Bifunctional Enzyme Activity at the Same Active Site: Study of 3α and 20β Activity by Affinity Alkylation of 3α,20β-Hydroxysteroid Dehydrogenase with 17-(Bromoacetoxy)steroids†

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ABSTRACT: The relationship between 3α and 20β activity in 3α,20β-hydroxysteroid dehydrogenase (3α,20β-HSD; EC 1.1.1.53) from *Streptomyces hydrogenans* was studied by affinity alkylation with 17-(bromoacetoxy)progesterone (17-BAP) and 5α-dihydrotestosterone 17-bromoacetate (DTB). Both 17-BAP and DTB are substrates for 3α,20β-HSD and must therefore bind at the enzyme active site. 17-BAP and DTB irreversibly and simultaneously inactivate the 3α and 20β activity of 3α,20β-HSD at pH 7.0 and 25 °C, in a time-dependent manner, producing first-order kinetic half-times of 25 and 67 h, respectively. Biphasic kinetics from inactivation of 3α,20β-HSD with DTB at pH 9.0, reported earlier [Edwards, C. A. F., & Orr, J. C. (1978) *Biochemistry 17*, 4370–4376], was found in the present investigation to be associated with (a) base-catalyzed hydrolysis ($t_{1/2}$ = 20 h) of DTB (10^{-5} M) to produce 5α-dihydrotestosterone (DT), a superior substrate relative to DTB for 3α,20β-HSD, and (b) base-catalyzed reactivation ($t_{1/2}$ = 0.25 h) of the affinity-alkylated 3α,20β-HSD, which obscures the inactivation kinetics ($t_{1/2}$ = 67 h at pH 7.0). Complete inactivation of 3α,20β-HSD at pH 7.0 requires 1 mol of newly synthesized 5α-[4-^{14}C]dihydrotestosterone 17-[2′-^{3}H]bromoacetate ([^{14}C,^{3}H]DTB) or 17-([2-^{3}H]bromoacetoxy)progesterone (17-[^{3}H]BAP) per mol of enzyme. Although 6β-(bromoacetoxy)progesterone (6β-BAP) is a 20-ketopregnene substrate and DTB is a 3-ketoandrostane substrate, each steroid simultaneously inactivates 3α and 20β activity. Following inactivation of 3α and 20β activity with 6β-BAP or DTB, at pH 7.0, reactivation of 3α,20β-HSD can be obtained by adjusting the pH to 9.0. Reactivation of 3α,20β-HSD at pH 9.0 involves base-catalyzed hydrolysis of the ester linkage between the steroid and alkylated amino acid residue at the active site as shown by experiments with [^{14}C,^{3}H]DTB in which 5α-[4-^{14}C]dihydrotestosterone and [2-^{3}H]glycolic acid are released from the affinity-radioalkylated enzyme. Most likely, a carboxy group of an aspartic or glutamic acid residue is alkylated by [^{14}C,^{3}H]DTB or 17-[^{3}H]BAP in the vicinity of the steroid D ring at the active site of 3α,20β-HSD as evidenced by release of [^{3}H]glycolic acid on treatment with base. Reactivation of 3α or 20β activity fails to occur at pH 9.0 following affinity alkylation, at pH 7.0, of 3α,20β-HSD with 17-BAP, 16β-(bromoacetoxy)progesterone, or 21-(bromoacetoxy)progesterone. Affinity alkylation of 3α,20β-HSD with DTB or 6β-BAP requires 1 mol of steroid to completely inactivate 1 mol of enzyme. Because it is unlikely that a single steroid molecule bound to one site can simultaneously occupy a second site, these results are consistent with the same active site containing both 3α and 20β activity. Two new models of steroid binding at the active site of 3α,20β-HSD can account for these findings.

The oxidoreductase 3α,20β-hydroxysteroid dehydrogenase (3α,20β-HSD; EC 1.1.1.53)[1] from *Streptomyces hydrogenans* specifically reduces 3-ketoandrostane and 20-ketopregnane steroids (Schmidt-Thome et al., 1962; Pocklington & Jeffery, 1968, 1969; Blomquist, 1973; Edwards & Orr, 1978). Affinity alkylation of 3α,20β-HSD was earlier accomplished with cortisone 21-iodoacetate (Ganguly & Warren, 1971). We recently described syntheses of 2α-, 6β-, 11α-, and 16α-(bromoacetoxy)progesterone (Strickler et al., 1975; Arias et al., 1973; Sweet et al., 1972) and 21-[(bromoacetyl)amino]-progesterone (Sweet et al., 1978) which affinity alkylate 3α,20β-HSD. First-order kinetics of enzyme inactivation were consistently obtained in all of these studies when incubations of the steroid bromoacetates with 3α,20β-HSD were conducted at pH 7.0 and 25 °C. Affinity radioalkylation experiments with ([2-^{3}H]bromoacetoxy)progesterone derivatives identified a histidyl residue near positions 16 and 21 of the steroid D ring, a cysteinyl residue near the 6 position of the B ring, and two different methionyl residues near positions 2 and 11 of the A and C rings, respectively, at the active site of 3α,20β-HSD. Recently, 5α-dihydrotestosterone bromoacetate, which is a substrate for 3α activity, was reported to inactivate 3α,20β-HSD at pH 9.0 (Edwards & Orr, 1978).

The present report describes syntheses of 17-([2-^{3}H]-bromoacetoxy)progesterone (17-[^{3}H]BAP) and 5α-[4-^{14}C]-dihydrotestosterone [2′-^{3}H]bromoacetate ([^{14}C,^{3}H]DTB) to affinity radioalkylate 3α,20β-HSD. Incubation of 5α-dihydrotestosterone bromoacetate (DTB) at pH 9.0 with 3α,20β-HSD caused enzyme inactivation by a biphasic kinetic process (Edwards & Orr, 1978). The chemistry of these complex kinetics was investigated with [^{14}C,^{3}H]DT in the present study. The discovery that 3α and 20β activity originate from a common catalytic region can be accounted for by two models of steroid binding at the active site of 3α,20β-HSD.

Experimental Section

Materials

Cortisone, progesterone, 17-hydroxyprogesterone, 17-acetoxyprogesterone, and 17β-hydroxy-5α-androsten-3-one were purchased from Steraloids Co. Nucleotides (NAD and

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[1] Abbreviations used: 3α,20β-HSD, 3α,20β-hydroxysteroid dehydrogenase; 17-[^{3}H]BAP, 17-([2-^{3}H]bromoacetoxy)progesterone; [^{14}C,^{3}H]DTB, 5α-[4-^{14}C]dihydrotestosterone [2′-^{3}H]bromoacetate; DTB, 5α-dihydrotestosterone bromoacetate; DT, 5α-dihydrotestosterone; [^{14}C]DT, 5α-[4-^{14}C]dihydrotestosterone; 17-AP, 17-acetoxyprogesterone; EGME, ethylene glycol monoethyl ether and buffers containing this solvent; 6β-BAP, 6β-(bromoacetoxy)progesterone; 16α-BAP, 16α-(bromoacetoxy)progesterone; 21-BAP, 21-(bromoacetoxy)progesterone.

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NADH), L-amino acids, Triton X-100, and inorganic chemicals were purchased from Sigma Chemical Co. 3α,20β-Hydroxysteroid dehydrogenase from *S. hydrogenans*, with a specific activity of 12–18 units/mg, was obtained from Boehringer Mannheim Corp. Glass-distilled water was used for all aqueous solutions. Organic chemicals and solvents were obtained from Fisher Scientific Co. Diphenyloxazole and 1,4-bis[2-(5-phenyloxazolyl)]benzene were obtained from New England Nuclear Corp. [2-^{3}H]Bromoacetic acid (110 mCi/mmol), [2-^{14}C]glycolic acid (250 μCi/mmol), and 17β-hydroxy-5α-[4-^{14}C]androstan-3-one (50 mCi/mmol) were purchased from Amersham Corp. Dialyses were performed with Union Carbide dialysis tubes from Scientific Products Corp. Dreiding molecular models with a bond length scale of 2.5 cm/Å were purchased from Fisher Scientific Co.

Methods

Melting points were determined in a Mel-Temp apparatus and are reported uncorrected. Infrared spectra were obtained with KBr pellets and were recorded with a Beckman Acculab 4 instrument. Ultraviolet absorption spectra were recorded in a Beckman Model 25 spectrophotometer. Nuclear magnetic resonance spectra of samples in deuteriochloroform, containing tetramethylsilane as an internal standard, were recorded in a Varian T-60 spectrometer. Elemental analyses were performed by Galbraith Laboratories, Knoxville, TN.

Synthesis of 17-([2-^{3}H]Bromoacetoxy)progesterone (17-[^{3}H]BAP). 17-([2-^{3}H]Bromoacetoxy)progesterone was synthesized by modification of a recently reported procedure (Samant & Sweet, 1977) represented by equation A of Figure 1. 17-Hydroxyprogesterone (170 mg, 0.376 mmol) was added to a mixture of 50 mg (0.35 mmol) of bromoacetic acid and 10 mCi of [2-^{3}H]bromoacetic acid (110 mCi/mmol) which had reacted with 0.5 mL of trifluoroacetic anhydride for 30 min. The resulting solution was heated at 70 ± 1 °C under reflux (anhydrous conditions) in a Scientific Products Temp-Blok Module Heater (Model H2025-1) for 1 h. After the reaction mixture was cooled to 25 °C, volatile components were evaporated with a stream of anhydrous nitrogen. Ether was added and evaporated with a stream of anhydrous nitrogen to remove traces of trifluoroacetic anhydride. The residue was dissolved in 5 mL of an ethanolic hydrobromic acid solution (0.85 mL of 48% aqueous HBr in 100 mL of absolute ethanol) and heated under reflux for 20 min. The reaction mixture was cooled, 23 mg of solid sodium bicarbonate was added, and the resulting mixture was stirred for 10 min. The suspension was concentrated to dryness with a stream of nitrogen. Analysis of the crude product by thin-layer chromatography (benzene–ethyl acetate, 95:5; Eastman silica gel G, no. 6060) showed it to contain 17-[^{3}H]BAP, 17-(trifluoroacetoxy)progesterone, and 17-hydroxyprogesterone. The residue was triturated with 1 mL of hot benzene, and the mixture was transferred to a 12 × 150 mm column containing 6 g of Merck silica gel G, packed under pressure with benzene–ethyl acetate (96:4) (Hunt & Rigby, 1967). The column was eluted under pressure with benzene–ethyl acetate (96:4) at a flow rate of 0.2 mL/min, collecting 1-mL fractions. The pooled 17-[^{3}H]BAP fractions were concentrated to dryness under reduced pressure, and the residue was crystallized from petroleum ether (trace of acetone) to give a crystalline product, mp 165–167 °C. Thin-layer chromatography, mixture melting point with nonradioactive 17-BAP, and NMR, infrared, and ultraviolet spectral properties showed the radioactive (13 mCi/mmol) product to be 17-[^{3}H]BAP.

Synthesis of 17β-Hydroxy-5α-[4-^{14}C]androstan-3-one 17-[2′-^{3}H]Bromoacetate ([^{14}C,^{3}H]DTB). The commercial benzene–ethanol solvent was removed from 0.01 mCi (50 mCi/mmol, 0.050 mg) of 5α-[4-^{14}C]dihydrotestosterone with a stream of nitrogen. Then 29 mg (0.1 mmol) of nonradioactive 5α-dihydrotestosterone in 2 mL of anhydrous CH_2Cl_2 was mixed with the isotopically labeled steroid in a 25-mL Erlenmeyer flask, and the solution was cooled in an ice bath. Bromoacetic acid (13.8 mg, 0.1 mmol) in 1.5 mL of CH_2Cl_2 (from a solution of 46 mg of bromoacetic acid in 5.0 mL of CH_2Cl_2) was mixed with 5 mCi (87 mCi/mmol) of bromo-[2-^{3}H]acetic acid and then transferred to the reaction vessel. Dicyclohexylcarbodiimide (22.9 mg, 0.11 mmol) in 0.63 mL of CH_2Cl_2 (from a solution of 182 mg of DCC in 5.0 mL of CH_2Cl_2) was added to the reaction mixture followed 5 min later by the addition of 0.005 mL of anhydrous pyridine. The reaction mixture was stirred at 0 °C, and at 30-min intervals aliquots were analyzed by thin-layer chromatography on silica gel developed with chloroform (Fisher Scientific Co. reagent grade solvent, containing 0.75% ethanol) and visualized with iodine vapor (and measurement of ^{14}C and ^{3}H activity in appropriate spots was carried out). The [^{14}C]DT spot (R_f 0.3) disappeared as the [^{14}C,^{3}H]DTB spot (R_f 0.5) increased in intensity. After 4.5 h the mixture was concentrated in a stream of nitrogen to 0.5 mL and then applied to a 0.9 × 10 cm chromatographic column (containing 6 g of silica gel G, slurried with $CHCl_3$ and packed under pressure). The column was eluted under pressure with chloroform (Hunt & Rigby, 1967), and the fractionated eluate was analyzed by TLC for [^{14}C,^{3}H]DTB. Fractions containing the product were pooled and concentrated to dryness in a stream of nitrogen to give 20 mg of pure (one spot, R_f 0.5, on TLC) [^{14}C,^{3}H]DTB with physical and spectroscopic properties identical with nonradioactive DTB. Specific activities of [^{14}C,^{3}H]DTB were ^{14}C = 184 μCi/mmol and ^{3}H = 10.3 mCi/mmol.

Enzyme Assays. Enzyme assays were conducted at 25 °C with the following solutions added to a final volume of 3.0 mL in matched 1 × 1 cm cuvettes: 2.6 mL of 0.05 M potassium phosphate buffer, pH 6.5; 0.100-mL aliquot of enzyme solution (250 μg of 3α,20β-HSD in 4.8 mL of 0.05 M potassium phosphate buffer, pH 7.0); 0.200 mL of cortisone (0.18 μmol) in ethanol; 0.100 mL of NADH (0.1 μmol) in 0.05 M potassium phosphate buffer, pH 7.0. The slope of the initial linear decrease in absorbance at 340 nm (due to oxidation of NADH) as a function of time was used to calculate enzyme activity. Assays were performed in triplicate and conducted at 25 ± 1 °C in a Beckman Model 25 recording spectrophotometer. Kinetic data were fitted by least mean squares with a Hewlett-Packard Model 97 preprogrammed calculator. Data from inactivation kinetics and stoichiometry of inactivation from radiolabeling experiments were similarly treated. Protein concentrations were determined according to the method of Lowry et al. (1951).

Amino Acid Analysis of Radioalkylated 3α,20β-HSD. Radioalkylated 3α,20β-HSD was digested with 6 N HCl in evacuated, sealed tubes at 110 °C for 22 h. Following lyophilization of the hydrolysate, the residue was dissolved in 0.3 mL of 0.2 M citrate buffer, pH 2.2, and amino acid analyses were performed with a Beckman Spinco Model 118C amino acid analyzer (Gransberg et al., 1969). The split stream effluent (flow rate 1.7 mL per min per tube) from the analyzer was fractionated with a Gilson FC-80H Mini Fractionator. Aliquots (0.05 mL) from each fraction were dissolved in 15 mL of scintillation solution [0.05% 2,5-diphenyloxazole, 0.01% 1,4-bis[2-(5-phenyloxazolyl)]benzene, and toluene–Triton X-100 (2:1)]. Radioactivity was measured in a Beckman Model LS 330 liquid scintillation spectrometer. Under these

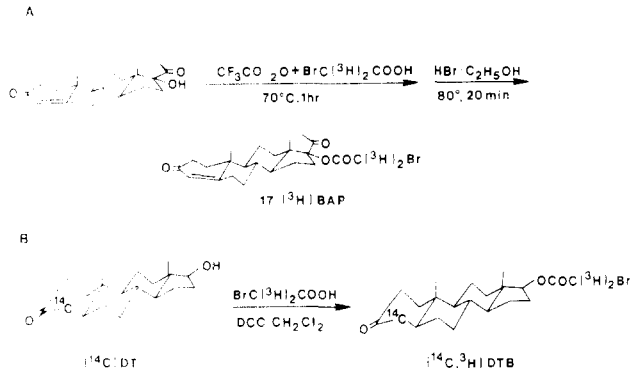

FIGURE 1: Synthesis of 17-[^{3}H]BAP and [^{14}C,^{3}H]DTB. (Equation A) 17-Hydroxyprogesterone was treated with a [2-^{3}H]bromoacetic acid–trifluoroacetic anhydride mixture at 70 °C for 1 h, and the intermediate obtained from this reaction was heated in ethanolic hydrobromic acid for 20 min. 17-[^{3}H]BAP was isolated by silica gel chromatography. (Equation B) 5α-[4-^{14}C]Dihydrotestosterone ([^{14}C]DT) was treated with [2-^{3}H]bromoacetic acid and dicyclohexylcarbodiimide (DCC) in methylene chloride to produce [^{14}C,^{3}H]DTB.

conditions the counting efficiency for tritium was 18.8%. Correction for quench was with external standardization. Differential measurement of ^{14}C and ^{3}H was performed according to the procedure of Bush (1964).

Results

Synthesis and Reactions of 17-([2-^{3}H]Bromoacetoxy)-progesterone (17-[^{3}H]BAP). [2-^{3}H]Bromoacetic acid in trifluoroacetic anhydride reacted with 17-hydroxyprogesterone at 70 °C and gave a complex mixture. The chemistry of this reaction was recently discussed in elaborate detail elsewhere (Samant & Sweet, 1977). Treatment of the mixture with ethanolic HBr, followed by silica gel chromatography, gave a 30% yield of 17-[^{3}H]BAP and a 40% recovery of starting material (A, Figure 1). The tritium-labeled product possessed physical and spectroscopic properties identical with nonradioactive 17-BAP (Samant & Sweet, 1977). 17-[^{3}H]BAP (10^{-4} M) or [^{14}C,^{3}H]DTB (10^{-4} M) reacted with 2-mercaptoethanol, L-cysteine, L-methionine, or L-histidine (10^{-4} M), in 40% ethanol–0.05 M potassium phosphate buffer at pH 7.0 and 25 °C, to produce the corresponding steroid–amino acid conjugates. Conjugation reactions were monitored by thin-layer chromatography with silica gel plates, developed with 1-butanol–acetic acid–water (12:3:5). The alkylating activities of 17-BAP and DTB with amino acids were similar to those of other (bromoacetoxy)progesterone analogues (Sweet & Warren, 1972; Arias et al., 1973).

Synthesis and Hydrolysis Kinetics of 5α-[4-^{14}C]Dihydrotestosterone 17-[2'-^{3}H]Bromoacetate ([^{14}C,^{3}H]DTB). Synthesis of DTB following the method of Edwards & Orr (1978) gave low yields accompanied by large amounts of a byproduct, 3,3-diethoxy-5α-androstan-17β-ol 17-bromoacetate (mp 104–107 °C), which was produced during recrystallization of DTB from hot ethanol. Recrystallization of DTB from acetone–petroleum ether prevented this problem. Efficient synthesis of [^{14}C,^{3}H]DTB (B, Figure 1) was obtained by modification of a method for preparing isotope-labeled progesterone bromoacetates (Sweet et al., 1978). [^{14}C,^{3}H]DTB had physical (mp 121–122 °C) and spectroscopic properties identical with those of the nonradioactive DTB.

Hydrolysis kinetics of [^{14}C,^{3}H]DTB at pH 9.0 were obtained with 500 mL of a solution containing 10^{-4} M [^{14}C,^{3}H]DTB (specific activities: ^{14}C = 184 μCi/mmol; ^{3}H = 10.3 mCi/mmol) in 10% ethanol–Tris buffer at pH 9.0, maintained at

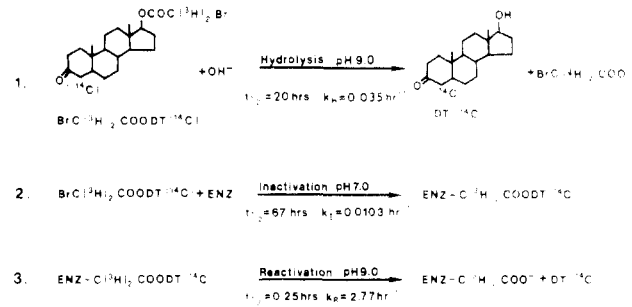

FIGURE 2: Equations for reactions of [^{14}C,^{3}H]DTB and 3α,20β-HSD. The equations represent (1) hydrolysis of [^{14}C,^{3}H]DTB (10^{-5} M) at pH 9.0 and 25 °C, (2) inactivation of 3α,20β-HSD (10^{-7} M) with [^{14}C,^{3}H]DTB (10^{-5} M) at pH 7.0 and 25 °C, and (3) reactivation of 3α,20β-HSD from eq 2 at pH 9.0 and 25 °C. The kinetic rate constants in eq 1–3 were calculated from the first-order half-lives of the experimentally determined hydrolysis of [^{14}C,^{3}H]DTB, inactivation with [^{14}C,^{3}H]DTB of 3α,20β-HSD, and reactivation of the inactivated enzyme, respectively. These k values were calculated from the formula $t_{1/2} = k^{-1}$ ln 2 (March, 1977b).

25 °C. At hourly intervals 5-mL aliquots of the reaction mixture were withdrawn and extracted 5 times with equal volumes of ether. The combined ethereal extracts were washed with water, dried, ($MgSO_4$), filtered, and concentrated in a stream of dry nitrogen to less than 0.5 mL. The residue was chromatographed on thin-layer silica gel plates developed with chloroform (0.75% ethanol). Zones corresponding to [^{14}C,^{3}H]DTB (R_f 0.5) and 5α-[4-^{14}C]dihydrotestosterone ([^{14}C]DT; R_f 0.3) were removed and quantitated by measuring ^{14}C activity. The ^{14}C activity was 22 000–24 000 dpm/TLC plate throughout the experiment. A plot of the percent DTB hydrolyzed as a function of time produced classical base-catalyzed ester hydrolysis kinetics ($B_{Ac}2$ mechanism; March, 1977a) at pH 9.0 (eq 1, Figure 2). Incubation of 10^{-4} M [^{14}C,^{3}H]DTB or 5α-dihydrotestosterone acetate in 0.05 M phosphate buffer, pH 7.0, at 25 °C, however, resulted in less than 5% hydrolysis during 28 days. Hydrolysis of DTB at pH 9.0 was kinetically similar to those of secondary (bromoacetoxy)progesterone derivatives (Sweet et al., 1972; Sweet & Warren, 1972; Arias et al., 1973; Sweet, 1976).

Both 5α-dihydrotestosterone 17-acetate and DTB were tested for substrate activity with 3α,20β-HSD. The results are summarized in Table I. DTB is a substrate for 3α,20β-HSD, and therefore this steroid must bind at the enzyme active site.

Stability of 3α,20β-HSD. Enzyme assays with 5α-dihydrotestosterone (DT; 3α activity) and progesterone (20β activity) as substrates (Table I) were conducted with aliquots from incubation mixtures of 3α,20β-HSD (10^{-7} M) at 25 °C in (a) 0.05 M phosphate buffer, pH 7.0, (b) 10% ethanol–0.05 M phosphate buffer, pH 7.0, (c) Tris buffer, pH 9.0, and (d) 10% ethanol–Tris buffer, pH 9.0. While 3α and 20β activity decreased at *equal* rates in each of the incubation mixtures, the stability of 3α,20β-HSD during 48 h of incubation was greatest in Tris buffer at pH 9.0 and least in 10% ethanol–phosphate buffer at pH 7.0. Simultaneous loss of 3α and 20β activity under a variety of different conditions further supports the contention (Blomquist, 1973; Edwards & Orr, 1978) that both activities are associated with a single enzyme.

Kinetic results from 3α,20β-HSD with the substrates progesterone, cortisone, or 5α-dihydrotestosterone are generally in agreement with those from other laboratories (Table I). The parameter of utilization efficiency (Π), introduced by White & Jeffery (1973) for 3α,20β-HSD, simplifies comparison of kinetic constants. Increasing Π values reflect decreasing substrate utilization efficiencies.

Table I: Kinetic Constants for 3α,20β-Hydroxysteroid Dehydrogenase

substrate (act.)	K_m (μM)	V_{max} (mol min^{-1} mg^{-1})	Π^a (min mg L^{-1})
progesterone (20β)	3.9	11.6	0.34
5α-pregnane-3,20-dione (3α, 20β)	4.5	10.5	0.43
cortisone (20β)	318	2.57	124[b]
	51	12.52	4.8
6β-(bromoacetoxy)-progesterone (20β)	20	3.72	5.37
17-hydroxyprogesterone (20β)	85.5	3.94	21.8[b]
	10.5	4.96	2.1
17-(bromoacetoxy)-progesterone (20β)	1830	0.41	4450[b]
5α-dihydrotestosterone (3α)	574	8.7	65.9[c]
	448	3.2	140[d]
5α-dihydrotestosterone acetate (3α)	137	1.1	124
5α-dihydrotestosterone bromoacetate (3α)			580[e]

substrate (act.)	K_m (μM)	V_{max} (mol min^{-1} mg^{-1})	Π^a (min mg L^{-1})	ref
progesterone (20β)	2.8	29	0.09	Edwards & Orr (1978)
	1.1	6.1	0.18	White & Jeffery (1973)
	2.2	20	0.22	Pocklington & Jeffery (1968)
cortisone (20β)	24	17.9	1.3	Edwards & Orr (1978)
	110	18	6.1[f]	Szymanski & Furfine (1977)
	29.3	10.9	2.7	Gibb & Jeffery (1973)
5α-dihydrotestosterone (3α)	460	1.29	356	Edwards & Orr (1978)
	112	14	8	Gibb & Jeffery (1973)
	289	19.6	14.7	Pocklington & Jeffery (1968)

[a] $\Pi = K_m/V_{max}$ is a parameter of utilization efficiency [see White & Jeffery (1973)]; enzyme-saturating concentrations of NADH (10^{-4} M) were used in all assays. [b] 50% EGME-0.05 M phosphate buffer, pH 6.5. [c] 0.05 M phosphate buffer, pH 7.0. [d] 0.05 M Tris buffer, pH 9.0. [e] Calculated from assays at a single substrate concentration. [f] 0.1 M Bicine buffer, pH 8.0.

Substrate Characteristics of 17-Acetoxy- and 17-(Bromoacetoxy)progesterone with 3α,20β-HSD. Assays with 3α,20β-HSD (1×10^{-7} M) and 17-acetoxy- (17-AP) or 17-(bromoacetoxy)progesterone (17-BAP) in 0.05 M potassium phosphate buffer containing 10% ethanol, at pH 6.5 and 25 °C, failed to produce measurable activity (NADH, 10^{-4} M) at the upper limit of steroid solubility (10^{-4} M). Enzyme assays containing 10^{-4} M 17-BAP or 17-AP and using cortisone (10^{-6}–10^{-5} M) as a substrate showed no measurable inhibition in the rate of cortisone reduction.

Solutions were sought in which 3α,20β-HSD is stable and also the solubility of 17-BAP and 17-AP is greater than 10^{-4} M. Mixtures of dioxane, acetonitrile, or tetrahydrofuran with 0.05 M phosphate buffer at pH 7.0 caused rapid loss of enzyme activity. But 3α,20β-HSD was stable in 50% ethylene glycol monomethyl ether (EGME) in 0.05 M phosphate buffer at pH 6.5 for over 72 h at 25 °C. Human placental 17β-hydroxysteroid dehydrogenase was also found to be remarkably stable in this system (R. C. Strickler, personal communication). Concentrations of 10^{-2} M 17-BAP and 17-AP can be obtained with 50% EGME–0.05 M phosphate buffer. Enzyme assays with 17-BAP and 17-hydroxyprogesterone in the EGME buffer system at pH 6.5 produced the kinetic results summarized in Table I. The presence of 50% EGME in the buffer solution increased the Π value of 5α-dihydrotestosterone and cortisone 10–20-fold over values obtained with 10% ethanolic buffers.

17-[^{3}H]BAP Inactivation of 3α,20β-HSD. To 3α,20β-HSD (250 μg, 2.5 nmol) in 4.8 mL of 0.05 M potassium phosphate buffer at pH 7.0 was added 17-BAP (0.135 mg, 0.3 μmol) in 0.2 mL of EGME, and the resulting mixture was incubated at 25 °C. A solution containing equimolar quantities of the enzyme and 17-AP in the same buffer system served as a control. Aliquots from inactivation and control mixtures were assayed for enzyme activity at 1-h intervals. Inactivation of 3α,20β-HSD under these conditions followed first-order kinetics with a $t_{1/2}$ of 25 h. Addition of 2-mercaptoethanol (15 molar excess relative 17-BAP) to the incubation mixture quenched the inactivation reaction, and thereafter enzyme activity remained constant. The presence of 1.2×10^{-5} M progesterone in the inactivation mixture produced a $t_{1/2}$ of inactivation of 59 h. Most likely, progesterone protects 3α,20β-HSD against inactivation by competing with 17-BAP for the active site.

Stoichiometry of 17-[^{3}H]BAP Inactivation. Inactivation kinetics from an incubation mixture of 2.7 mg (6 μmol) of 17-[2-^{3}H]BAP (13 mCi/mmol) and 5 mg (0.05 mol) of 3α,20β-HSD in 100 mL of buffer were identical with those described above. During 25, 50, and 75% of enzyme inactivation, 30-mL aliquots were removed from the incubation mixture and treated with 2-mercaptoethanol (50 μmol). Each sample was dialyzed against distilled water, adjusted to pH 7.5–8.0, until radioactivity in the dialysate remained at background (40 cpm). The retentates were lyophilized, the residues were dissolved in 2 mL of water, and then protein and ^{3}H radioactivity contents were measured. The decrease in enzyme activity was linear with incorporation of radioactivity throughout the time course of inactivation. One mole of [2-^{3}H]carboxymethyl group was incorporated per mole of inactivated enzyme during inactivation of 3α,20β-HSD by 17-[2-^{3}H]BAP.

Amino Acid Residue Radioalkylated by 17-[^{3}H]BAP. A series of 25, 50, and 80% 17-[^{3}H]BAP affinity-radioalkylated 3α,20β-HSD samples from the above experiments were lyophilized and prepared for amino acid analysis as described under Methods. 1,3-Bis(carboxymethyl)histidine, 1-(carboxymethyl)histidine, 3-(carboxymethyl)histidine, ε,ε-bis-(carboxymethyl)lysine, and *S*-(carboxymethyl)cysteine were added as internal standards. The split stream effluent was fractioned and quantitated for tritium radioactivity. Ninhydrin and radioactive elution profiles were superimposed. More than 95% of the radioactivity from 25, 50, or 80% radioalkylated 3α,20β-HSD emerged in a single peak which did not coincide with any of the internal standards (panel I, Figure 3). The retention time of the tritium peak approximated that of glycolic acid (Sweet et al., 1978; Strickler et al., 1975). Analysis and fractionation of an 80% ^{3}H-radioalkylated and hydrolyzed enzyme sample, containing authentic [^{14}C]glycolic acid, produced ^{14}C and ^{3}H profiles with coincident peaks of tritium and carbon-14 activity (panel II, Figure 3).

Confirmation of a ([^{3}H]Carboxymethyl)oxy Ester Formed by 17-[^{3}H]BAP at the Active Site of 3α,20β-HSD. 3α,20β-HSD (0.02 μmol) was incubated with 17-[2-^{3}H]BAP (0.9 mg, 0.27 μmol) in 35 mL of 50% EGME–phosphate buffer, pH 7.0 at 25 °C, for 63 h to obtain 85% inactivation. 2-Mercaptoethanol (20 mg, 0.3 mmol) was added, and the resulting solution was dialyzed with water until no radioactivity

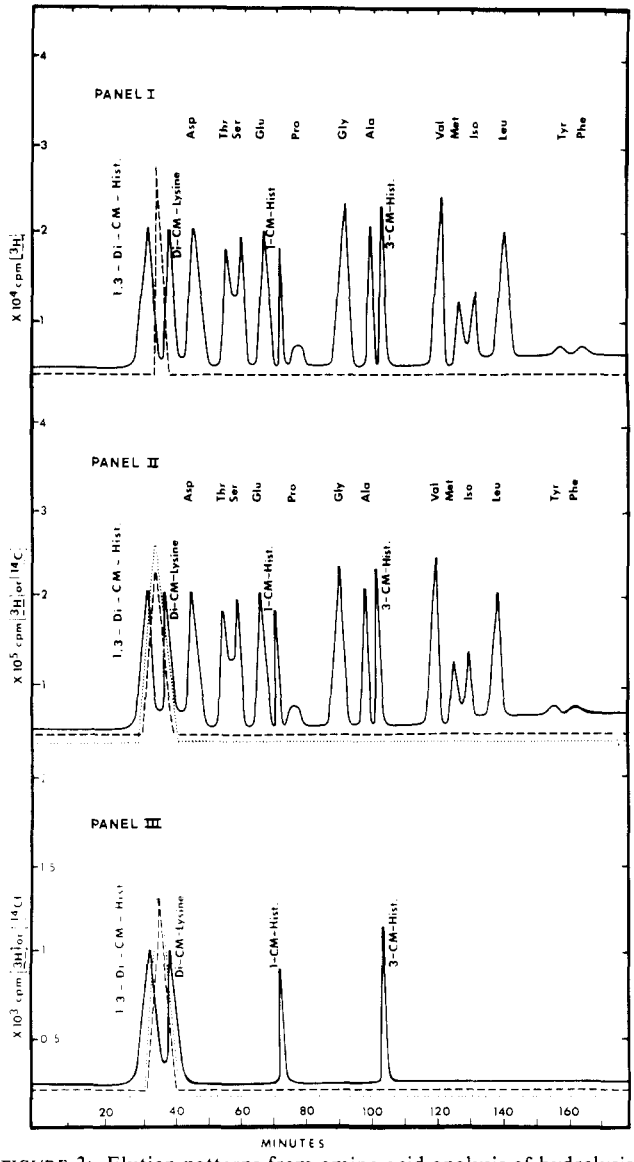

FIGURE 3: Elution patterns from amino acid analysis of hydrolysis products from inactivation with 17-[^{3}H]BAP of 3α,20β-HSD. A mixture containing authentic 1,3-bis(carboxymethyl)histidine, 1-(carboxymethyl)histidine, 3-(carboxymethyl)histidine, ϵ,ϵ-bis(carboxymethyl)lysine, and *S*-(carboxymethyl)cysteine was added to each sample, just before amino acid analysis, as an internal standard. Panel I shows the ^{3}H profile (broken line) superimposed on the ninhydrin profile. This analysis was obtained from 50% inactivated enzyme which had been hydrolyzed with 6 N HCl for 22 h at 110 °C. Panel II shows the ^{3}H (broken line) and ^{14}C (dotted line) profiles superimposed on the ninhydrin profile from a 22-h acid hydrolysate of 80% inactivated enzyme. Authentic [2-^{14}C]glycolic acid (0.05 mCi) was added to the hydrolysate before amino acid analysis. Panel III shows the ^{3}H (broken line) and ^{14}C (dotted line) profiles superimposed on the ninhydrin profile from an alkaline hydrolysis of 90% inactivated enzyme, with 0.2 N NH_4OH. The analytical sample derived from the "dialysate" chamber described under Results contained the standards described above and authentic [2-^{14}C]glycolic acid.

was detected in the dialysate. A 5-mL aliquot from this mixture was placed in the "*retentate*" chamber of the dual-chamber Technilab Instruments Model 262 dialysis apparatus, and 5 mL of distilled water adjusted to pH 9.0 with 0.2 N NH_4OH was placed in the "*dialysate*" chamber. In a second experiment 5 mL of 0.2 N NH_4OH was placed in the "*dialysate*" chamber. The dialysis apparatus was continuously agitated at 25 °C. At hourly intervals a 0.100-mL aliquot from each chamber was quantitated for radioactivity. The pH 9.0 "*dialysate*" chamber contained near background amounts of radioactivity (less than 50 cpm) after 48 h of equilibration. But after 4 h of equilibration of the 0.2 N NH_4OH dialysis experiment, 20% of the total radioactivity was present in the "*dialysate*" chamber. These results are similar to those reported earlier by Takahaski et al. (1967), who demonstrated that ribonuclease-T, inactivated by [^{14}C]iodoacetic acid, contained a ([^{14}C]carboxymethyl)oxy ester residue at the enzyme active site. The ([^{14}C]carboxymethyl)oxy ester of ribonuclease-T could not be hydrolyzed at pH 9.0, but 0.1 N NaOH produced complete hydrolysis at 25 °C within 7 h with formation of [^{14}C]glycolic acid.

The solution which contained a radioactive fragment in the "*dialysate*" chamber from 0.2 N NH_4OH treatment of 17-[^{3}H]BAP-radioalkylated 3α,20β-HSD was lyophilized. The residue was dissolved in 0.250 mL of 0.2 M sodium citrate buffer, pH 2.2, containing [^{14}C]glycolic acid (115 000 cpm), 1,3-bis(carboxymethyl)histidine, and ϵ,ϵ-bis(carboxymethyl)-lysine. The mixture was resolved in an amino acid analyzer, and the ninhydrin, ^{3}H, and ^{14}C profiles from the fractionated effluent were superimposed (panel III, Figure 3). The [^{14}C]glycolic acid fractions contained *all* of the ^{3}H radioactivity.

The proportion of ^{3}H label present as an ([^{3}H]carboxymethyl)oxy ester at the active site of 3α,20β-HSD was determined by placing a solution of 85% affinity-radioalkylated 3α,20β-HSD (5 mg, 5 000 000 cpm/mg) in 5 mL of water to which 0.125 mL of 30% NH_4OH solution was added in the "*retentate*" chamber of the Technilab dialysis apparatus. [2-^{14}C]Glycolic acid (12 500 cpm/mL) in 5 mL of 0.2 N NH_4OH was placed in the "dialysate" chamber, and the apparatus was agitated at 25 °C. Aliquots (0.100 mL) were removed from each chamber at hourly intervals and quantitated for ^{3}H and ^{14}C activity. Nearly identical profiles from the flux of ^{14}C and ^{3}H radioactivity contents in the two chambers were obtained during 24 h. This result suggests that all of the [^{3}H]carboxymethyl group was derived from 17-[^{3}H]BAP bound to 3α,20β-HSD through a ([^{3}H]carboxymethyl)oxy ester linkage.

Attempts to identify an aspartic or glutamic acid residue alkylated by 17-[^{3}H]BAP at the active site of 3α,20β-HSD, according to an earlier procedure using nagarase digestion (Takahashi et al., 1967), were unsuccessful. The tritiated peptide fragments resisted resolution on an analytical 170-cm Sephadex G-25 column. The presence of a hydrophobic steroid molecule attached to the peptides is believed to have interfered with the separation.

[^{14}C,^{3}H]DTB Inactivation of 3α,20β-HSD: pH 7.0 vs. pH 9.0. Incubation of 3α,20β-HSD (10^{-7} M) with [^{14}C,^{3}H]DTB (10^{-5} M) in 10% ethanol–phosphate buffer, pH 7.0 at 25 °C, produced a time-dependent and irreversible loss in 3α and 20β activity. Changes in 3α and 20β activity were measured simultaneously with DT and progesterone as substrates. Control incubation mixtures consisted of 3α,20β-HSD (10^{-7} M) and dihydrotestosterone 17-acetate (DTA; 10^{-5} M) in 10% ethanol–phosphate buffer, pH 7.0. A logarithmic plot of 3α or 20β activity as a function of time produced a linear tracing (panel I, Figure 4). Thus, inactivation of 3α,20β-HSD with DTB at pH 7.0 followed first-order kinetics with $t_{1/2}$ (3α activity) = $t_{1/2}$ (20β activity) = 67 h. Incubation of DTB (10^{-5} M) with 3α,20β-HSD (10^{-7} M) in Tris buffer at pH 9.0 and 25 °C produced biphasic kinetics similar to those recently published (Edwards & Orr, 1978). But in our hands 3α and 20β activities were found to decrease at nearly equal rates, and enzyme inactivation plateaued at 50% during the 72-h incubation period (panel II, Figure 4). The reactions which

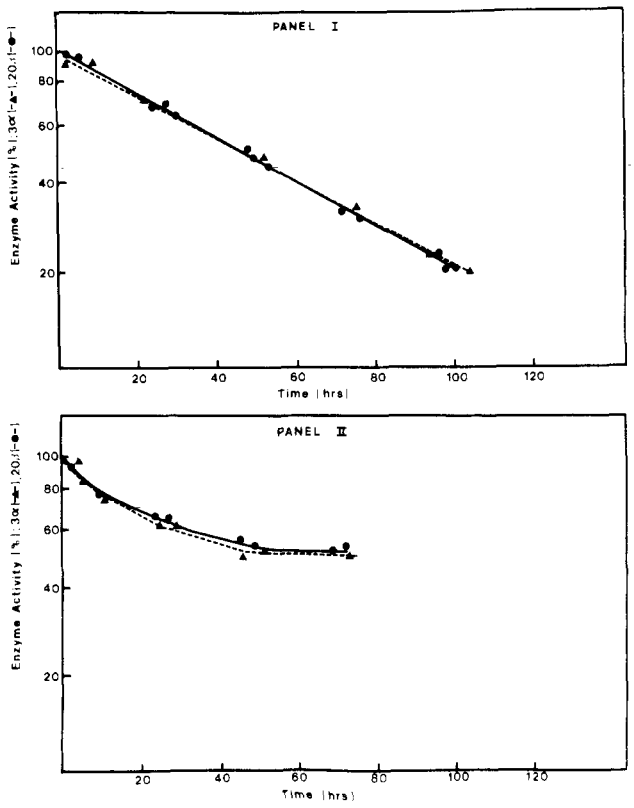

FIGURE 4: [^{14}C,^{3}H]DTB inactivation of 3α,20β-HSD (pH 7.0 vs. pH 9.0). Logarithmic plots of 3α (▲) and 20β (●) enzyme activity (percent of control) as a function of incubation time are shown. (Panel I) [^{14}C,^{3}H]DTB (10^{-5} M) was incubated with 3α,20β-HSD (10^{-7} M) in 10% ethanol–0.05 M phosphate buffer at pH 7.0 and 25 °C. (Panel II) [^{14}C,^{3}H]DTB (10^{-5} M) was incubated with 3α,20β-HSD (10^{-7} M) in 10% ethanol–0.05 M Tris buffer at pH 9.0 and 25 °C. See Results for experimental details.

contribute to these biphasic kinetics are discussed below. Incubation of 6β-(bromoacetoxy)progesterone (6β-BAP; 10^{-5} M) with 3α,20β-HSD (10^{-7} M) at pH 9.0 produced biphasic kinetics similar to those obtained with DTB, except that the inactivation plateaued at 70% enzyme activity during the initial 2 to 3 h of incubation.

Stoichiometry of Inactivation. Following 35, 55, and 73% inactivation of 3α,20β-HSD with [^{14}C,^{3}H]DTB in the above reaction at pH 7.0, 10-mL aliquots were removed from the incubation mixture, treated with 15 μmol of 2-mercaptoethanol, and dialyzed against frequent changes of 0.005 M phosphate buffer at pH 7.0 and 25 °C until no radioactivity could be measured in the dialysate. Protein content and ^{3}H activity were measured in the retentate. Extrapolation of the tracing to 100% inactivation showed that 1 mol of [^{14}C,^{3}H]-DTB completely inactivated 1 mol of 3α,20β-HSD.

[^{3}H]Glycolic Acid from the 6 N HCl Hydrolysate. The procedure for initial identification of the amino acid alkylated by [^{14}C,^{3}H]DTB was essentially the same as that used for 17-[^{3}H]BAP above. Following 6 N HCl digestion and amino acid analysis of the hydrolysate, fractionation of the analyzer effluent produced a ^{3}H activity profile which was superimposed on the ninhydrin amino acid profile. Authentic carboxymethylated amino acids were added as internal standards to the radiolabeled hydrolysate prior to analysis. The major peak of ^{3}H radioactivity (over 80% of the tritium content) coincided with [^{14}C]glycolic acid, showing that DTB reacted with an aspartic or glutamic acid residue at the active site of 3α,20β-HSD.

Reactivation of 3α,20β-HSD Affinity Alkylated with DTB or 6β-BAP. Following inactivation (>80%) of 3α,20β-HSD with DTB (or 17-BAP) at pH 7.0 and 25 °C, the incubation mixtures were treated with 2-mercaptoethanol and the pH was increased to 9.0 by adding 0.1 N NaOH. Control incubations contained 5α-dihydrotestosterone acetate (or 17-acetoxyprogesterone) under corresponding conditions. DTB-inactivated 3α,20β-HSD recovered 50–60% of 3α and 20β activity, as shown in Figure 5. But 17-BAP-inactivated enzyme could not be reactivated under these conditions. Inactivation–reactivation experiments with 6β-(bromoacetoxy)progesterone (or 11α-(bromoacetoxy)progesterone) and 3α,20β-HSD produced results similar to those obtained with DTB [see also Sweet (1976)]. But 3α,20β-HSD inactivated with 16α-(bromoacetoxy)progesterone (16α-BAP) or 21-(bromoacetoxy)progesterone (21-BAP) at pH 7.0 did not undergo reactivation at pH 9.0.

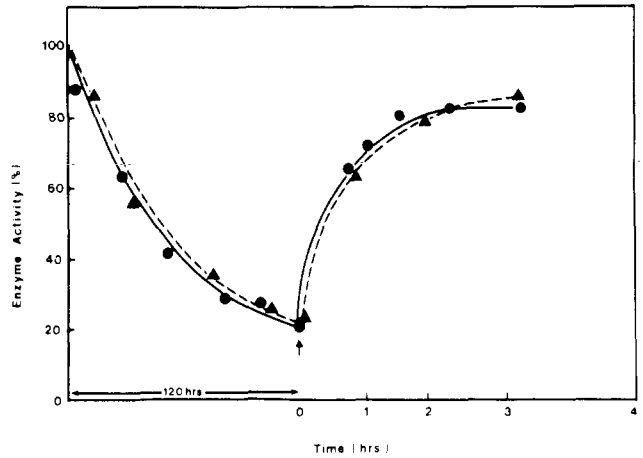

FIGURE 5: [^{14}C,^{3}H]DTB inactivation of 3α,20β-HSD at pH 7.0 followed by reactivation at pH 9.0. Both 3α (▲) and 20β (●) activities were assayed during inactivation of 3α,20β-HSD with [^{14}C,^{3}H]DTB at pH 7.0 and 25 °C. After 120 h 80% inactivation was obtained; then 15 μmol of 2-mercaptoethanol was added to quench the reaction. Reactivation of 3α,20β-HSD took place following adjustment of the reaction mixture to pH 9.0 (arrow) with 0.1 N NaOH. Enzyme activity (percent of control) is plotted on a linear scale as a function of time. Each point represents the mean of determinations from three experiments.

Release of [^{14}C]DT from Affinity-Radiolabeled 3α,20β-HSD. A 75-mL incubation mixture containing 3α,20β-HSD inactivated to over 90% with [^{14}C,^{3}H]DTB at pH 7.0 and 25 °C (see [^{14}C,^{3}H]DTB Inactivation of 3α,20β-HSD, above) was treated with 110 μL of 2-mercaptoethanol. The resulting solution was extracted with several 100-mL portions of ether to remove unreacted steroid. Each of the ethereal extracts was concentrated to dryness, and ^{3}H content was measured by scintillation counting. When the ethereal extracts contained no measurable tritium, the aqueous portion which contained the radioalkylated enzyme was extracted with several additional volumes of ether. The clear aqueous portion was adjusted to pH 9.0 with 0.1 N NaOH and extracted with 100-mL portions of ether at hourly intervals. The ether extracts were concentrated to dryness under a stream of nitrogen, and ^{3}H and ^{14}C activity was measured in the residues. During this experiment over 70% of the ^{14}C content from the aqueous solution was extracted by ether, but ^{3}H activity in the extract was less than 0.01% of that associated with the affinity-radioalkylated 3α,20β-HSD. A portion (600 cpm of ^{14}C activity) of the ether-extracted radioactivity was applied to a TLC plate together with authentic nonradioactive 5α-dihydrotestosterone (DT) and DTB. The chromatogram was developed with chloroform (0.75% ethanol) and visualized with iodine vapor. Then the chromatogram was cut into 10 sections, and each

section was extracted with 10 mL of scintillation fluid. More than 90% of the ^{14}C activity was present in the section containing DT. Thus, after affinity radioalkylation with [^{14}C,^{3}H]DTB of 3α,20β-HSD at pH 7.0, [^{14}C]DT was released from the enzyme by hydrolysis at pH 9.0.

Recovery of [^{3}H]Glycolic Acid from the pH 9.0 Hydrolysate. The above aqueous portion from which [^{14}C]DT had been extracted with ether and which contained 2.5×10^6 cpm of ^{3}H was dialyzed against an equal volume of distilled water. Aliquots of the dialysate were analyzed for radioactivity. After 24 h the ^{3}H activity was nearly evenly distributed between the retentate and dialysate. Authentic [^{14}C]glycolic acid was added to a portion of the retentate or dialysate, which was fractionated with an amino acid analyzer. Analysis of the ^{3}H and ^{14}C activity from the fractionated effluent showed that over 90% of the ^{3}H activity comigrated with [^{14}C]glycolic acid. Thus, incubation at pH 9.0 of 3α,20β-HSD that had been inactivated with [^{14}C,^{3}H]DTB caused hydrolytic release of both [^{14}C]DT and also [^{3}H]glycolic acid from the affinity-radioalkylated enzyme. The base-catalyzed release of [^{3}H]-glycolic acid from 3α,20β-HSD which was affinity radioalkylated with [^{14}C,^{3}H]DTB shows that the [^{3}H]bromoacetate side chain reacts with an ϵ-carboxylic amino acid residue at the active site. This conclusion is based on the criterion of Takahashi et al. (1967), discussed above in the results with 17-[^{3}H]BAP.

Discussion

The following terminology is introduced to describe certain reactions between (bromoacetoxy)steroids and 3α,20β-HSD in precise chemical terms. *Affinity alkylation* is defined as the formation of a covalent bond between a chemically reactive substrate (alkylating agent) and an active-site amino acid, promoted by specific binding between the substrate and enzyme. The term *affinity radioalkylation* describes covalent bond formation between an isotopically labeled and chemically reactive steroid and an active-site amino acid residue promoted by substrate–enzyme binding. The new terminology replaces the chemically less descriptive terms *affinity labeling* and *affinity radiolabeling* which have appeared in our earlier publications.

Several laboratories reported that the steroid oxidoreductase from *S. hydrogenans* exhibits 3α- and 20β-hydroxysteroid dehydrogenase activity (Pocklington & Jeffery, 1968, 1969; Gibb & Jeffery, 1971; Blomquist, 1973; Edwards & Orr, 1978). Convincing evidence that two different activities are derived from the same enzyme was provided by comigration of 3α and 20β activity during electrophoresis (Blomquist, 1973; Edwards & Orr, 1978). The present results confirmed the 3α and 20β activities (Table I) and provided additional evidence that they are due to a single enzyme in a series of pH- and alcohol-induced inactivation experiments. The simultaneous loss of 3α and 20β activity obtained with 1 mol of DTB or 6β-BAP per mol of 3α,20β-HSD during affinity alkylation at pH 7.0 and 25 °C is also consistent with a single enzyme having two different activities. Moreover, after DTB (or 6β-BAP) completely inactivates 3α,20β-HSD at pH 7.0, 3α and 20β activity can be restored at the same rate at pH 9.0 (Figures 4 and 5). This agrees with a single enzyme theory, discussed in greater detail below.

A 0.05 M Tris buffer system at pH 9.0 for affinity alkylation with DTB of 3α,20β-HSD was recently used by Edwards & Orr (1978) and produced complex inactivation kinetics. The present results show that two reactions, which cannot occur at pH 7.0 (eq 1 and 3, Figure 2), influence the *apparent* kinetics at pH 9.0 of inactivation of 3α,20β-HSD by DTB. Hydrolysis of DTB at pH 9.0 produces DT (and bromoacetate) which is a superior substrate relative to DTB (Table I). Increasing amounts of DT, produced at the expense of DTB, progessively reduce the rate of enzyme inactivation by competing for the active site of 3α,20β-HSD with the affinity-alkylating steroid. A second reaction, pH-dependent reactivation of the inactivated enzyme, complicates the kinetics of inactivation with DTB at pH 9.0. Although the rates of inactivation by DTB of 3α,20β-HSD at pH 7.0 and 9.0 are probably similar, the rate of affinity alkylation of the enzyme at pH 9.0 cannot be measured directly because $k_R \simeq 270k_I$ (Figure 2). The complex biphasic inactivation kinetics (panel II, Figure 4) represent a composite of three reactions (Figure 2). By contrast, DTB hydrolysis and 3α,20β-HSD reactivation do not occur at pH 7.0. Simple first-order kinetics from inactivation with DTB of 3α,20β-HSD are obtained with a simultaneous decrease in 3α and 20β activity (panel I, Figure 4).

Kinetic studies using fluorescence techniques revealed two different types of steroid binding for 3α,20β-HSD with corticosteroid 21-aldehydes (Szymanski & Furfine, 1977). One type of steroid binding is catalytically productive for 20β activity at the active site. The other nonproductive type of binding was considered to occur at a noncatalytic site. These workers also concluded that steroid binding at the active site of 3α,20β-HSD was not ordered by cofactor, contradicting earlier conclusions (Betz & Warren, 1968; Betz & Taylor, 1970). Results from the present work indicate that cofactor binding is not prerequisite for steroid binding since affinity alkylation of 3α,20β-HSD with (bromoacetoxy)steroids occurs in the absence of cofactor. However, the *required* presence of cofactor to obtain affinity alkylation *would* be a novel criterion for substrate binding ordered by cofactor.

Evidence for the same active site of 3α,20β-HSD containing both 3α and 20β activity was obtained from inactivation–reactivation studies in the present work. Reactivation of affinity-alkylated, steroid-specific enzymes has been previously described. One report mentioned that following rapid (20 min) inactivation of placental 17β-hydroxysteroid dehydrogenase by estradiol 3-iodoacetate, at pH 7.6 and 37 °C, a slow (48 h) "spontaneous" reactivation due to phenolic ester hydrolysis was observed (Pons et al., 1976). A previous report from our laboratory showed that when 3α,20β-HSD was inactivated with 6β-(bromoacetoxy)progesterone (6β-BAP) at pH 7.0 and 25 °C the enzyme could be reactivated according to a first-order, pH-dependent process by adjusting the reaction mixture to pH 8.0, 8.5, or 9.0 (Sweet, 1976). Reactivation required that the steroid be bound through an ester linkage to the enzyme during inactivation. In the present work both 3α and 20β activities were simultaneously measured during affinity alkylation of 3α,20β-HSD with DTB (Figure 5) or 6β-BAP. DTB-inactivated 3α,20β-HSD was reactivated at pH 9.0 faster than DTB is hydrolyzed (eq 1 and 3, Figure 2). DTB and 6β-BAP produced different inactivation kinetic rates with 3α,20β-HSD ($t_{1/2}$ = 67 h for DTB; $t_{1/2}$ = 0.5 h for 6β-BAP), but 3α and 20β inactivations proceeded simultaneously at pH 7.0. Also, 1 mol of steroid produced complete inactivation per mol of enzyme in each case. Reactivation at pH 9.0 proceeded rapidly ($t_{1/2}$ = 0.5 h) with 3α and 20β activity restored simultaneously. Affinity alkylation of 3α,20β-HSD by a single C_{21} or C_{19} steroid simultaneously inactivates both 3α and 20β activity under different conditions. Therefore, the two activities must originate from the same active site.

The conspicuous differences between rates of base-catalyzed hydrolysis of DTB and reactivation of affinity-alkylated

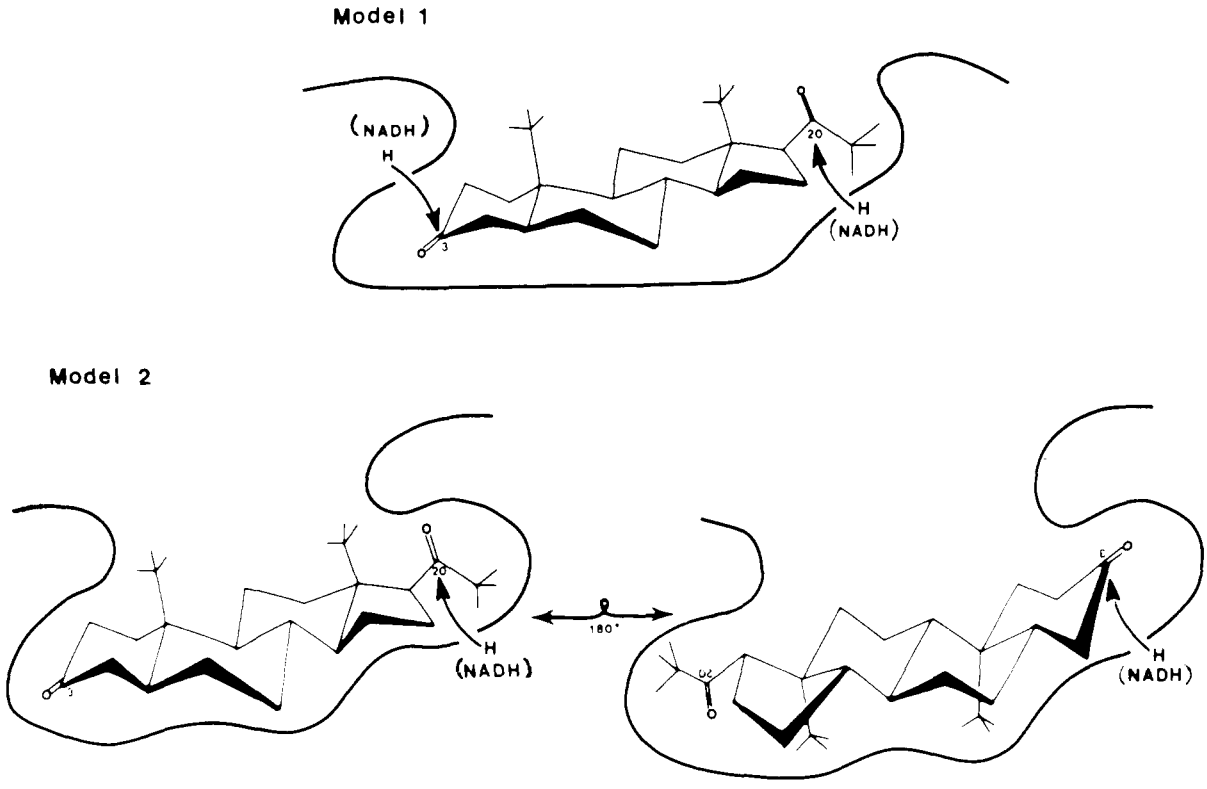

FIGURE 6: Two models proposed for steroid binding at the active site of 3α,20β-HSD. Model 1 represents a single steroid binding mode with the C-3 or C-20 carbonyl group receiving a 3β- or 20α-hydride from appropriately located NADH molecules. This model can account for the bifunctional activity of the active site of 3α,20β-HSD if a steroid substrate is restricted to a single enzyme–substrate complex. Model 2 represents two different enzyme–substrate complexes related through 180° rotation of the steroid at the active site. The C-3 or C-20 carbonyl group can receive a 3β- or 20α-hydride from the same NADH molecule with formation of the appropriate hydroxysteroid at a single catalytic region. The 3α or 20β activity depends upon both the orientation of the steroid at the active site and its tendency to accept a hydride.

3α,20β-HSD (Figure 2) are interesting. We are unaware of analogous examples involving similar hydrolysis rate differences. However, the rate enhancement of affinity alkylation by an enzyme active site has been described. Lawson & Schramm (1962, 1965) showed that irreversible inactivation of chymotrypsin is pH dependent and involves participation by a neighboring amino acid residue to achieve alkylation of an active-site methionine. The mechanism described by Lawson & Schramm for pH-dependent, affinity alkylation of chymotrypsin by *p*-nitrophenyl *N*-(bromoacetyl)-α-aminoisobutyrate involves acylation of a serine residue followed by alkylation of the neighboring methionine. We propose that the increase in the rate of hydrolytic reactivation of 3α,20β-HSD, which has been affinity alkylated by DTB or 6β-BAP, may be promoted by participation of a neighboring amino acid group at the enzyme active site. Intramolecular catalysis leading to rate enhancement of hydrolysis reactions is a well-known phenomenon (Bender, 1971).

After affinity alkylation of 3α,20β-HSD with 17-BAP at pH 7.0 no reactivation of 3α or 20β activity was observed during 24 h at pH 9.0. This is consistent with results from affinity radioalkylation experiments with 17-[^{3}H]BAP in which hydrolysis of the steroid–([^{3}H]carboxymethyl)oxy–amino acid linkage that is required for reactivation does not occur at pH 9.0. Indeed, 0.1 N hydroxide must be used to effect the hydrolysis (conditions under which enzyme activity is irreversibly lost). Steric hindrance (von Euw & Reichstein, 1947; Fieser, 1950; Samant & Sweet, 1977) at the tertiary 17-(carboxymethyl)oxy bridge may prevent ester hydrolysis at lower base concentrations in contrast to the results with [^{14}C,^{3}H]DTB in which [^{14}C]DT is readily hydrolyzed from the affinity-radioalkylated 3α,20β-HSD at pH 9.0.

When 16α-BAP or 21-BAP was used to affinity alkylate 3α,20β-HSD at pH 7.0, in contrast to results with DTB and 6β-BAP, enzyme reactivation was not obtained at pH 9.0. Most likely this results from carboxymethylation by 16α-BAP and 21-BAP of the imidazole ring of a histidine residue at the active site of 3α,20β-HSD (Ganguly & Warren, 1971; Sweet et al., 1972). Histidine has been implicated as an amino acid which participates in hydrogen transfer at the active sites of Δ^5-3-ketosteroid isomerase (Malhotra & Ringold, 1965; Talalay, 1965; Jones & Wigfield, 1969; Benson et al., 1971), lactate dehydrogenase (Ringold, 1966), and glutamate dehydrogenase (Baker, 1967). In the present work the failure to obtain reactivation of 3α,20β-HSD at pH 9.0 following affinity alkylation with 16α-BAP or 21-BAP (bound to the active site by base-labile ester linkages) suggests that alkylation of the histidine residue impairs its ability to participate in hydrogen transfer at the active site. Accordingly, it is useful to consider impairment of catalytic function of an active-site amino acid, modified by affinity alkylation, when applying catalytic competence (Groman et al., 1975) as a criterion of "affinity labeling".

Reaction of four different D-ring substituted (bromoacetoxy)steroids with a common amino acid (e.g., histidine) is seemingly possible. A bromoacetoxy reagent group at the 16α or 21 position on progesterone alkylates a histidine residue. The present results show that 1 mol of 17-BAP or DTB inactivated 3α,20β-HSD by reaction with a carboxylic amino acid residue at the active site. The failure of the 17-(bromoacetoxy)steroids to react with histidine may result from their binding at the active site of 3α,20β-HSD differently than the 16α- or 21-substituted steroids. Two types of steroid binding at the enzyme active site may occur which can account

for these differences (discussed below).

Following complete inactivation of 3α,20β-HSD with 1 mol of DTB or 6β-BAP at pH 7.0, 3α and 20β activities were restored in a time-dependent manner at pH 9.0. The relative rates of 3α and 20β inactivation at pH 7.0, or reactivation at pH 9.0, were practically identical. When a single molecule of 6β-BAP reacts with a cysteine residue at the active site of 3α,20β-HSD,the presence of the steroid blocks both 3α and 20β activity. Both activities are restored following hydrolytic release of the steroid from the active site. These results are consistent with the steroid binding region at the enzyme active site having two cofactor binding regions at opposite ends of the steroid molecule (model 1, Figure 6) or a *single* cofactor binding region and two different steroid binding orientations at the active site (model 2, Figure 6).

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