Synthesis of 2α-, 6α-, and 6β-Bromoprogesterone and Study of the Binding Site of 20β-Hydroxysteroid Dehydrogenase†

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ABSTRACT: To extend the application of affinity labeling for the characterization of macromolecular steroid binding sites we have synthesized 2α-, 6α- and 6β-bromoprogesterone and studied their reactions with various amino acids and with 20β-hydroxysteroid dehydrogenase from *Streptomyces hydrogenans*. Of all the amino acids studied, the bromoprogesterones react only with cysteine. All the bromoprogesterones are capable of reversible binding at the enzyme active site as they serve as substrates for the enzyme. Both 6α- and 6β-bromoprogesterone inactivate the enzyme in a time-dependent and irreversible manner. Progesterone slows the rate of inactivation and excess 2-mercaptoethanol stops, but does not reverse it. 2α-Bromoprogesterone does not inactivate the enzyme despite the fact that it clearly reacts with model sulfhydryl compounds. Radioactive 6β-bromoprogesterone was synthesized from progesterone-*1,2-t*. Inactivation of the enzyme was accompanied by radiolabeling. Studies of stoichiometry indicate that inactivation results from affinity labeling of a single sulfhydryl group at the enzyme active site. After inactivation and hydrolysis a single major fraction of radioactivity is seen on thin-layer chromatography. It has a mobility identical with that of the reaction product of 6β-bromoprogesterone and cysteine and can be cocrystallized with this compound. These observations are compatible with a mechanism whereby the steroid moieties of 6α- and 6β-bromoprogesterone deliver the reagent groups to the binding site of 20β-hydroxysteroid dehydrogenase where it reacts with a cysteine residue at that site. These compounds are offered to study certain steroid binding sites of high affinity present in receptor proteins of target organs.

Previous efforts in this laboratory have effected the synthesis of several affinity-labeling steroids. One, 4-mercuri-17β-estradiol (Chin and Warren, 1968), was shown to display long-acting estrogenic activity (Muldoon and Warren, 1969). Another, 4-mercuri-17α-estradiol, is a long-acting antiestrogen (Ellis and Warren, 1971). Further, 2-diazoestrone sulfate (Chin and Warren, 1970) and cortisone 21-iodoacetate (Ganguly and Warren, 1971) have been synthesized and shown to affinity label various steroid binding sites. We have presumed that such compounds may be useful for determination of the molecular mechanisms of steroid action, for elucidation of the amino acid residues at steroid binding sites and even for use as antisteroids.

Using cortisone 21-iodoacetate, Ganguly and Warren (1971) demonstrated the presence of histidine at the catalytic site of 20β-hydroxysteroid dehydrogenase from *Streptomyces hydrogenans*. To further elucidate the topography of the steroid binding site of this macromolecule, we have synthesized 2α-, 6α-, and 6β-bromoprogesterone. Studies with these compounds indicate that the steroid binding site contains a cysteine residue capable of reacting with 6α- and 6β-bromoprogesterone and therefore presumably present at that region of the site, approximated on binding, by the 6 position of the steroid.

Experimental Section

Materials

Reagent grade salts and inorganic acids were purchased from the Mallinckrodt Co. Reagent grade organic solvents were purchased from Fisher Scientific and distilled prior to use. Anhydrous organic solvents were freshly prepared according to Fieser and Fieser (1967, 1969). Progesterone and 3β-hydroxy-5-pregnen-20-one acetate were obtained from the Steraloids Co. Progesterone-*1,2-t* (33.5 Ci/mmole) was obtained from New England Nuclear. Cortisone, amino acids, reduced glutathione, NADH, and 20β-hydroxysteroid dehydrogenase from *S. hydrogenans* were purchased from Sigma. The enzyme was found to be over 90% pure by disc electrophoresis on polyacrylamide. Pronase (*Streptomyces griseus* protease), B grade, was obtained from Calbiochem. Aminopeptidase was obtained from Henly and Co., New York. Ethylene glycol, bromine, aluminum isopropoxide, and *p*-toluenesulfonic acid were obtained from Eastman Organic Chemicals. Sodium methoxide and ethyl oxalate were obtained from the Matheson Co. *N*-Bromosuccinimide was purchased from the Matheson Co. and recrystallized from water. Triton X-100, 2-mercaptoethanol, and ninhydrin aerosol were obtained from Sigma. 2,5-Diphenyloxazole and 1,4-bis[2-(5-phenyloxazolyl)]benzene were obtained from Amersham-Searle. The scintillation solution was made with 0.5% 2,5-diphenyloxazole and 0.01% 1,4-bis[2-(5-phenyloxazolyl)]benzene in a mixture of toluene–Triton X-100 (2:1). 5,5′-Dithiobis(2-nitrobenzoic acid) was obtained from Aldrich and crystallized from alcohol before use. Glass-redistilled water was used for all solutions. Thin-layer chromatography utilized Eastman 6060 silica gel sheets.

Methods

Melting points were determined on a Mel-Temp apparatus and are reported without correction. Optical rotation values were determined with a Polyscience SR 5 polarimeter and optical rotatory dispersion curves were recorded on a Jasco Model ORD/UV-5 spectropolarimeter. Ultraviolet absorption spectra were recorded on a Cary 14 spectrophotom-

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eter. Infrared spectra were measured with a Beckman IR-8 spectrophotometer. The labeled compounds were counted in a Packard Tri-Carb 3320 liquid scintillation spectrometer. Elemental analyses were done by Galbraith Laboratories, Knoxville, Tenn.

Synthesis of 6β-Bromoprogesterone. 6β-Bromoprogesterone was prepared as described by Sondheimer *et al.* (1953). The stereochemistry of this compound was proved by Adams *et al.* (1956). 6β-Bromoprogesterone has an ultraviolet absorption maximum at 246 mμ and a negative Cotton effect in the optical rotatory dispersion curve: $[\alpha]_{309}^{25}$ (*c* 1 mg/ml, chloroform, trough) −720°, mp 142–145° dec.

Synthesis of 6α-Bromoprogesterone. 6α-Bromoprogesterone was prepared according to Ringold *et al.* (1962). This compound has an ultraviolet absorption maximum at 237 mμ and a positive Cotton effect in the optical rotatory dispersion curve: $[\alpha]_{286}^{25}$ (*c* 1 mg/ml, chloroform, peak) +160°, mp 162°. *Anal.* Calcd for $C_{21}H_{29}BrO_2$: C, 64.09; H, 7.42; Br, 20.31. Found: C, 63.98; H, 7.37; Br, 20.25.

Synthesis of 2α-Bromoprogesterone. The synthesis of 2α-bromoprogesterone involves six steps as shown in Figure 1. The three intermediates, 3β-hydroxy-5-pregnen-20-one acetate ethylene ketal, 3β-hydroxy-5-pregnen-20-one ethylene ketal, and 4-pregnen-3,20-dione 20-ethylene ketal, were prepared from 3β-hydroxy-5-pregnen-20-one acetate (pregnenolone acetate) by the sequence as described by Gut (1956). Physical properties were found to be similar to those he reported.

For preparation of 2-ethyloxalyl-4-pregnen-3,20-dione 20-ethylene ketal, a solution of 25 ml of 1.0 N methanolic sodium methoxide in 100 ml of dry benzene was distilled until 76 ml of distillate was collected. The residue was diluted with 40 ml of benzene and the solution treated with 54 ml (50 mmoles) of ethyl oxalate. All solid immediately dissolved. Then 4-pregnene-3,20-dione 20-ethylene ketal (4.3 g, 24 mmoles) was added and magnetically stirred for 24 hr. The mixture was diluted with 240 ml of ether and stirring continued for 2 hr, and the precipitate was collected with a Büchner funnel and dried under reduced pressure. The filtrate was evaporated to dryness, triturated with ether, filtered, and dried under reduced pressure. The two crude solid products were combined, dissolved in 10 ml of water and adjusted to pH 5 by 1.0 N HCl. The yellow amorphous precipitate was collected and dried over P_2O_5: yield, 82%; mp 73–75°; λ_{max}^{MeOH} 240 mμ (ε, 12,500); infrared spectrum (KBr) 1675 (3 C=O), 1620 (C=C), 1060, and 1040 cm^{-1} (ketal); R_F 0.64 on thin-layer chromatography with acetone–MeOH (80:20) as solvent. *Anal.* Calcd for $C_{27}H_{38}O_6$: C, 70.75; H, 8.38. Found: C, 70.40; H, 8.40.

For bromination of 2-ethyloxalyl-4-pregnene-3,20-dione 20-ethylene ketal to 2α-bromo-4-pregnene-3,20-dione 20-ethylene ketal, 2-ethyloxalyl-4-pregnene-3,20-dione ethylene ketal (1.35 g, 3.26 mmoles) and potassium acetate (0.64 g, 6.52 mmoles) in 20 ml of methanol was chilled to about 5° with stirring. Bromine (13.2 g) in 100 ml of CCl_4 was added dropwise over a period of 50 min. The resulting turbid, yellow solution was treated with 20 mg of phenol and 3.26 ml of 1.0 N methanolic sodium methoxide. The solution was allowed to reflux for 10 min on the steam bath and then chilled in an ice bath. The yellow crystals were collected by filtration and the mother liquor was concentrated to about 5.0 ml to yield additional crystals. The combined crystals of 2α-bromo-4-pregnene-3,20-dione 20-ethylene ketal were recrystallized from acetone–hexane: yield, 75%; mp 127–129° dec; infrared spectrum (KBr) 1675 (3 C=O), 1615 (4 C=C), 1065, and 1040 cm^{-1} (ketal); R_F 0.59 on thin layer chromatography with $CHCl_3$–hexane (85:15) as solvent. *Anal.* Calcd for $C_{23}H_{33}BrO_5$: C, 63.20; H, 7.55; Br, 18.12. Found: C, 62.71; H, 7.39; Br, 18.16.

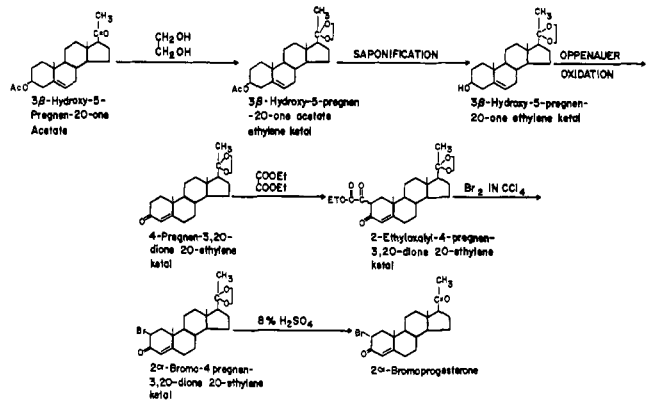

FIGURE 1: Scheme of synthesis of 2α-bromoprogesterone.

For conversion of 2α-bromo-4-pregnene-3,20-dione 20 ethylene ketal to 2α-bromoprogesterone (2α-bromo-4-pregnene-3,20-dione), a solution of 2α-bromo-4-pregnene-3,20-dione 20-ethylene ketal (1.0 g, 2.28 mmoles) in 70 ml of methanol and 12 ml of 8% sulfuric acid (v/v) was refluxed for 10 min, poured into 200 ml of salt water, and extracted with 200 ml of ethyl acetate. After drying over sodium sulfate, the solvent was removed with a Rinco evaporator. The residue was recrystallized from methanol: yield, 61%, mp 148–149° dec; $[\alpha]_{25}^{D}$ +192° (chloroform); λ_{max}^{MeOH} 244 mμ (ε 15,200); infrared spectrum (KBr) 1710 (20 C=O), 1685 (3 C=O), and 1615 cm^{-1} (4 C=C), no absorbance between 1200 and 1040 cm^{-1}. All the physical properties agree with the results of Allen and Weiss (1960). *Anal.* Calcd for $C_{21}H_{29}BrO_2$: C, 64.09; H, 7.42; Br, 20.31. Found: C, 63.92; H, 7.37; Br, 20.29.

Synthesis of Radioactive 6β-Bromoprogesterone. A solution of progesterone-*1,2-t* (3.65 mCi) in 3 ml of anhydrous carbon tetrachloride was mixed with unlabeled progesterone (90 mg, 0.236 mmole). The specific activity was determined to be 16.1 Ci/mole. *N*-Bromosuccinimide (50 mg, 0.30 mmole) was added and the mixture was refluxed for 1 hr. After cooling to room temperature, the reaction mixture was filtered with a fritted Büchner funnel. The filtrate was dried with nitrogen and the residue triturated with hexane and recrystallized twice from 3 ml of acetone: yield, 10.0 mg (9%); specific activity 8.75 Ci/moles; mp 147–150° dec; single spot on thin-layer chromatogram with R_F value identical with unlabeled 6β-bromoprogesterone.

Synthesis of Progesterone-6-S-L-cysteine. 6β-Bromoprogesterone (500 mg, 1.25 mmoles) was dissolved in 100 ml of 95% ethanol through which a flow of nitrogen was maintained for 30 min. Cysteine·HCl (160 mg, 1.0 mmole) was added over a period of 30 min and the pH value was adjusted to 8.0 with 1.0 N NaOH. The solution was then kept at room temperature for 3 hr under nitrogen with the pH value maintained at 8.0 throughout the time. After cooling at 0–5° for 3 hr, a precipitate of NaBr and NaCl was removed by filtration. The filtrate was evaporated to dryness with a Rinco evaporator. Progesterone-6-*S*-L-cysteine in the residue was extracted with absolute ethanol at 50–60°. Undissolved materials containing unreacted cysteine·HCl, NaBr, and NaCl were filtered out and the filtrate was adjusted to pH 7.0 with

concentrated HCl. More precipitate of NaCl was removed and the filtrate was kept at 0–5° overnight. The yellow precipitate of progesterone-6-*S*-L-cysteine was collected with a Büchner funnel. The filtrate was condensed to half of its original volume and left at 0–5° overnight to obtain the second crop. The combined crude product was recrystallized from ethanol–ether: yield, 18%; mp 201–203° dec; λ_{max}^{MeOH} 240 mμ (ε 9300); infrared spectrum (KBr) 1715 (20 C=O), 1675 (3 C=O), 1590, 1410 (COO^-), 1527, 1465 cm^{-1} (NH_3^+), and no band at 2550 cm^{-1} indicating absence of the SH group; R_F 0.75 on thin-layer chromatography with acetone–MeOH (80:20) as solvent. The single spot both absorbs ultraviolet light and is ninhydrin positive. *Anal.* Calcd for $C_{24}H_{35}NSO_4$: C, 66.54; H, 8.15; N, 3.23; S, 7.39. Found: C, 65.71; H, 8.04; N, 3.32; S, 7.52. Based upon these findings and reasons presented in the discussion, the compound is tentatively designated as progesterone-6-*S*-L-cysteine.

Reaction of the Bromoprogesterones with Amino Acids. Solutions, 2.5×10^{-4} M, of each of the bromoprogesterones were incubated with 1.25×10^{-3} M cysteine, methionine, histidine, *N*-acetylhistidine, lysine, tyrosine, *N*-chloroacetyltyrosine, tryptophan, *N*-chloroacetyltryptophan, aspartic acid, or alanine in 0.1 M potassium phosphate buffer–MeOH (50:50) (pH 7.0) at room temperature for 12 hr. The reaction mixtures were dried and the residues extracted with 1.0 ml of methanol. Control solutions of each of the bromoprogesterones and amino acids (separately) were treated in a similar way. Any new compounds produced from coupling of the bromoprogesterones and amino acids can be detected by thin-layer chromatography as fractions of differing mobility which show both absorbance of ultraviolet light and ninhydrin color. Chromatograms were developed with butanol–acetic acid–water (4:1:2) to examine the mobility of amino acids and with chloroform–hexane (9:1) to examine the mobility of steroids.

Inactivation of 20β-Hydroxysteroid Dehydrogenase by the Bromoprogesterones. 20β-Hydroxysteroid dehydrogenase (0.1 μM) was preincubated with the appropriate bromoprogesterone (1.0 μM) in 0.05 M potassium phosphate buffer (pH 7.0) containing 10% ethanol. The enzyme was assayed first at zero time and then at suitable intervals until more than 90% enzyme activity was lost. The enzyme assay was carried out in 0.05 M potassium phosphate buffer (pH 6.5) at 25 ± 1° in a final volume of 3.0 ml using 0.2 μmole of NADH as cofactor and, unless otherwise stated, 0.36 μmole of cortisone as substrate (Betz, 1968). The reaction was initiated by addition of an aliquot of the preincubation solution containing approximately 1 μg of the enzyme. Assays were done with a Beckman DU spectrophotometer equipped with a Gilford power supply and a Honeywell recorder. Enzyme activity was estimated from the initial linear decrease in absorbance at 340 mμ. Control preincubations were also carried out.

Analyses of Amino Acids and Labeled Compound of the Inactivated 20β-Hydroxysteroid Dehydrogenase. After 20β-hydroxysteroid dehydrogenase was inactivated by radioactive 6β-bromoprogesterone, the reaction was stopped by addition of a fiftyfold excess of 2-mercaptoethanol. The inactivated enzyme solution then was dialyzed against glass-redistilled water at 5° until no significant radioactivity was found in the outer compartment. Contents of the bag were lyophilized and hydrolyzed first with 2% (w/w) pronase for 4 hr, followed with aminopeptidase M overnight at room temperature (R. Bradshaw and P. Newmann, 1971, personal communication). The process then was repeated and the enzymatic hydrolysis stopped by addition of 0.2 M citrate buffer (pH 2.2). Before hydrolysis, aliquots of the enzyme were taken to determine protein content and radioactivity for stoichiometric study. After hydrolysis, the enzyme was extracted with a minimum amount of methanol and an aliquot of the solution was submitted to thin-layer chromatography to identify the labeled compound. The remaining portion was used to confirm the identity of the labeled compound by cocrystallization.

Results

Stereochemistry of 6α-Bromoprogesterone. Ringold *et al.* (1962) did not state how they proved the stereochemistry of the compound that they identified as 6α-bromoprogesterone. We suggest this structure since (1) with strong acid, 6β-bromoprogesterone will isomerize to the more thermodynamically stable 6α isomer; (2) the compound has a lower ultraviolet absorption maximum (237 mμ) than that of progesterone (240 mμ) whereas a 6β-bromo derivative of Δ^4-3-keto steroid generally has an ultraviolet absorption maximum at 246–248 mμ; and (3) the compound shows a reversal of the Cotton effect of 6β-bromoprogesterone.

Reaction of the Bromoprogesterones with Amino Acids. Cysteine is the only amino acid found to react with the bromoprogesterones at pH 7.0, 25°. It reacts with all three bromoprogesterones but the reaction rates are different. On thin-layer chromatography new spots were always produced with mobilities between the parent bromoprogesterone and cysteine. In the case of 6β-bromoprogesterone and cysteine, the reaction product had the mobility of progesterone-6-*S*-L-cysteine.

Reactions of the Bromoprogesterones with 5,5′-Dithiobis-(2-nitrobenzoic Acid) and Reduced Glutathione. 5,5′-Dithiobis-(2-nitrobenzoic acid) (0.1 μmole) was reacted with reduced glutathione (0.1 μmole) in 2.8 ml of 0.05 M potassium phosphate buffer (pH 7.0) to produce the yellow anion. Then the appropriate bromoprogesterone (0.05 μmole) in 0.2 ml of ethanol was added and the decrease of absorbance at 412 mμ was recorded with a Cary 14 spectrophotometer. In another experiment, reduced glutathione (0.1 μmole) was reacted with the appropriate bromoprogesterone in 0.05 M potassium phosphate buffer (pH 7.0) under an atmosphere of nitrogen. At different intervals, 0.2 ml of the reaction mixtures was added to 2.8 ml of a solution containing 0.2 μmole of 5,5′-dithiobis(2-nitrobenzoic acid) to determine the unreacted glutathione. The results are shown in Figures 2 and 3. It can be seen that in both cases 6β-bromoprogesterone reacts much more rapidly than the 2α and 6α derivatives to alkylate the model sulfhydryl compounds. With glutathione, the reactions follow second-order kinetics with the rate constants for 6β-6α-, and 2α-progesterone in the ratio 15:3:1.

Evidence That the Bromoprogesterones Are Substrates of 20β-Hydroxysteroid Dehydrogenase. Assays carried out as described above with NADH (0.2 μmole) and each bromoprogesterone in a 3.0-ml total volume revealed that all the three are substrates of 20β-hydroxysteroid dehydrogenase. This indicates that each binds at the enzyme active site. As shown in Figure 4, the Michaelis constants of 6β-, 6α-, and 2α-bromoprogesterone estimated by the method of Lineweaver and Burk (1934) are 8.20×10^{-6}, 1.43×10^{-5}, and 3.92×10^{-6} M, respectively. The V_{max} values are 3.7, 5.8, and 3.9 nmoles per min, respectively. Under similar conditions, progesterone has $K_m = 3.90 \times 10^{-6}$ M and V_{max} = 3.9 nmoles/min.

Inactivation of 20β-Hydroxysteroid Dehydrogenase by the

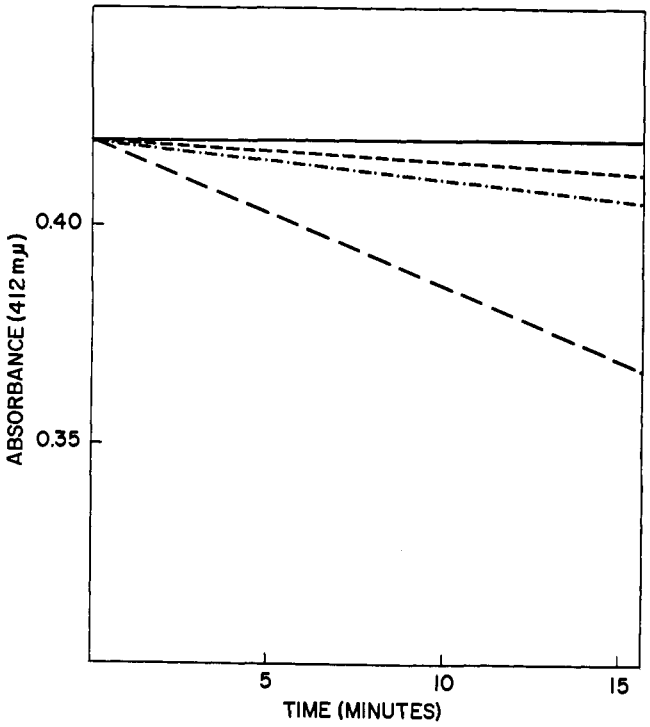

FIGURE 2: Reaction of 0.05 μmole of progesterone or bromoprogesterones with 0.1 μmole of colored anionic form of 5,5′-dithiobis-(2-nitrobenzoic acid). Steroids in 0.2 ml of ethanol were added separately to the preformed colored solution of reduced glutathione and 5,5′-dithiobis(2-nitrobenzoic acid) in 2.8 ml of 0.05 M potassium phosphate buffer, pH 7.0, 25°. The absorption spectra were recorded on a Cary 14 spectrophotometer. (—) Progesterone, (----) 2α-bromoprogesterone, (-●-) 6α-bromoprogesterone, and (– –) 6β-bromoprogesterone.

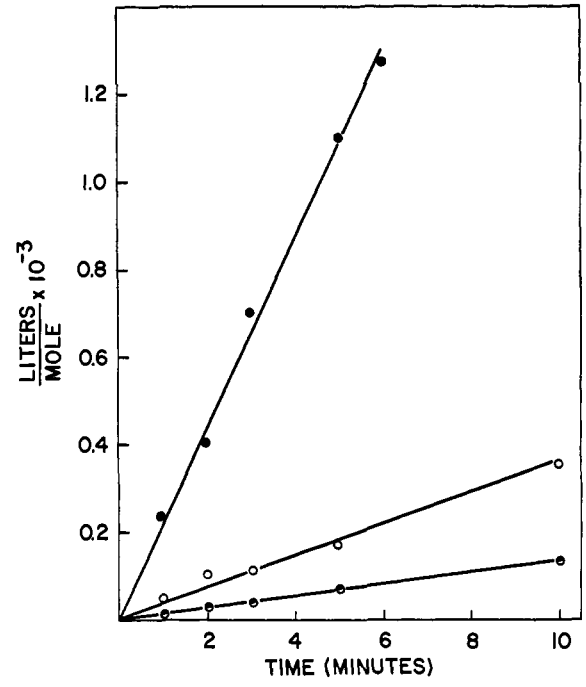

FIGURE 3: Second-order plots for the reaction of 0.05 μmole of bromoprogesterones (A) with 0.05 μmole of reduced glutathione (B) in 0.05 M potassium phosphate buffer, pH 7.0, 25° for times shown. Unreacted glutathione was determined by reaction with 5,5′-dithiobis(2-nitrobenzoic acid) at 412 mμ. Reaction A + B → x with [A] = [B]; the ordinate represents [x]/[A] ([A] − [x]). (●) 6β-Bromoprogesterone, (○) 6α-bromoprogesterone, and (◓) 2α-bromopregesterone.

Bromoprogesterones. It can be seen from Figure 5 that the inactivation of 20β-hydroxysteroid dehydrogenase by the bromoprogesterones follows pseudo-first-order kinetics. The half life of the enzyme is 2 hr with 6β-bromoprogesterone and 48 hr with 6α-bromoprogesterone as determined by extrapolating the plot. 2α-Bromoprogesterone does not inactivate the enzyme.

It can also be seen from the same figure that in the presence of twice the amount of progesterone (2.0 μM), the inactivation rate by 6β-bromoprogesterone was slowed so that the enzyme has a half-life of 4 hr. This gives strong evidence that 6β-bromoprogesterone is reacting at the enzyme active site from which it is partially excluded by progesterone itself.

The irreversibility of the inactivation of 20β-hydroxysteroid dehydrogenase was shown by mixing fivefold excess of 2-mercaptoethanol after 50% inactivation effected by 6β-bromoprogesterone. The process of inactivation was stopped but no reversal was seen, indicating that a covalent linkage is formed between the steroid molecule and some active amino acid residue.

Treatment of 20β-Hydroxysteroid Dehydrogenase with Tritiated 6β-Bromoprogesterone. 20β-Hydroxysteroid dehydrogenase (5 mg as a suspension) in saturated ammonium sulfate was centrifuged at 60,000*g* for 15 min. The pellet was taken up in 48 ml of 0.05 M potassium phosphate buffer (pH 7.0) and the solution was centrifuged again at 60,000*g* to separate any undissolved matter. The exact concentration of protein was shown to contain 5.0 mg (50 nmoles) of enzyme by the method of Lowry *et al.* (1951). Ethanol (25 μl) was added to a 0.5 ml aliquot of the resulting solution, which was used as a control. A solution containing 6β-bromoprogesterone-*1,2-t* (0.2 mg, 0.50 μmole) in 2.5 ml of ethanol was added to the remaining 47.5 ml of protein solution. An aliquot was counted and the reaction mixture was shown to contain 4.00×10^6 cpm.

When 25% inactivation was obtained, one-fifth of the reaction mixture was taken out and mixed with 50 μl of 1.4×10^{-2} M 2-mercaptoethanol to stop the inactivation. When 50% inactivation was attained, another one-fifth of the sample was removed and 50 μl of 2-mercaptoethanol was added. When the enzyme was 75% inactivated, 150 μl of 2-mercaptoethanol was added to the remaining mixture. The three enzyme samples were then separately dialyzed and lyophilized as described previously. The dry protein samples were dissolved in 1.8, 1.9, and 3.6 ml of water. These sample contained 0.17, 0.19, and 1.03 mg for the 25, 50, and 75% inactivated enzyme samples, respectively. Total radioactivity was 3600, 7400, and 54,000 cpm, respectively, which shows approximately a ratio of 1:2:15. If one molecule of tritiated 6β-bromoprogesterone (specific acitivity 8.75 Ci/mole) binds with one sulfhydryl group per molecule of enzyme, total radioactivity expected would be 3700, 7900, and 60,000 cpm, respectively. The results thus indicate that 6β-bromoprogesterone reacts with the sulfhydryl residue at the enzyme binding site and the molar reaction ratio is one to one.

Identification of the Active Amino Acid Residue at the Steroid Binding Site of 20β-Hydroxysteroid Dehydrogenase. The aqueous solution (1 ml) of 75% inactivated enzyme (0.3 mg) was lyophilized and dissolved in 0.2 ml of 0.1 M potassium phosphate buffer (pH 8.0). Pronase (1 μl; 5 mg in 1 ml of 0.1 M potassium phosphate buffer) was added and the digestion was allowed to proceed for 4 hr at room temperature. Amino

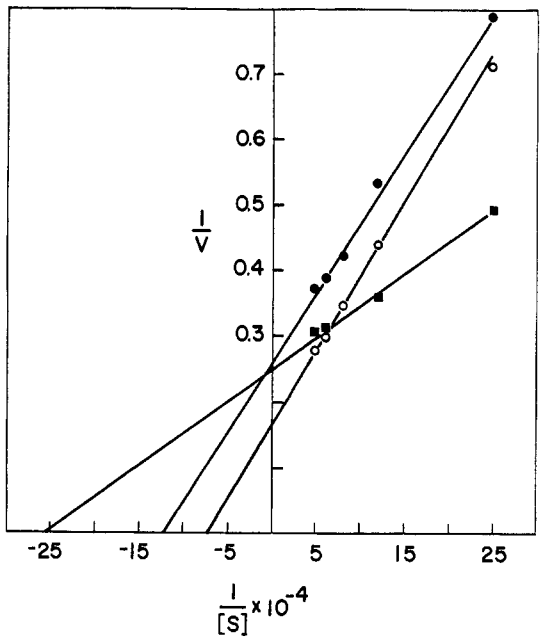

FIGURE 4: Double-reciprocal plot of reduction of the bromoprogesterones by 20β-hydroxysteroid dehydrogenase using assay conditions described in the text. V, velocity in nmoles of NAD^+ generated per min. [S], molar concentration of substrate. (●) 6β-Bromoprogesterone, (○) 6α-bromoprogesterone, and (■) 2α-bromoprogesterone.

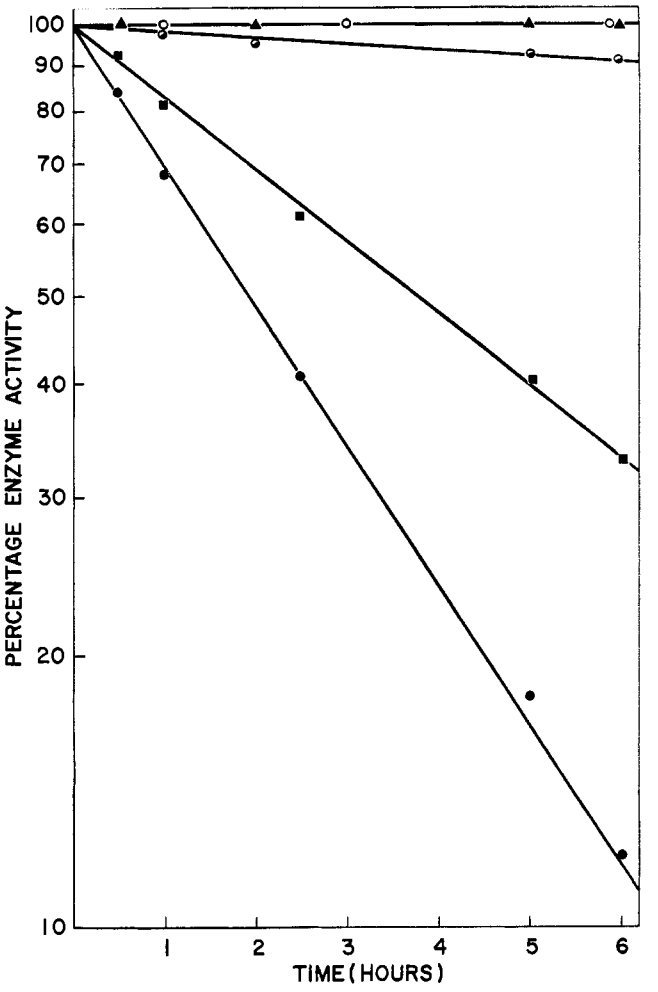

FIGURE 5: Pseudo-first-order plots for the effect of progesterone and bromoprogesterones on the activity of 20β-hydroxysteroid dehydrogenase. Preincubations conducted with 50 μg of enzyme in 48 ml of 0.05 M potassium phosphate buffer, pH 7.0, 25°, to which was added at zero time 0.05 μmole of steroids in 2 ml of ethanol separately. At times indicated, 0.1 ml of this solution was assayed as described in the text using near saturated concentrations of substrate and cofactor. (▲) Progesterone, (○) 2α-bromoprogesterone, (◒) 6α-bromoprogesterone, (●) 6β-bromoprogesterone, and (■) 0.05 μmole of 6β-bromoprogesterone and 0.10 μmole of progesterone.

peptidase M (50 μl; 10,000 mU/ml) then was added and the reaction mixture was left overnight at room temperature. After the hydrolysis was repeated, the reaction was stopped by addition of 1 ml of 0.2 M citrate buffer (pH 2.2). The aqueous solution was lyophilized again and the remaining residue was extracted with 0.2 ml of methanol at room temperature. Then 20 μl of the alcoholic solution was applied to Eastman 6060 thin-layer sheet as were standard 6β-bromoprogesterone, progesterone-6-*S*-L-cysteine, cysteine, and other amino acids. The chromatogram was developed with butanol–acetic acid–water (4:1:2) at room temperature. Steroids were observed under ultraviolet light and amino acids were detected with ninhydrin spray. The labeled product of the enzyme hydrolysate was identified by cutting the chromatogram into 1-cm pieces, scraping the silica gel layer into 10 ml of scintillation solution, and counting. It can be seen from Figure 6 that the major radioactive peak of the hydrolysate was identical in mobility with standard progesterone-6-*S*-L-cysteine. If the radioactivity of the labeled hydrolysate was corrected for quenching effects (25.80%) with an external standard, then the whole hydrolysate with 75% inactivation was shown to have 56,000 cpm. For 1.03×10^{-5} mmole of 20β-hydroxysteroid dehydrogenase, if there is only one sulfhydryl group per enzyme molecule reacting with tritiated 6β-bromoprogesterone at the active site, and assuming the enzyme is 100% pure and 100% inactivated, the theoretical total counts should be 84,000 cpm.

Confirmation of the Labeled Amino Acid Residues by Co-crystallization. The remaining portion of the 75% inactivated enzyme hydrolysate was chromatographed on thin-layer sheet against standard progesterone-6-*S*-L-cysteine. The whole strip of silica gel layer (without ninhydrin spray) which was identical with the R_F value of progesterone-6-*S*-L-cysteine (with ninhydrin spray) was scraped into 5 ml of methanol. The methanol extract was filtered and 50 mg of progesterone-6-*S*-L-cysteine were added. The steroid then was recrystallized four times from ethanol–ether as described previously. In each recrystallization the concentration was determined gravimetrically and radio-activity determined by scintillation counting. Table I indicates that the specific activities of progesterone-6-*S*-L-cysteine remain almost the same during the four recrystallizations.

Discussion

The physical properties of 6β-bromoprogesterone agree with those in the literature (Sondheimer *et al.*, 1953; Adams and Patel, 1956; Rao and Gollberg, 1963). 6α-Bromoprogesterone was proved to have its relative configuration by chemical isomerization from its 6β isomer ultraviolet spectrum and the optical rotatory dispersion curve. 2α-Bromoprogesterone was synthesized by a process different from that of Allen and Weiss (1960). The physical properties of the compound obtained from two different processes agreed but the present process is simpler and gives a better yield.

TABLE I: Cocrystallization of the Tritiated Enzymatic Hydrolysate of 20β-Hydroxysteroid Dehydrogenase with Progesterone-6-*S*-L-cysteine.

Times of Cocrystallization	Progesterone-6-*S*-L-cysteine (mg)	Cpm	Sp Act. (cpm/mg)
0	17.0	5600	330
1	14.0	4400	314
2	9.8	3080	310
3	7.2	2200	302

Starting with tritiated progesterone having a specific activity of 16.1 Ci/moles, the resulting 6β-bromoprogesterone is 8.75 Ci/mole. The loss of radioactivity in preparation may be due to resonance of the progesterone free radical initiated by *N*-bromosuccinimide. Further, the exchange of a tritium with a proton at position 2 may occur by enolization. It is also possible that a secondary hydrogen isotope effect slows down the rate of bromination in progesterone-*1,2-t* molecules (Melander, 1960; Collins and Bowman, 1970) so reduces the specific activity.

Of the amino acids tested, the bromoprogesterones react only with cysteine. The reaction follows second-order kinetics and almost certainly results from an SN2 mechanism. With simple sulfhydryl compounds, 6β-bromoprogesterone reacts at a rate five times that of 6α-bromoprogesterone. This discrepancy in rates is what one would expect because of the steric hindrance imposed by the C_{10} angular methyl group. With 20β-hydroxysteroid dehydrogenase the rate of inactivation by 6β-bromoprogesterone is 24 times that by 6α-bromoprogesterone. It is tempting to speculate that these rate ratios result from the spatial location of the enzyme cysteine at a region of the steroid binding site which approximates the 6α position of the bound steroid to a greater degree than the 6β position. One cannot go further than speculate in this regard because the affinity constants of the two steroids for the enzyme are not really known.

Nevertheless, all three bromoprogesterones can carry out the reversible binding step at the active site of the enzyme as shown by the fact that they serve as substrates. Progesterone markedly slows the inactivation of the enzyme by 6β-bromoprogesterone as to be expected if they compete for the active site. Inactivation is demonstrated to be irreversible by the fact that no activity is recovered on dialysis or after addition of 2-mercaptoethanol.

By the techniques of thin-layer chromatography and cocrystallization, the major radioactive peak of the enzyme hydrolysate is identified to be progesterone-6-*S*-L-cysteine, and the stoichiometry indicates that one molecule of 6β-bromoprogesterone binds covalently with one sulfhydryl residue at the active site of 20β-hydroxysteroid dehydrogenase. We attempted to use the automatic amino acid analyzer to identify the radioactive product of the enzyme hydrolysate as was done in our laboratory previously (Ganguly and Warren, 1971), but as progesterone-6-*S*-L-cysteine is absorbed by the column too tightly to be eluted out, the process is not useful in this experiment.

From the above observations, we conclude that the active-site cysteine residue of 20β-hydroxysteroid dehydrogenase resides at a point on the protein near the 6 position of the bound steroid. It is pertinent to note that Sondheimer *et al.* (1953) and Fieser and Romero (1953) demonstrated that reaction of 6β-bromoprogesterone with potassium acetate resulted in 2-acetoxyprogesterone. These authors carried out the reaction with anhydrous potassium acetate in glacial acetic acid under reflux, conditions favoring enolization of the 3-keto group and subsequent attack at C_2. Under our conditions, enolization should be minimal and the SN2 attack at C_6 should predominate.

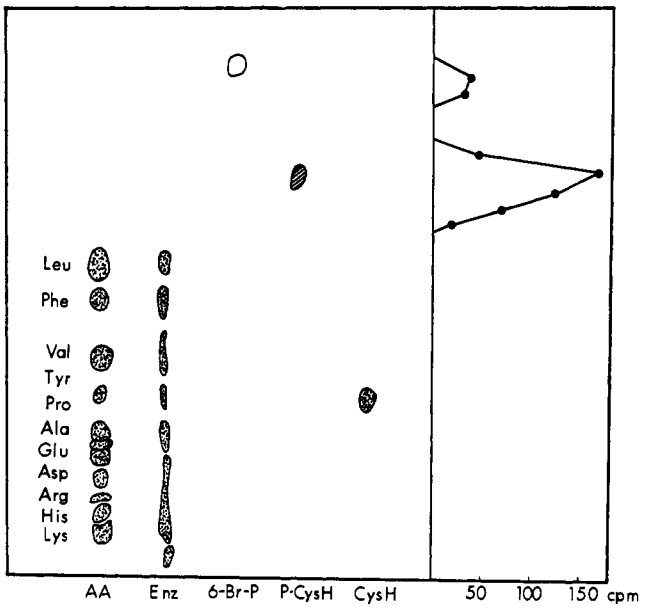

FIGURE 6: Distribution of radioactivity on thin-layer chromatography of hydrolysate of 20β-hydroxysteroid dehydrogenase after treatment with tritiated 6β-bromoprogesterone. (●) Ninhydrin positive, (○) ultraviolet light absorbent, (⊘) ninhydrin positive and ultraviolet light absorbent. AA, standard amino acids; Enz, enzyme hydrolysate; 6-Br-P, 6β-bromoprogesterone; P-CysH, progesterone-6-*S*-L-cysteine; CysH, L-cysteine.

Previous studies with 20β-hydroxysteroid dehydrogenase indicate that the sulfhydryl residues of the native enzyme are unreactive to several sulfhydryl reagents (Betz, 1968; Ganguly and Warren, 1971). Only after denaturation in 6 M guanidine hydrochloride did sulfhydryl groups become reactive and under these conditions the enzyme appears to contain two groups per mole (Betz, 1968). Thus, it would appear that 6β- and 6α-bromoprogesterone are uniquely capable of conveying the reactive group to an otherwise inaccessible sulfhydryl group. While it must be considered that the progesterone-6-*S*-L-cysteine isolated after hydrolysis could arise by reaction of bromoprogesterone with a cysteine residue present in the small contaminant or with free cysteine bound noncovalently at the active site, the inability of cortisone 21-iodoacetate to alkylate a cysteine residue in the enzyme (Ganguly and Warren, 1971) and the inhibition of the rate of inactivation by progesterone make such a possibility appear extremely remote.

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Specific Modification of Methionine-192 of α-Chymotrypsin by an Affinity Label Exploiting the Orienting Properties of the Linear Acetylenic Group†

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ABSTRACT: The practicability of incorporating acetylenic bonds into affinity labels in order to exploit their rodlike properties for improving the orientation of an alkylating function toward a selected protein nucleophile has been demonstrated by the facile irreversible inhibition of α-chymotrypsin by 6-bromo-1-phenylhex-4-yn-3-one. 6-Bromo-1-phenylhex-4-yn-3-one was designed to achieve optimum orientation of its propargylic bromide alkylating function toward both histidine-57 and methionine-192. However, the latter function was alkylated selectively owing to its 100-fold greater reactivity with propargylic bromides. Rate of inhibition, pH–rate profile, competitive inhibition, effect on K_m(app) and k_3, amino acid analytical, and diagonal peptide-mapping studies established that 6-bromo-1-phenylhex-4-yn-3-one was a methionine-192-specific, active-site-directed, irreversible inhibitor. The calculated values of its K_i (10 mM) and rate of inhibition constant (2.8×10^{-3} sec^{-1}) show it to be one of the best methionine-192 of α-chymotrypsin directed affinity labels yet evaluated. The data demonstrate that the linear geometry of the acetylenic bond can be used to good advantage in improving the degree of specificity achievable in a desired protein modification and that propargylic (and by analogy allylic) bromide functions represent a valuable addition to the list of alkylating groups normally employed for protein modification.

Use of affinity labels (active-site-directed reagents, site-specific reagents) to effect specific modifications of enzymes and immunoglobulins has already proven of great value (Baker, 1967; Singer, 1967; Glazer, 1970; Shaw, 1970). Although the spectrum of modifications which can now be achieved using affinity labels is quite broad, considerable scope still remains for improving the functional group and stereospecificity (or -selectivity) of such reagents and in this regard we had become intrigued by the possibility of exploiting the unique geometries of cis and trans ethylenic and linear acetylenic functions (either alone or in appropriate combinations) to achieve more precise orientation of an alkylating function toward a selected protein nucleophile. The practicability of exploiting the rodlike properties of acetylenic bonds to improve orientation in affinity labels has now been demonstrated by the irreversible inhibition of CT[1] by the dihydrocinnamylpropargylic bromide BPH (**5**, see Scheme I). This compound was designed to alkylate methionine-192 specifically and was found to be very effective in this regard. The data obtained also confirm that propargylic (and *ipso facto* allylic) bromide functions represent useful additions to the list of convenient alkylating agents employed in protein modification.

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[1] Abbreviations used are: CT, α-chymotrypsin; BPH, 6-bromo-1-phenylhex-4-yn-3-one; BPH-CT, BPH-inactivated α-chymotrypsin; hve, high voltage electrophoresis.