.2
Tyr	16.2
Phe	17.4
Trp	12.4

[a] Not determined.

Additional experiments with 17β-hydroxyandrostan-3-one and progesterone at equimolar concentrations or at similar relative concentrations (relative concentration = [substrate]/K_m) gave less than additive velocities in both cases; however, nonclassical kinetics of progesterone reduction precluded further interpretation of these results.

Thermal Inactivation. The rate of enzyme inactivation was measured at 52 °C. The results indicated no difference in the rates of loss of 3α- and 20β-HSD activities at 52 °C. The slope of the regression line for activity with progesterone was

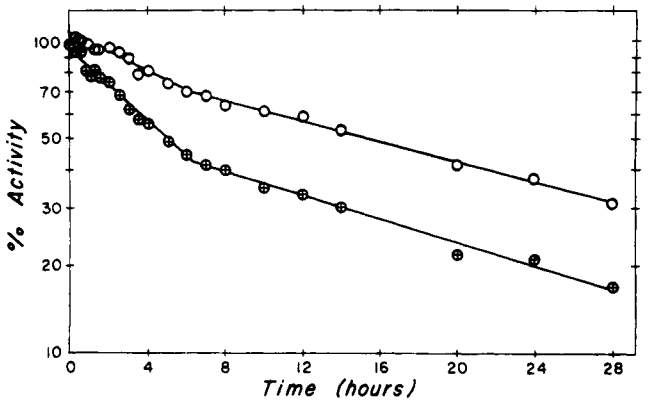

FIGURE 3: Inactivation with 17β-bromoacetoxyandrostan-3-one: (⊕-⊕) 20β-HSD activity, assayed with progesterone; (O-O) 3α-HSD activity, assayed with 17β-hydroxyandrostan-3-one.

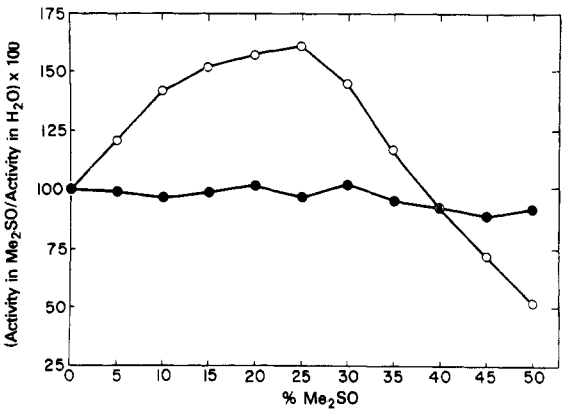

FIGURE 4: Effect of dimethyl sulfoxide on 3α- and 20β-HSD activities: (O) 20β-HSD activity, assayed with pregn-5-ene-3β,20β-diol; (●) 3α-HSD activity, assayed with 3α-hydroxyandrostan-17-one.

−0.0150 enzyme unit/min (R^2 = 0.97), while the slope with 17β-hydroxyandrostan-3-one was −0.0154 enzyme unit/min (R^2 = 0.98).

Loss of Activity with Haloacetoxysteroids

Three haloacetoxysteroids were synthesized from substrates for the 3α- and 20β-HSD activities and tested for their abilities to inactivate the 3α- and 20β-HSD activities. The first two compounds, cortisone 21-iodoacetate and 16α-bromoacetoxyprogesterone, were made from 20-ketone substrates. The third compound, 17β-bromoacetoxyandrostan-3-one, was synthesized from a 3-ketone substrate.

(*a*) *Cortisone 21-Iodoacetate.* The enzyme was incubated with cortisone 21-iodoacetate to determine the rates of inactivation of the 3α- and 20β-HSD activities (see Experimental Procedure). In two separate trials, the 3α- and 20β-HSD activities were not inactivated by cortisone 21-iodoacetate during a few hours of incubation. After 20 h, however, approximately 20% loss of both 3α- and 20β-HSD activities was found; in the absence of cortisone 21-iodoacetate, the enzyme was stable. The enzyme was also stable during 20 h of incubation when NADH was added to the incubation mixture along with the steroid.

(*b*) *16α-Bromoacetoxyprogesterone.* Inactivation of the enzyme with 16α-bromoacetoxyprogesterone gave similar rates of loss of the 3α- and 20β-HSD activities. The half-time for inactivation was 9 h for both activities and R^2 = 0.98. No inactivation was observed in control incubations in which (a) the haloacetoxysteroid was omitted or (b) bromoacetate anion was present instead of the bromoacetoxysteroid. The observed inactivation was thus due to the bromoacetoxysteroid. In the presence of 16α-bromoacetoxyprogesterone, neither 17β-hydroxyandrostan-3-one nor progesterone conferred any protection from inactivation of the enzyme. In contrast, NADH in the reaction mixture conferred complete protection from inactivation.

(*c*) *17β-Bromoacetoxyandrostan-3-one.* Incubation of 3α,20β-HSD with 17β-bromoacetoxyandrostan-3-one (Figure 3) caused biphasic inactivation. The 20β-HSD activity was lost twice as rapidly as the 3α-HSD activity during the first 7 h of incubation; after 7 h, both activities diminished at approximately equal rates. The preferential inactivation of the 20β-HSD activity suggests that 3α- and 20β-hydroxysteroids might not occupy the same site on the enzyme. Control incubations showed no inactivation of enzyme either (a) in the absence of bromoacetoxysteroid or (b) when bromoacetic acid was substituted for the steroid.

The results of experiments on the inactivation by haloacetoxysteroids are summarized as follows. Cortisone 21-iodoacetate, reported to inactivate 20β-HSD rapidly ($t_{1/2}$ = 3 h; Ganguly and Warren, 1971), inactivated our preparation only 20% in 20 h. 16α-Bromoacetoxyprogesterone inactivated the 3α- and 20β-HSD at equal rates but much more slowly than reported by Sweet and co-workers (1972). The new compound, 17β-bromoacetoxyandrostan-3-one, related to a 3-ketone substrate, inactivated the 20β-HSD activity more rapidly than the 3α-HSD activity and in an apparently biphasic process.

Effects of Dimethyl Sulfoxide (Me_2SO). Me_2SO at concentrations from 0 to 50% (v/v) was used in assays with NAD^+ and steroid alcohols to reexamine the preliminary results of Rapaport and Orr (1973a), which showed increasing 3α-HSD and decreasing 20β-HSD activities with increasing Me_2SO concentrations. In the present experiments (Figure 4), the 3α-HSD activity was essentially unaffected up to 50% Me_2SO; in contrast, the 20β-HSD activity rose to a maximum at 25% Me_2SO and then fell sharply.

Effect of Temperature on the Reaction Rates. A series of assays was done at various temperatures in the absence of Me_2SO but using the same substrate and enzyme concentrations as in the experiments with Me_2SO. Assay mixtures, complete except for the enzyme, were equilibrated in covered cuvettes in the spectrophotometer before the assays were started. The 3α-HSD activity increased more rapidly than the 20β-HSD activity with increasing temperature.

From the Arrhenius plot obtained for the 20β-HSD activity assayed with pregn-5-ene-3β,20β-diol, an activation energy of 570 cal/mol and a frequency factor of 8.65×10^9 M^{-1} s^{-1} were calculated (R^2 = 0.96). For the 3α-HSD activity assayed with 3α-hydroxyandrostan-17-one, the activation energy was 1400 cal/mol and the frequency factor was 3.12×10^{14} M^{-1} s^{-1} (R^2 = 0.99). These results indicated a differential effect of temperature on the rate-determining steps. It has not yet been established that the rate-determining step is the same for both activities.

Discussion

The lack of 17β-HSD activity in our crystalline enzyme was unexpected because this activity is present in commercial preparations of 3α,20β-HSD (Rapaport and Orr, 1973a) and has been detected after gel electrophoresis of crude enzyme fractions at the same mobility as the 3α- and 20β-HSD activities. The work of Markert et al. (1975) indicates the presence of a 17β-HSD activity that is induced independently of the 3α,20β-HSD activity in *S. hydrogenans*; the localization of this 17β-HSD in gels has not been reported. It is possible that the 17β-HSD activity reported by Markert corresponds

to that observed in crude fractions by us and in commercial preparations by others.

In spite of the large amount of study that has been directed at the 3α,20β-HSD, the relationship between the active-site region for the 3α- and 20β-hydroxysteroids had not been well defined. It was assumed, without much supporting evidence, that the 3α,20β-HSD was one enzyme rather than a mixture of a 3α-HSD and a 20β-HSD. As a result of the present work, it appears that this assumption is correct; in addition, it is most reasonable to conclude that the 3α,20β-HSD has interacting sites for 3α- and 20β-hydroxysteroids.

In our studies, the 3α- and 20β-HSD activities did not separate at any stage of the purification. No differences in behavior of the two activities were found during inactivation at 52 °C or inactivation by cortisone 21-iodoacetate or 16α-bromoacetoxyprogesterone. As was already reported for the commercial enzyme, combinations of substrates for the 3α- and 20β-HSD activities gave less than additive reaction velocities. These results suggested that the 3- and 20-ketosteroids have interacting, perhaps, even overlapping, binding sites.

Competition experiments showed that the substrates 17β-hydroxyandrostan-3-one and progesterone gave less than additive velocities as measured by NADH disappearance; likewise, 17β-hydroxyandrostan-3-one and cortisone gave less than additive velocities and demonstrated mutual inhibition. The simplest interpretation of these experiments is that substrates for 3α- and 20β-HSD activities compete for one site at which they both react with pyridine nucleotide, although separate allosteric sites for the two activities cannot be excluded. There appear to be only four binding sites per tetrameric enzyme. Another interpretation is that nonproductive binding of a substrate for 3α-HSD in the active-site region of an enzyme specific for 20β-hydroxysteroids was partially blocking the 20β-activity and vice versa. Nonproductive binding might occur if hydrophobic steroid molecules were attracted to hydrophobic active-site regions of steroid dehydrogenases, without being able to react. More information could probably have been gained by measuring the extent of transformation of each substrate by methods such as gas chromatography.

Differences in behavior between the 3α- and 20β-HSD activities were also found. During inactivation with 17β-bromoacetoxyandrostan-3-one, the 20β-HSD activity was lost more rapidly than the 3α-HSD activity. After the addition of increasing amounts of Me_2SO to the assay, the 3α-HSD activity remained essentially constant, while the 20β-HSD activity rose and then fell.

Equal rates of inactivation of the 3α- and 20β-HSD activities were found in incubations of enzyme with cortisone 21-iodoacetate and 16α-bromoacetoxyprogesterone, both synthesized from substrates for the 20β-HSD activity. In contrast, 17β-bromoacetoxyandrostan-3-one, synthesized from a substrate for the 3α-HSD activity, gave a complex inactivation pattern, with the 20β-HSD activity being inactivated twice as rapidly as the 3α-HSD activity during the first 7 h of incubation with the haloacetoxysteroid.

The inactivation of both 3α- and 20β-HSD activities at equal rates by cortisone 21-iodoacetate and 16α-bromoacetoxyprogesterone and the lack of inactivation of both activities when NADH was added with the haloacetoxysteroids would indicate interacting and possibly overlapping binding sites for the two types of substrates. The same results could have been obtained with two different enzymes, but this is unlikely, as it would require us to assume that these two enzymes are coinduced, copurify, are coincidental on electrophoretic gels, and have the same N-terminal amino acid. Indiscriminate modification of nucleophilic residues of the enzyme is unlikely, because NADH completely protected the enzyme from inactivation by these two haloacetoxysteroids.

The pattern of inactivation of the 3α- and 20β-HSD activities by 17β-bromoacetoxyandrostan-3-one was complex, with 20β-HSD activity lost twice as rapidly as 3α-HSD activity during the first 7 h; after 7 h, almost equal rates of inactivation were obtained. If the 3α- and 20β-HSD activities were independent, more rapid inactivation of the 3α- than the 20β-HSD activity would be predicted for the haloacetate of a substrate for 3α-HSD, depending upon the recognition of the haloacetoxysteroid as a substrate for 3α-HSD, which was not tested.

The differences between the 3α- and 20β-HSD activities at various concentrations of Me_2SO indicate some differential effect on the binding sites or on the steroids. Determination of K_m and V values for 3α- and 20β-hydroxysteroids in 25% Me_2SO, the concentration that gave the largest difference between the 3α- and 20β-HSD activities, might elucidate the effect of Me_2SO on the reaction. Alterations of enzyme conformation in Me_2SO could be detected by measuring the circular dichroism difference spectrum of the enzyme during titration with Me_2SO (Villanueva et al., 1974).

Differences between the 3α- and 20β-HSD activities in Me_2SO were found both in our experiments and in those of Rapaport and Orr (1973a). Whereas we found no effect of Me_2SO on 3α-HSD activity, they found increasing 3α-HSD activity with increasing Me_2SO concentration; further, we found 20β-HSD activity to increase, reaching a maximum of 25% Me_2SO, yet they found progressive decreases in 20β-HSD activity with increasing Me_2SO concentrations. Both studies used the same substrates and similar techniques. The differences between our results and those of Rapaport and Orr were probably at least partly due to the enzyme used.

Experiments on the effect of temperature on the assay gave an activation energy of 570 cal/mol for pregn-5-ene-3β,20β-diol and 1400 cal/mol for 3α-hydroxyandrostan-17-one. Since only one substrate for each activity was used, it is not known if differences in activation energy reflect properties characteristic of 3α- and 20β-hydroxysteroids in general or of the specific substrates used. Because the axial 3α-hydroxyl group causes 1,3-diaxial interaction with consequent strain in ring A, the higher activation energy for the 3α-hydroxysteroid may well reflect general differences in activation energy for 3α- and 20β-hydroxysteroids.

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References

Blomquist, C. H. (1972), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 31,* 841.

Blomquist, C. H. (1973), *Arch. Biochem. Biophys. 159,* 590.

Gabriel, O. (1971), *Methods Enzymol. 22,* 578.

Ganguly, M., and Warren, J. C. (1971), *J. Biol. Chem. 246,* 3646.

Gibb, W., and Jeffery, J. (1971), *Eur. J. Biochem. 23,* 336.

Gibb, W., and Jeffery, J. (1973), *Biochem. J. 135,* 881.

Holmquist, B., and Vallee, B. L. (1973), *Biochemistry 12,*

4409.
Hübener, H. J. (1963), in Methods of Enzymatic Analysis, Bergmeyer, H. U., Ed., Academic Press, Weinheim, p 477.
Hübener, H. J., and Sahrholz, F. G. (1960), *Biochem. Z. 333,* 95.
Hübener, H. J., Sahrholz, F. G., Schmidt-Thomé, J., Nesemann, G., and Junk, R. (1959), *Biochim. Biophys. Acta 35,* 270.
Lindner, F., Junk, R., Nesemann, G., and Schmidt-Thomé, J. (1958), *Hoppe-Seyler's Z. Physiol. Chem. 313,* 117.
Lowry, O. H., Rosebough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.
Markert, C., Betz, B., and Träger, L. (1975), *Z. Naturforsch. C 30C,* 266.
Maurer, H. R. (1971), Disc Electrophoresis and Related Techniques of Polyacrylamide Gel Electrophoresis, Walter de Gruyter, New York, N.Y., p 32.
Nesemann, G., Hübener, H. J., Junk, R., and Schmidt-Thomé, J. (1960), *Biochem. Z. 333,* 88.
Pocklington, T., and Jeffery, J. (1968), *Eur. J. Biochem. 7,* 63.
Pocklington, T., and Jeffery, J. (1969), *Eur. J. Biochem. 9,* 142.
Rapaport, E., and Orr, J. C. (1973a), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 32,* 2336.
Rapaport, E., and Orr, J. C. (1973b), *Proc. Plenary Sess., Int. Congr. Biochem. 9th,* abstract 2d27.
Sweet, F., Arias, F., and Warren, J. C. (1972), *J. Biol. Chem. 247,* 3423.
Träger, L. (1973), *Hoppe-Seyler's Z. Physiol. Chem. 354,* 1077.
Villanueva, G. B., Batt, C. W., and Brunner, W. (1974), *J. Chem. Soc., Chem. Commun.,* 766.
White, I. H., and Jeffery, J. (1972), *Eur. J. Biochem. 25,* 409.

Purification, Subunit Structure, and Amino Acid Composition of Avian Erythrocyte Adenosine Monophosphate Deaminase†

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ABSTRACT: Adenosine monophosphate deaminase has been purified over 6000-fold from chicken erythrocytes to a specific activity of 976 units/mg in 25% yield. The procedure involves ammonium sulfate fractionation, chromatography on DE-52 cellulose, further ammonium sulfate fractionation, and chromatography on phosphocellulose. Electrophoresis on polyacrylamide gels containing sodium dodecyl sulfate showed that the subunit size of the erythrocyte isozyme corresponds to a molecular weight identical to that of the breast muscle isozyme (i.e., 69 000). Antibody directed against the purified erythrocyte AMP deaminase showed a single precipitin band on Ouchterlony plates when tested against the RBC enzyme but did not cross-react with muscle AMP deaminase. Cosedimentation of native muscle and erythrocyte AMP deaminases on sucrose velocity gradients showed that the molecular weights of these isozymes are virtually identical. The purified erythrocyte isozyme is highly specific for 5′-AMP; the apparent rates of deamination of adenosine, 5′-ADP, and 5′-ATP were less than 1% of the rates observed with 5′-AMP. The substrate concentration required to attain 50% V_{max} ($S_{0.5}$) in the absence of potassium ion is 4.5 mM. Kinetic studies performed in the presence and absence of potassium ion yielded data that are consistent with theoretical curves for a four-subunit allosteric model, and K_1 (the dissociation constant for the R conformational state) estimated from these data is 1.8×10^{-3} M 5′-AMP. V_{max} is unchanged over the range of [KCl] from 0 to 200 mM. Substrate saturation data obtained at various concentrations of phytic acid, a potent allosteric inhibitor of the erythrocyte deaminase, indicate that phytate binds most readily to the conformation having the lower affinity for substrate. Phytate inhibition is reversed at saturating levels of 5′-AMP and is also sharply diminished in the presence of KCl; e.g., when assayed at 2 mM 5′-AMP the concentrations of phytate required for 50% inhibition of enzymatic activity in the absence and presence of 150 mM KCl were 1 μM and 4 mM, respectively. The characteristics of phytate inhibition indicated that the mechanism of inhibition is not due to chelation of Zn(II). The amino acid composition of chicken erythrocyte AMP deaminase is similar but not identical to the isozyme from avian breast muscle. The subunit molecular weight of the red blood cell isozyme based on amino acid composition is in good agreement with the subunit molecular weight determined by sodium dodecyl sulfate–gel electrophoresis.

AMP deaminase is an allosteric enzyme that exists in several molecular forms. Catalytic and immunological differences (Henry and Chilson, 1973), as well as developmental studies (Sammons and Chilson, 1977; Kaletha and Zydowo, 1971), provide indirect evidence for functional differences among these isozymes and suggest that they are regulated differently. However, the molecular bases for the AMP deaminase isozymes are not known. A major reason for the lack of structural detail regarding these isozymes is that purification to homogeneity, in quantities sufficient for molecular studies, is made difficult by the fact that AMP deaminase activity is generally very low in nonmuscle tissues (Conway and Cooke, 1939; Lowenstein, 1972). Until recently, skeletal muscle was the only tissue from which highly purified preparations had been ob-

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