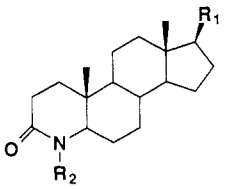
Jenkins, W. T. (1982) *Adv. Enzymol. Relat. Areas Mol. Biol. 53*, 307.
Koshland, D. E. (1970) *Enzymes (3rd Ed.) 1*, 341.
Krish, K. (1971) *Enzymes (3rd Ed.) 5*, 43.
Levy, M., & Ocken, P. (1967) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 26*, 816.
Mentlein, R., & Heymann, E. (1984) *Biochem. Pharmacol. 33*, 1243.
Morrison, J. F. (1982) *Trends Biochem. Sci. (Pers. Ed.) 7*, 102.
Ocken, P. R. (1967) Ph.D. Thesis, New York University, New York, NY.
Penefsky, H. S. (1979) *Methods Enzymol. 56*, 527.
Stone, S. R., & Hofsteenge, J. (1986) *Biochemistry 25*, 4622.
Williams, J. W., & Morrison, J. F. (1979) *Methods Enzymol. 63*, 437.

# 3β-Hydroxy-Δ⁵-steroid Dehydrogenase/3-Keto-Δ⁵-steroid Isomerase from Bovine Adrenals: Mechanism of Inhibition by 3-Oxo-4-aza Steroids and Kinetic Mechanism of the Dehydrogenase

Martin Brandt and Mark A. Levy*
*Department of Medicinal Chemistry, Smith Kline & French Laboratories, King of Prussia, Pennsylvania 19406*
*Received June 16, 1988; Revised Manuscript Received August 17, 1988*

ABSTRACT: Several 3-oxo-4-aza steroids (**1**) have been identified as inhibitors of the 3β-hydroxy-Δ⁵-steroid dehydrogenase/3-keto-Δ⁵-steroid isomerase catalyzed conversion of pregnenolone to progesterone. By kinetically decoupling the two enzyme activities isolated from bovine adrenal cortex, it has been demonstrated that inhibition by **1** occurs through interference of both activities. A preferred ordered association of substrates to the 3β-hydroxy-Δ⁵-steroid dehydrogenase in which the cofactor binds prior to steroid was determined by isotope exchange at equilibrium. With this result, the dead-end inhibition patterns of **1** with the dehydrogenase were interpreted to originate from a preferred association of inhibitor within an enzyme ternate containing NADH; this proposal is supported by data from multiple inhibition analysis indicating synergistic binding of NADH and **1**. Similarly, inhibition of the 3-keto-Δ⁵-steroid isomerase by the 3-oxo-4-aza steroids was enhanced in the presence of the positive effector NADH. On the basis of pH profiles upon $V_m$, $V_m/K_m$, and $1/K_i$ for both enzyme activities, inhibition is proposed to result from the structural similarity of **1** to intermediate states formed upon enzyme catalysis.

Over the last several years, members of a 3-oxo-4-aza series of steroids, compound **1**, have been described as potent re-




**1**

versible inhibitors of hepatic and prostatic steroid 5α-reductases (Rasmusson et al., 1986, 1984). It has been proposed that such inhibitors, if selective for steroid 5α-reductase, could be employed as therapeutic agents for the treatment of benign prostatic hypertrophy, acne, and male pattern baldness through blockade of 5α-dihydrotestosterone biosynthesis—the androgen responsible for progression of these disorders (Brooks et al., 1986a). Members of the 4-aza series of steroids currently are being evaluated toward this end (Brooks et al., 1986a,b).

Recent reports have suggested that one of these 4-aza steroids might not demonstrate unique specificity for steroid 5α-reductase. For example, Cooke and Robaire (1986) have reported that *N*,*N*-diethyl-4-methyl-3-oxo-4-aza-5α-androstane-17β-carboxamide (4-MA)[1] blocks the conversion of pregnenolone to progesterone in testis microsomes. In addition, Chan et al. (1987) have observed inhibition of progesterone synthesis by 4-MA in porcine granulosa cells. These results are consistent with interference by 4-MA of one or both of the reactions catalyzed by the enzymes 3β-hydroxy-Δ⁵-steroid dehydrogenase and 3-keto-Δ⁵-steroid isomerase.

The sequential enzyme activities of 3β-hydroxy-Δ⁵-steroid dehydrogenase (EC 1.1.1.145) and 3-keto-Δ⁵-steroid isomerase (EC 5.3.3.1) catalyze requisite steps in the biogenesis of androgenic, progestogenic, and estrogenic steroidal hormones and, among other metabolites, the corticosteroids. While proteins which catalyze these reactions can be uniquely isolated from bacterial sources (Talalay & Wang, 1955; Batzold et al., 1976), the microsomal-derived activities from mammalian tissues such as ovine adrenals (Ford & Engel, 1974), bovine ovaries, (Cheatum & Warren, 1966), rat testes (Ishii-Ohba et al., 1986a), and rat adrenals (Ishii-Ohba et al., 1986b, 1987) appear to reside within a single protein, possibly utilizing the same steroid binding site for both activities. In this regard, the 3-keto-Δ⁵-steroid isomerase from these sources requires NAD(H) as a positive allosteric effector (Oleinick & Koritz, 1966; Neville & Engel, 1968; Ishii-Ohba et al., 1986a). Oxidation by the 3β-hydroxy-Δ⁵-steroid dehydrogenase is

* Author to whom correspondence should be addressed at Smith Kline & French Laboratories, Research and Development Division, P.O. Box 1539, King of Prussia, PA 19406-0939.

[1] Abbreviations: 4-MA, *N*,*N*-diethyl-4-methyl-3-oxo-4-aza-5α-androstane-17β-carboxamide; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid: DHEA, dehydroepiandrosterone; ADIOL, 5α-androstane-3β,17β-diol; DHT, 5α-dihydrotestosterone.

0006-2960/89/0428-0140$01.50/0 

considered to be the rate-determining step for conversion of 3β-hydroxy $\Delta^5$-steroids (such as pregnenolone) to 3-keto $\Delta^4$-steroids (progesterone) (Marston et al., 1985).

Our interest in modulating pathways of steroid biosynthesis through the inhibition of nicotinamide-dependent oxidoreductases prompted an investigation into the interactions of the 3-oxo-4-aza steroids with 3β-hydroxy-$\Delta^5$-steroid dehydrogenase and 3-keto-$\Delta^5$-steroid isomerase. Upon consideration of the chemical mechanisms of these enzymes, we surmised that inhibition by 3-oxo-4-aza steroids (**1**) might derive from the preferential association of such a compound to a discrete form of the enzyme. In this regard, we proposed that the resulting complex might consist of a ternate comprised of enzyme, inhibitor, and reduced or oxidized cofactor. Herein, we report our efforts toward elucidation of the mechanism of this inhibition upon the bovine adrenal 3β-hydroxy-$\Delta^5$-steroid dehydrogenase and 3-keto-$\Delta^5$-steroid isomerase. In studying the inhibition by **1**, we also have established the kinetic mechanism of the dehydrogenase reaction.

## Experimental Procedures

### *Materials*

[4-$^{14}$C]Pregnenolone (57.2 mCi/mmol), [4-$^{14}$C]dehydroepiandrosterone (52.0 mCi/mmol), [4-$^{14}$C]-5α-dihydrotestosterone (52.0 mCi/mmol), [9,11-$^3$H(N)]androsterone (53.5 Ci/mmol), [1,2-$^3$H(N)]-5α-dihydrotestosterone (51.6 Ci/mmol), Aquasol 2, and Econosolve II were obtained from New England Nuclear (Boston, MA). [1α,2α-$^3$H(N)]-Androstanediol (60 Ci/mmol) and [4-$^3$H]nicotinamide adenine dinucleotide ($NAD^+$, 3.2 Ci/mmol) were purchased from Amersham. 5-Pregnene-3,20-dione and 5-androstene-3,17-dione were purchased from Steraloids (Wilton, NH). Fatty acid free bovine serum albumin (BSA) was purchased from Boehringer Mannheim Biochemicals. DEAE-cellulose (DE-52) was procurred from Whatman. Emulgen 913 was obtained from Kao-Atras Co., Tokyo, and DEAE-Sepharose was purchased from Pharmacia, Inc. All other chemicals and enzymes were obtained from Sigma Chemical Co. Protein concentrations were estimated by the method of Bradford (1976) with the Bio-Rad protein assay. Radioactivity was determined with either a Beckman LS-5801 or LS-3801 scintillation counter, calibrated for correction to disintegrations per minute (dpm) with Beckman $^{14}$C and $^3$H standards or with a System 2000 Bioscan imaging scanner (Bioscan, Washington, DC). Single-wavelength UV/vis spectral changes were monitored on a Gilford 260 spectrophotometer. The 4-aza steroids were synthesized by Dr. Dennis Holt and Jill Erb according to procedures previously described by Rasmusson et al. (1984, 1986). Radiolabeled steroids were purified by thin-layer chromatography. Prior to use, the [$^3$H]$NAD^+$ was purified by ion-exchange chromatography on a column (0.7 × 2.0 cm) of DE-52 according to a procedure similar to that outlined for the exchange reactions below. Analyses of enzymatic reactions were performed on either plastic-backed, silica TLC plates (Krieselgel 60 $F_{254}$, Merck) or prechanneled glass-backed plates with a preabsorbing region (Si250F-PA 19C, Baker).

### *Methods*

*Preparation of 3β-Hydroxy-$\Delta^5$-steroid Dehydrogenase/3-Keto-$\Delta^5$-steroid Isomerase from Bovine Adrenal Cortex.* Except where specified, all procedures involving enzyme preparation and manipulations were conducted on ice or at 4 °C. Bovine adrenal glands, obtained from Moyer Packing Co., Souderton, PA, were kept in ice-cold saline (0.9% NaCl) prior to dissection and isolation of the cortex. Each gland was bisected along its longitudinal median, and the cortex was removed with minimal contamination of the surrounding medullary tissue. The fresh cortex tissues (118 g) from 3 lb of adrenal glands were suspended in 3 volumes of pH 7.4 buffer containing 250 mM sucrose, 50 mM Tris, 25 mM KCl, and 5 mM $MgCl_2$ (STKM medium) and treated to three successive 10-s pulses with a Brinkmann Polytron tissue homogenizer. The supernatant obtained from a 15-min, 700*g* centrifugation (RC-5 Sorvall centrifuge) of this homogenate was filtered through cheesecloth and recentrifuged at 10000*g* for 25 min. The microsomes in this second supernatant were isolated by centrifugation at 100000*g* (Beckman L8-M ultracentrifuge) for 60 min. The combined pellets were washed by resuspension in SKTM and isolated by a second 60-min 100000*g* centrifugation. The combined microsomal pellets were suspended in SKTM buffer to a final protein concentration of 10 mg/mL. No loss of dehydrogenase or isomerase activity in the suspended microsomes was observed over several weeks upon storage in 20-mL aliquots at −80 °C.

The 3β-hydroxy-$\Delta^5$-steroid dehydrogenase and 3-keto-$\Delta^5$-steroid isomerase activities were partially purified from the bovine adrenal cortex microsomes by a modification of the procedure for the rat adrenal enzymes (Ishii-Ohba et al., 1986b). Since dehydrogenase and isomerase activities are not separated by these procedures, rapid screening of solutions and column fractions for the desired activities could be accomplished by monitoring for the 3-keto-$\Delta^5$-steroid isomerase activity. While being continuously stirred, aliquots of 5% sodium cholate (prepared in pH 7.5 20 mM potassium phosphate containing 20% glycerol, 100 μM EDTA, 10 μM $NAD^+$, and 0.1 mM dithiothreitol) were slowly added to the suspension of microsomes giving a final concentration of 0.6% cholate. After this was stirred for an additional 30 min, the solubilized proteins were isolated by centrifugation for 60 min at 100000*g*. The resulting supernatant contained greater than 90% of the 3-keto-$\Delta^5$-isomerase activity. This solution was adjusted to 20% glycerol and then applied to a 60-mL column of DEAE-Sepharose CL-6B equilibrated with 20 mM potassium phosphate buffer, pH 7.5, containing 20% glycerol, 0.1 mM EDTA, 0.2% sodium cholate, 0.1 mM dithiothreitol, and 0.01 mM $NAD^+$. The column was washed successively with an additional 125–150 mL of the equilibration buffer, 200 mL of the same solution containing 0.4% Emulgen 913, and an additional 200–300 mL of buffer containing 1.0% Emulgen 913. 3β-Hydroxy-$\Delta^5$-steroid dehydrogenase and 3-keto-$\Delta^5$-steroid isomerase activities coeluted just prior to the 1% Emulgen 913 wash. The combined fractions containing the desired activities were concentrated by ultrafiltration using an Amicon PM-10 membrane to a protein concentration of 1 mg/mL. Aliquots (1 mL) were stored at −80 °C; no loss of enzyme activity was observed for up to 1 year.

*Enzyme Assays and Inhibition Studies.* Activity of the 3-keto-$\Delta^5$-isomerase was monitored at 248 nm ($\epsilon$ = 16 300 $M^{-1}$ $cm^{-1}$) (Ishii-Ohba et al., 1986a,b) corresponding to formation of a conjugated 3-keto-4-ene steroid from 5-pregnene-3,20-dione in the presence of 10 μM $NAD^+$ or NADH. Enzyme (5 μg of protein) was equilibrated at 30 °C for 5 min in 100 mM potassium phosphate, pH 7.5, with 20% glycerol, 0.1 mM EDTA, and nicotinamide adenine dinucleotide prior to initiation of activity by the addition of substrate in acetonitrile. The content of acetonitrile in the cuvette, which did not exceed 2% of the final 0.5-mL incubation volume, caused no inhibition of enzyme activity. Initial rates of 3-keto-4-ene formation were linear over a 5-min time period. With pregnene-3,20-dione as substrate, the specific activity for the isomerase was determined to be 1 μmol/(min·mg). For inhibition studies,

compounds were added to the cuvette as acetonitrile solutions within the aforementioned limits.

3β-Hydroxy-$\Delta^5$-steroid dehydrogenase activity was monitored by employing several different substrates. Dehydroepiandrosterone (DHEA) and pregnenolone were utilized for activities which were coupled to the isomerase. 5α-Androstane-3β,17β-diol (ADIOL) and 5α-dihydrotestosterone (DHT) were used in assays to decouple the dehydrogenase activity from that of the isomerase. For all assays, radiolabeled substrates (and inhibitors when so designated) in ethanol were deposited in test tubes, and the solvent was removed in a Savant rotary evaporator. Buffer (100 mM potassium phosphate, pH 7.5), cofactors ($NAD^+$ or NADH), and enzyme were then added to a final volume of 0.5 mL. All incubations were conducted at 30 °C and were initiated by addition of enzyme which had previously been diluted in the incubation buffer containing 10 mg/mL BSA to stabilize the activity. Nonvarying substrate concentrations were maintained at 1.0 μM steroid and 125 μM cofactor. Following a 10-min incubation, the assay was quenched with 4 mL of ethyl acetate containing 0.15 μmol each of unlabeled substrate and product as carrier steroid. The resulting radiolabeled steroids were isolated and analyzed on a Bioscan imaging scanner, as previously described (Levy et al., 1987). When coupled to the isomerase, the total dehydrogenase activity was taken as the sum of the 3-keto products (3-keto-5-ene and 3-keto-4-ene steroids). As specified in the text, a cofactor regenerating system (NADH → $NAD^+$) consisting of lactate dehydrogenase (2.5 μg; 1.9 units) with a final concentration of 5 mM pyruvate was added to the incubations. All incubations were set up such that no more than 15% of initial concentration of substrates was consumed. A specific activity was determined for the 3β-hydroxy-$\Delta^5$-steroid dehydrogenase of 52 nmol/(min·mg) with pregnenolone as substrate.

*Dependence of Kinetic Parameters on pH.* A constant ionic strength buffer ($\mu$ = 0.10 M) consisting of succinic acid, imidazole, and diethanolamine (0.33:0.44:0.44) (Ellis & Morrison, 1982) was prepared as a stock solution. Aliquots of this stock buffer were adjusted to final pH with HCl or NaOH and then diluted to a working solution of $\mu$ = 0.01 M. Enzyme activities for the 3β-hydroxy-$\Delta^5$-steroid dehydrogenase were determined with [$^{14}$C]DHT as the variable substrate with a constant concentration of 5 μM NADH in a final incubation volume of 0.5 mL. The effects of pH upon enzyme inhibition by **1b** were performed at constant concentrations of [$^{14}$C]DHT (1.0 μM) and NADH (5 μM) while concentrations of the inhibitor were varied. The 3-keto-$\Delta^5$-steroid isomerase assay utilized 5-progesterone as substrate containing 10 μM NADH. Additional details of the assays followed the procedures described above, with activities normalized to the amount of enzyme used per assay. Initial velocity data were fitted with the HYPER program (Cleland, 1979); apparent inhibition constants at each pH were determined by Dixon analysis (Dixon, 1953). The pH dependence of $V_m$, $V_m/K_m$, and $1/K_{i,app}$ were fit to eq 4 and 5 by the BELL and HBBELL programs (Cleland, 1979, 1982).

*Determination of Equilibrium Constant between ADIOL and DHT.* Reactions to determine the equilibrium constant with ADIOL and DHT were conducted at 30 °C in 0.5 mL of 100 mM potassium phosphate, pH 7.5, containing 0.5–2.0 μM [$^3$H]ADIOL [(0.2–2.0) × $10^6$ dpm] or [$^{14}$C]DHT [(0.5–2.0) × $10^5$ dpm] and 0.5–5.0 μM cofactor. Aliquots (50 μL) were removed at 5-min intervals and, following separation by TLC, were analyzed for their DHT:ADIOL ratio. Equilibrium was achieved within 10 min with 10 μg of enzyme and within 20 min with 2.5 μg of enzyme. The final concentration of $NAD^+$ and NADH was determined from the change in the steroid concentration where the stoichiometry of steroid and cofactor products formed from the initial conditions was assumed to be equal.

*Isotopic Steroid Exchange at Equilibrium.* Reactions were performed at 30 °C in 100 mM potassium phosphate containing 10 mg/mL BSA, pH 7.5, in a total volume of 2 mL. All steroids were deposited in test tubes, as described for the enzyme assays, prior to addition of the other reaction components. Concentrations of one substrate/product pair (either steroid or nicotinamide) were varied while an equilibrium ratio was maintained (DHT/ADIOL = 1.0; $NAD^+$/NADH = 10.0); the concentration of the other substrate/product pair was held constant at its resulting equilibrium ratio, thereby maintaining chemical equilibrium. When the steroid exchange rates upon varying total cofactor concentration were determined, the concentrations of ADIOL and DHT were held constant at 1 μM each. Following the addition of enzyme to a final concentration of 4 μg/mL, the samples were incubated for 20 min to ensure that redistribution of the radiolabeled compound did not result from net synthesis. Radioisotope exchange was initiated upon addition of 5 μL of [$^3$H]DHT (250 000 dpm). The quantity of isotope added accounted for an insignificant amount of the total steroid present. Aliquots (400 μL) were removed every 30 s and immediately added to a mixture of 4.0 mL of ethyl acetate and 0.5 mL of 100 mM potassium phosphate, pH 7.5, containing unlabeled carrier steroids. The samples were vortexed, and the layers were separated by low-speed centrifugation. The ethyl acetate was transferred to a second test tube and concentrated to dryness in the Savant rotary evaporator. The residue, solubilized in a minimum volume (50 μL) of chloroform, was applied to plastic-backed silica TLC plates which were developed twice in chloroform–acetone (9:1). The radioactive DHT and ADIOL, located with a Bioscan imaging scanner, were cut from the plates and placed in scintillation vials. Following the addition of 2 mL of ethyl acetate and 10 mL of Econofluor II, the radioactive content of the isolated compounds was determined.

*Isotopic Cofactor Exchange at Equilibrium.* The procedures to determine cofactor exchange at equilibrium were modeled after those described by Schultz et al. (1977). All reactions of cofactor exchange were performed as described for the steroid exchange except the incubations were carried out in 0.5 mL of 10 mM potassium phosphate, pH 7.5. Redistribution of the radiolabel was initiated by the addition of 10 μL of [$^3$H]$NAD^+$ (500 000 dpm). Aliquots (100 μL) were removed every 2.5 min and quenched into a mixture of 4 mL of chloroform and 600 μL of 10 mM ammonium bicarbonate. A 0.5-mL sample of the aqueous layer was applied to a column of DE-52 (0.7 × 0.3 cm) which had been equilibrated with 2.0 mL of 10 mM ammonium bicarbonate. $NAD^+$ was eluted from the column in a 2.0-mL wash with the equilibration buffer; a second wash with 2.5 mL of 500 mM ammonium bicarbonate removed the NADH from the resin. To each sample was added 15 mL of Aquasol 2 for determination of radiochemical content. A parallel set of control reactions not containing steroid were performed to compensate for the cofactor affinity to protein coeluting from the DE-52. No exchange was observed in the absence of substrates or enzyme.

*Calculation of Exchange Velocity.* The exchange rate ($R$) was calculated by eq 1 describing the first-order process for

$$R = \frac{-[A][C]\{\ln (1 - F)\}}{t\{[A] + [C]\}} \quad (1)$$

isotopic exchange in which [A] and [C] are the concentrations of the reactants involved in the exchange reaction, $F$ is the fraction of theoretical isotopic equilibrium obtained, and $t$ is the reaction time to termination of exchange (in minutes) following the addition of the tracer (Boyer, 1959; Jomain-Baum & Schramm, 1978).

*Data Processing.* Data from initial velocity, dead-end inhibition, product inhibition, and multiple inhibition experiments were fit to appropriate rate equations whenever possible with the Fortran programs described by Cleland (1977, 1979). The EQORD program used to fit initial velocity ($v$) data for the 3$\beta$-hydroxy-$\Delta^5$-steroid dehydrogenase is described by eq 2,

$$v = \frac{V_m AB}{K_{ia}K_b + K_b A + AB} \quad (2)$$

where $A$ and $B$ are the reactant concentrations of the two substrates, $V_m$ is the maximum velocity, $K_{ia}$ is the dissociation constant of A from its binary enzyme complex, and $K_b$ is the Michaelis constant for substrate B. Multiple inhibition experiments were fit to eq 3 (Yonetani & Theorell, 1964;

$$v_i = v_0/[1 + I/K_i + J/K_j + IJ/(\beta K_i K_j)] \quad (3)$$

Northrop & Cleland, 1974) with SUPERFIT, an in-house curve fitting routine.[2] For eq 3, $I$ and $J$ are the concentrations of the two inhibitors, $v_0$ and $v_i$ are the velocities in the absence and presence of compounds I and J whose respective dissociation constants are $K_i$ and $K_j$, and $\beta$ is an experimentally derived term that represents the degree of binding cooperativity between the two inhibitors. Data for pH profiles that decreased with a slope of 1 at both low and high pH were fitted by eq 4 (BELL); profiles that decreased with a slope of 1 at high pH were fitted by eq 5 (HBBELL) (Cleland, 1982). In eq 4

$$\log y = \log [C/(1 + H/K_a + K_b/H)] \quad (4)$$

$$\log y = \log [C/(1 + K_b/H)] \quad (5)$$

and 5, $K_a$ and $K_b$ are the dissociation constants at low and high pH, respectively, of functional groups involved in catalysis or binding, $H$ is the hydrogen ion concentration, and $C$ is the pH-independent value of $y$, the parameter being fitted. All other equations have been previously described (Levy et al., 1987). The patterns summarized under Results are those to which the data were best fit according to previously established criteria (Cleland, 1979).

## Results

*Initial Velocity Kinetics.* Initial velocity kinetic constants and kinetic patterns for the 3$\beta$-hydroxy-$\Delta^5$-steroid dehydrogenase were determined with several steroid substrates; DHEA, pregnenolone, and ADIOL were used to monitor 3$\beta$-hydroxyl oxidation, and DHT was employed to evaluate the corresponding reverse reaction of 3-keto reduction. To evaluate the interactions of inhibitors with the dehydrogenase independent of 3-keto-$\Delta^5$-steroid isomerase activity, compounds not containing unsaturation at C-5 (ADIOL and DHT) proved to be convenient substrates. Use of these steroids allowed for kinetic evaluation in both catalytic directions.

Since the products formed by the 3$\beta$-dehydrogenase from DHEA and pregnenolone are substrates for the 3-keto-$\Delta^5$-steroid isomerase, total 3$\beta$-hydroxy-$\Delta^5$-steroid dehydrogenase activity was taken to be the sum of the resulting 3-keto $\Delta^5$- and $\Delta^4$-steroids. Each of these products was separable by TLC and was identified by comigration with authentic compounds. With both of these 3$\beta$-hydroxy $\Delta^5$-steroid substrates, the conjugated ketone represented the major product. Upon initial evaluation of the 3-hydroxy $\Delta^5$-substrates, nonlinear initial velocity patterns were observed. This deviation from linearity was shown to result from product inhibition by NADH and was overcome by inclusion of a cofactor regenerating system (NADH → NAD$^+$).

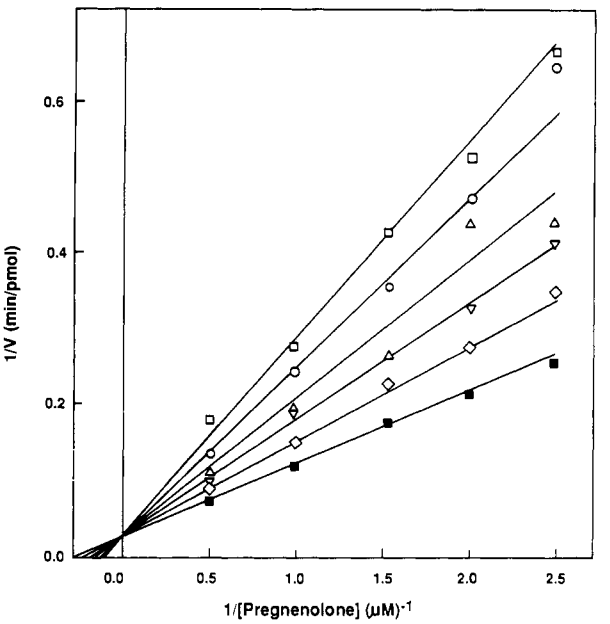



FIGURE 1: Initial velocity pattern for 3$\beta$-hydroxy-$\Delta^5$-steroid dehydrogenase. The activity of bovine adrenal 3$\beta$-hydroxy-$\Delta^5$-steroid dehydrogenase (0.13 $\mu$g) was determined at varying concentrations of pregnenolone and NAD$^+$ at pH 7.5; the data were analyzed by eq 2 as described in the text. The concentrations of NAD$^+$ are 30 (□), 37.5 (○), 50 (△), 65 (▽), 90 (◇), and 150 $\mu$M (■).

Table I: Kinetic Constants for Substrates with 3$\beta$-Hydroxy-$\Delta^5$-steroid Dehydrogenase

| steroid | cofactor | $K_{ia}$ ($\mu$M)[a] | $K_b$ ($\mu$M)[a] | $V_{max}$ [nmol/(min·mg)] |
|---|---|---|---|---|
| DHEA | NAD$^+$ | 132 ± 18 | 0.6 ± 0.1 | 80 ± 5 |
| ADIOL | NAD$^+$ | 116 ± 31 | 0.5 ± 0.1 | 120 ± 8 |
| pregnenolone | NAD$^+$ | 122 ± 13 | 1.9 ± 0.3 | 54 ± 4 |
| DHT | NADH | 2.8 ± 0.5 | 1.4 ± 0.4 | 76 ± 11 |

[a] The values of $K_{ia}$ and $K_b$ were obtained by fitting the initial velocity data to eq 2 as described in the text. For these analyses, the cofactor is represented by $K_{ia}$ and the steroid substrate by $K_b$.

Data obtained by varying the concentrations of both steroid and nicotinamide cofactor were analyzed by the SEQUEN, PINGPNG, and EQORD programs described by Cleland (1979). The initial velocity patterns of all four steroid substrates were best fit to the equilibrium ordered model (EQORD, eq 2). As required by this nonsymmetrical model, the point of intersection of the curves moved from left of the ordinate to upon the axis (Figure 1) when the reciprocal of substrate concentration, plotted upon the abscissa, was changed from A (NAD$^+$) to B (3$\beta$-hydroxy steroid). The kinetic constants derived from the analyses for the four steroids are summarized in Table I. The low dissociation constant determined for NADH ($K_{ia}$ = 2.8 ± 0.5 $\mu$M) when DHT is the steroid substrate ($K_b$ = 1.4 ± 0.4 $\mu$M) correlates with the product inhibition observed when the reaction is monitored in the opposite direction.

[2] SUPERFIT is a numerical analysis system based upon the SAS software package (SAS Institute, Inc.) which has been developed at Smith Kline & French Laboratories. Fitting of experimental data to nonlinear models utilizes the Marquardt algorithm (Marquardt, 1983).

Table II: Inhibition of 3β-Hydroxy-Δ⁵-steroid Dehydrogenase by 4-Aza-3-keto Steroids (**1**)

| compound | $R_1$ | $R_2$ | double bond | percent inhibition[a] | $K_{i,app}$ (nM)[b] |
|---|---|---|---|---|---|
| **1a** | $-CH(CH_3)CH_2OH$ | $-CH_3$ | | 97 | 30 |
| **1b** | $-C(O)N[CH(CH_3)_2]_2$ | $-CH_3$ | | 98 | 34 |
| **1c** | $-C(O)N[CH(CH_3)_2]_2$ | $-CH_3$ | | 88 | 56 |
| **1d** | $-CH(CH_3)CO_2H$ | $-CH_3$ | | 78 | 233 |
| **1e** | $-CH(CH_3)CH_2OH$ | $-CH_3$ | $\Delta^{5(6)}$ | 26 | 445 |
| **1f** | $-CH(CH_3)CH_2OH$ | $-H$ | $\Delta^{5(6)}$ | 21 | >1000 |
| **1g** | $-C(O)NHC(CH_3)_3$ | $-H$ | $\Delta^{1(2)}$ | 10 | >5000 |

[a] Percent inhibition represents the total amount of Δ⁵- and Δ⁴-progesterone isomers formed in the presence of 1 μM compound **1** relative to control without inhibitor; substrate concentrations were 1 μM pregnenolone and 125 μM $NAD^+$. [b] The apparent inhibition constants ($K_{i,app}$) were obtained as described in the text from Dixon analyses (Dixon, 1953) at constant concentrations of pregnenolone (1 μM) and $NAD^+$ (125 μM).

As previously reported for the bovine (Neville & Engel, 1968) and rat (Ishii-Ohba et al., 1986b) adrenal 3-keto-Δ⁵-steroid isomerases, $NAD^+$ and NADH were found to act as positive effectors for 3-keto-5-ene to 3-keto-4-ene conversion. No significant difference in catalytic efficiency was observed for the isomerization of 5-pregnene-3,20-dione in the presence of an invariable concentration (10 μM) of $NAD^+$ [$V_m/K_m$ = 11.6 nmol·(mg·min·μm)$^{-1}$] or NADH [$V_m/K_m$ = 11.4 nmol·(mg·min·μm)$^{-1}$]. By following the reduction in absorbance at 340 nm corresponding to concomitant oxidation of NADH, it was concluded that minimal reduction of 5-pregnene-3,20-dione to pregnenolone occurs during the 5-min incubation evaluating 3-keto-Δ⁵-steroid isomerase activity with this effector. This specificity results from the higher turnover rate of the isomerase relative to that of the dehydrogenase. Upon evaluation of initial velocity data with the HYPER program, the $K_a$ values for 5-pregnen-3,20-dione in the presence of 10 μM $NAD^+$ and NADH were determined to be 28 ± 15 μM ($n$ = 6) and 28 ± 13 μM ($n$ = 3), respectively.

*Equilibrium Constant between ADIOL and DHT.* The equilibrium constant between DHT/NADH and ADIOL/$NAD^+$ in the presence of the 3β-hydroxy-Δ⁵-steroid dehydrogenase was determined to be 0.10 ± 0.02 for an average of 13 experiments. There was no significant difference in this value upon initiating equilibrium from either direction of reaction or upon varying the amount of enzyme.

*Isotope Exchange at Equilibrium.* For exchange of both the ADIOL/DHT (B/C, Scheme I) and the $NAD^+$/NADH (A/D, Scheme I) pairs, the course of reaction was a first-order function both of time and of enzyme concentration. Enzyme concentrations and incubation times were adjusted in all isotope exchange experiments such that these linear relationships were maintained.

An increase in the reaction rate at equilibrium between ADIOL and DHT was observed upon increasing the total steroid concentrations (Figure 2). The maximum observed rate of this exchange, at constant concentration of $NAD^+$/NADH, was 50 pmol/min [12.5 nmol/(min·mg)]; solubility limits of the steroids precluded an extension of this curve to determine its upper plateau. The exchange rate between $NAD^+$ and NADH similarly increased with steroid concentrations up to 2 μM ADIOL but decreased upon further elevation of steroid levels. Even at the highest steroid concentrations, the $NAD^+$/NADH rate of exchange continued to decline, not having attained a plateau, having reached a minimum of 15% of the maximum rate at 15 μM ADIOL. Similarly, the steroid exchange rate upon varying cofactor concentrations, while maintaining constant levels of steroids, increased as a hyperbolic function to a constant value of 13.3 nmol/(min·mg). The half-maximum rate of steroid exchange upon varying cofactor concentrations ($NAD^+$/NADH = 10) was determined to occur at an $NAD^+$ concentration of 14 μM. These results indicate that the mechanism follows an ordered

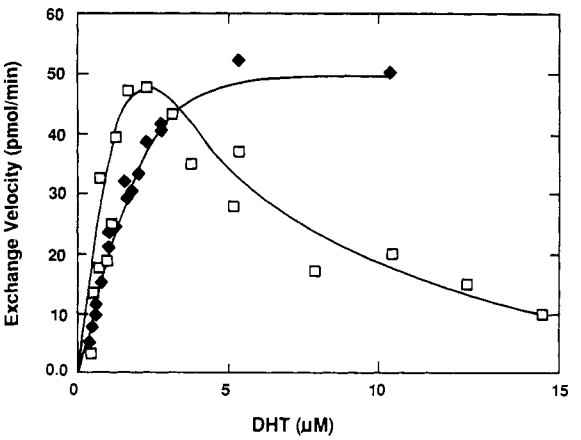



FIGURE 2: Effect of steroid concentration on 3β-hydroxy-Δ⁵-steroid dehydrogenase catalyzed exchange rates of $^3H$-labeled steroid and $^3H$-labeled cofactor at chemical equilibrium. Steroid (◆) and cofactor (□) exchange rates at equilibrium were determined at increasing total steroid concentration with the DHT/ADIOL concentration ratio maintained at 1:1. For all experiments, the cofactor concentrations were held at 10 μM $NAD^+$ and 1 μM NADH.

association of substrates with the nicotinamide adenine dinucleotide binding prior to the steroid.

*Inhibition of Enzyme Activities by 3-Oxo-4-aza Steroids.* Several 3-oxo-4-aza steroids (**1**) were found to be potent inhibitors of 3-hydroxy-Δ⁵-steroid dehydrogenase/3-keto-Δ⁵-steroid isomerase catalyzed formation of progesterone from pregnenolone. With each of the compounds investigated, a reduction in the total of dehydrogenase products (the sum of Δ⁵-pregnene-3,17-dione and progesterone) was observed with no change in the relative distribution of the Δ⁵- and Δ⁴-3-keto isomeric products. Since the relative amount of Δ⁵-pregnendione did not increase in the presence of the 4-aza steroids, the inhibition of progesterone formation appeared to originate from a preferred blockage of the dehydrogenase activity or an equivalent blockage of both activities. With pregnenolone as a substrate for the 3β-dehydrogenase, apparent inhibition constants were determined for compounds **1a**–**1g** (Table II). The fully saturated 3-oxo-4-aza steroids possessing a 4-methyl functionality, such as **1a**, **1b**, and **1c**, proved the more potent inhibitors ($K_{i,app}$ = 30–56 nM); introduction of an ionic functionality at C-17 (**1d**), unsaturation at C-5 or C-1 (**1e**, **1f**, and **1g**), and removal of 4-methyl (**1f** and **1g**) significantly decreased the interaction with the enzyme. When dehydrogenase activity was monitored independently of the isomerase with ADIOL, inhibition by **1a** and **1b** proved comparable to that with pregnenolone. Evaluation of dehydrogenase activity following a 750-fold dilution of enzyme preincubated with **1b** (600 nM) and $NAD^+$ (125 μM) demonstrated full recovery of activity; thus, inhibition of the 3β-hydroxy-Δ⁵-steroid dehydrogenase by **1** is reversible. Neither time-dependent inhibition nor slow-binding phenomenon was observed with **1a**, **1b**, or **1g**.

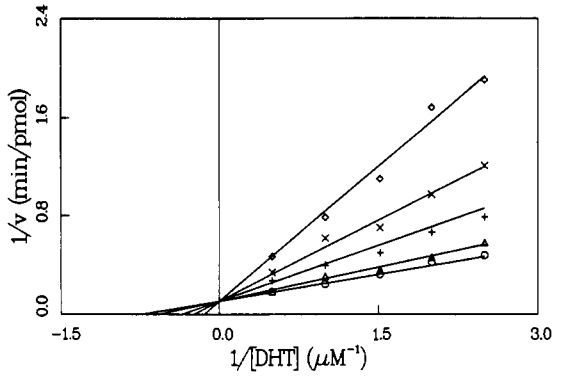



FIGURE 3: Dead-end inhibition pattern of 3β-hydroxy-Δ⁵-steroid dehydrogenase with **1b** versus DHT. 3β-Hydroxy-Δ⁵-steroid dehydrogenase activity (0.2 μg) was assayed with DHT as variable substrate in the presence of 125 μM NADH. The concentrations of inhibitor **1b** were 0 (○), 2 (△), 8 (+), 15 (×), and 30 nM (◇). The data were analyzed by the COMP program.

As demonstrated with **1a** ($K_{is}$ = 200 nM) and **1b** ($K_{is}$ = 230 nM) in the presence of 10 μM $NAD^+$, the 4-aza steroids were shown to block the 3-keto-Δ⁵-steroid isomerase catalyzed conversion of Δ⁵-pregnenedione to progesterone. As suggested above, the interaction of **1** and the isomerase, in the presence of $NAD^+$ as its positive effector, was considerably weaker than that of inhibitor with the dehydrogenase.

*Dead-End Inhibition Analysis.* When 3β-hydroxy-steroid dehydrogenase activity was monitored with the alcohol substrates (pregnenolone or ADIOL), the dead-end inhibition patterns of **1a** and **1b** versus steroid substrate fit best to an intersecting (noncompetitive) model; these plots were transformed to parallel (uncompetitive) patterns upon removal of NADH, a product inhibitor, by inclusion of a cofactor regenerating system. The inhibition constants derived for **1a** ($K_{ii}$ = 9 ± 1 nM) and **1b** ($K_{ii}$ = 17 ± 3 nM) versus ADIOL in the presence of the regenerating system demonstrate that inhibitor affinity for the dehydrogenase is greater than originally estimated by Dixon analysis (Table I). Comparable values were obtained with pregnenolone as substrate ($K_{ii}$ = 7 ± 2 nM and 15 ± 2 nM, respectively). By utilizing DHT as substrate to monitor inhibition in the reverse catalytic direction (ketone to alcohol), the dead-end inhibition patterns with **1a** ($K_{is}$ = 6 ± 1 nM) and **1b** ($K_{is}$ = 9 ± 1 nM) both proved to be competitive versus steroid (Figure 3).

Theoretical dead-end inhibition patterns for a reversible, ordered bisubstrate reaction (Segel, 1975) are presented in Table IIIB. Equating DHT (Table IIIA) with compound C (Table IIIB), the observed dead-end inhibition patterns with **1** would be consistent with the preferred association of **1** within an enzyme complex that contains NADH.

In order to confirm that the 4-aza compounds associate to a preferred enzyme complex (E–NADH), the cooperativity of binding between **1** and NADH was tested. Kinetic data obtained with the dehydrogenase at varying concentrations of NADH and **1**, while fixed concentrations of ADIOL (1.0 μM) and $NAD^+$ (125 μM) were maintained, were analyzed by eq 3. In this equation, the experimentally derived β term represents the degree of binding cooperativity between two inhibitors, I and J. The calculated value of β was determined to be less than one (β < 1) for both **1a** (β = 0.5) and **1b** (β = 0.7) (data not shown); hence, association of **1** and NADH to the enzyme is synergistic (Northrup & Cleland, 1974).

Several of the 3-oxo-4-aza steroids, including **1a** and **1b**, were shown to be competitive inhibitors versus steroid substrate with the 3-keto-Δ⁵-steroid isomerase. The same patterns were obtained with either $NAD^+$ or NADH as positive effectors for enzyme activity. However, in the presence of NADH, affinity of **1a** ($K_{is}$ = 37 ± 4 nM) and **1b** ($K_{is}$ = 50 ± 6 nM) for the isomerase was five to six times greater than that with $NAD^+$.

Table III: Dead-End Inhibition Patterns for 3β-Hydroxy-Δ⁵-steroid Dehydrogenase

| inhibitor | variable substrate[a] | fixed substrate[a,b] | kinetic pattern[c] |
|---|---|---|---|
| | (A) Experimental Results | | |
| **1a, 1b** | pregnenolone | $NAD^+$ | intersecting (NC) |
| **1a, 1b** | ADIOL | $NAD^+$ | intersecting (NC) |
| **1b** | pregnenolone | $NAD^+$ (+RS) | parallel (UC) |
| **1a, 1b** | ADIOL | $NAD^+$ (+RS) | parallel (UC) |
| **1a, 1b** | DHT | NADH | intersecting (C) |
| **1a, 1b** | $NAD^+$ (+RS) | ADIOL | parallel (UC) |
| **1b** | NADH | DHT | parallel (UC) |
| | (B) Theoretical Patterns for Ordered Mechanism[d] | | |
| I | A | B | parallel (UC) |
| I | B | A | parallel (UC) |
| I | C | D | intersecting (C) |
| I | D | C | parallel (UC) |

[a] The entry in parentheses (+RS) indicates that a cofactor regenerating system, as described under Methods, was included in the experiment. [b] As nonvariable substrate, the initial concentrations for the adenine dinucleotides and the steroids were 125 μM and 1 μM, respectively. [c] The kinetic patterns were analyzed by the computer programs described by Cleland (1977, 1979); the best fits of data to competitive (C), noncompetitive (NC), or uncompetitive (UC) models versus the variable substrate are as indicated. [d] The abbreviations for substrate and inhibitor refer to those used in Scheme III and correlate with the compounds in Scheme III: A = $NAD^+$, B = 3β-hydroxy steroid (ADIOL, pregnenolone), C = 3-keto steroid (DHT), D = NADH, and I = inhibitor (compound **1**).

*pH Dependence of Kinetic Constants for 3β-Hydroxy-Δ⁵-steroid Dehydrogenase and 3-Keto-Δ⁵-steroid Isomerase.* The pH dependence upon $V_m$ and $V_m/K_m$ has been investigated to determine the importance of ionizable groups in catalysis and steroid binding. As demonstrated in Figure 4A for the 3β-hydroxy-Δ⁵-steroid dehydrogenase, two ionizable groups ($pK_a$ = 5.6 ± 0.3 and $pK_b$ = 7.6 ± 0.3) were detected in the $V_m/K_m$ profile. A single protonation event with $pK_b$ = 8.2 ± 0.3 was shown to be involved in catalysis from the pH dependence on $V_m$ (Figure 4B). Similarly, the pH profile for inhibition of the 3β-hydroxy-Δ⁵-steroid dehydrogenase by **1b**, as depicted in Figure 4C, indicates that a single protonation event with $pK_b$ = 8.5 ± 0.2 enhances binding of inhibitor to the enzyme. The pH profiles upon $V_m$ ($pK_b$ = 8.3 ± 0.2), $V_m/K_m$ ($pK_a$ = 6.3 ± 0.3; $pK_b$ = 7.7 ± 0.2), and $1/K_i$ ($pK_b$ = 7.8 ± 0.2) for the 3-keto-Δ⁵-steroid isomerase were remarkably similar to those for the 3β-dehydrogenase (data not shown). Each inflection was best represented by single protonation/deprotonation events. No loss of enzyme activities was observed upon preincubation within the extreme pH ranges followed by dilution and activity assay at pH 7.5. Thus, the lower values for $V_m$ within the highest and lowest pH ranges do not result from irreversible denaturation of the enzymes.

## DISCUSSION

When both variable reactants are enzyme substrates, an equilibrium ordered initial velocity pattern originates from the rapid equilibrium addition of substrate to the enzyme.[3] Since

[3] An equilibrium ordered initial velocity pattern for a bisubstrate enzyme should be contrasted to the more commonly observed case in which one of the variable reactants is an activator, such as a metal cation, that is not consumed in the reaction. For these latter systems, the activator cannot dissociate from the enzyme upon binding of substrate and does not leave the enzyme between each catalytic cycle.

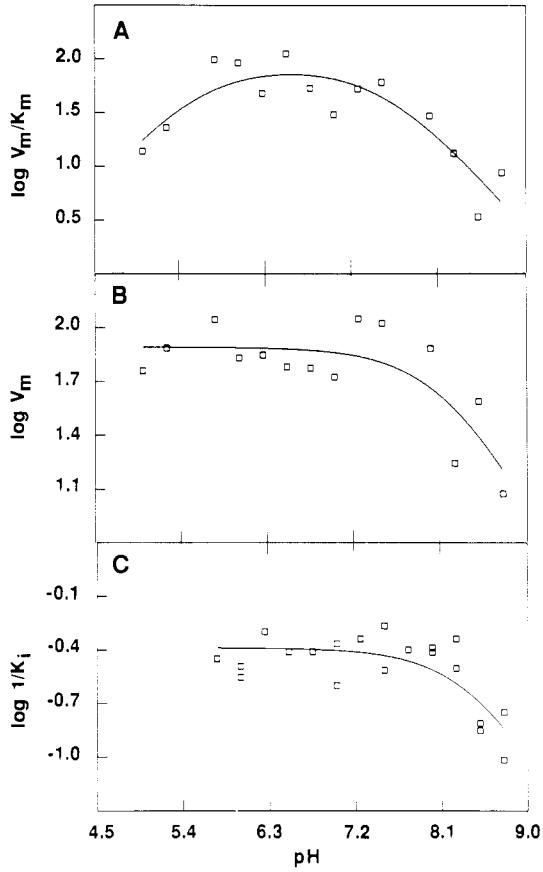
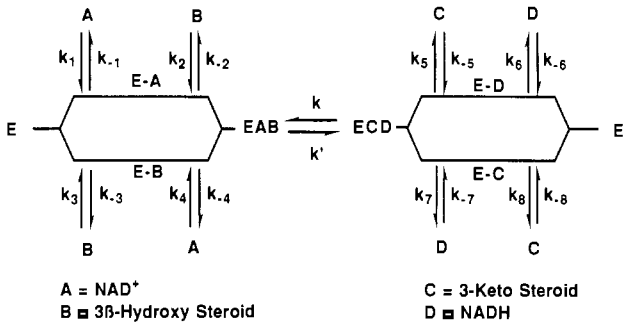



FIGURE 4: Variation of kinetic parameters of 3$\beta$-hydroxy-$\Delta^5$-steroid dehydrogenase upon pH. Apparent values of $V_m/K_m$ and $V_m$ for ADIOL and $1/K_i$ for the 3-oxo-4-aza steroid (**1b**) were determined throughout the indicated pH ranges in constant ionic strength buffer ($\mu$ = 0.01). The curve presented in (A), log ($V_m/K_m$) [nmol·(mg·min·$\mu$M)$^{-1}$] vs pH, was fit to eq 4 (BELL) and demonstrates inflection points at pH 5.6 and 7.6; the curves in (B), log $V_m$ [nmol·(mg·min)$^{-1}$] vs pH, and (C), log ($1/K_i$) (nM$^{-1}$) vs pH, were fitted to eq 3 (HBBELL) with single inflection points at pH 8.2 and 8.5, respectively.

the association of substrate A to enzyme (E) is at equilibrium, the Michaelis constant for substrate A ($K_a$) at saturating concentration of second substrate (B) must be half the enzyme level, or essentially zero; a fit to the equilibrium ordered model is observed only if release of A from the E–A complex is faster than interconversion of the internal complexes (EAB to ECD) and release of the first product. A true equilibrium ordered mechanism is rare since fully ordered mechanisms are not common and since the first substrate to add is often a slowly dissociating molecule (Cleland, 1977). In practice, such a pattern indicates that $K_a$ is immeasurably small compared to $K_{ia}$ such that the sequential model reduces to that for equilibrium ordered (eq 2). Thus, for the 3$\beta$-hydroxy-$\Delta^5$-steroid dehydrogenase, the rate constant for dissociation of enzyme and the first substrate bound (E–A) appears greater than the maximum enzyme velocity.

To confirm the ordered kinetics of the 3$\beta$-hydroxy-$\Delta^5$-steroid dehydrogenase, isotope exchange at equilibrium was undertaken. Because of the low values of the Michaelis constants (Table I) for the substrates, isotope exchange technically was easier to carry out than the determination of product inhibition patterns would have been for elucidating the order of substrate binding to the enzyme. In addition, such results distinguish more readily between compulsory, preferred, or random orders of substrates binding (Cleland, 1970). To avoid interference of the reaction catalyzed by 3-keto-$\Delta^5$-steroid isomerase with any 3-oxo $\Delta^5$-steroid, ADIOL and DHT were selected as the steroid substrates. In this way, the only radiolabeled steroids

Scheme I




detected were those directly involved in the equilibrium exchange reaction.

Compulsory binding of cofactor prior to steroid (upper pathway, Scheme I) requires that upon increasing the concentration of the steroid pair the rate of ADIOL (B) and DHT (C) interconversion would increase to a constant level whereas the cofactor exchange rate should decrease to zero. If the ordered pathway is preferred, increasing concentrations of the steroid pair should decrease the cofactor exchange rate to a constant level. For random binding of substrates such that either might bind to the enzyme in the absence of the other (Scheme I, upper and lower paths), the exchange rate of neither cofactor nor steroid would be depressed by elevated concentrations of the other (Boyer & Silverstein, 1963).

The results of isotope exchange at equilibrium, presented in Figure 2, are consistent with the ordered binding of cofactor prior to steroid dominating the kinetic mechanism. Although the rate of cofactor exchange was not forced to zero, the decline in this velocity continued even at the most elevated levels of steroid that were technically attainable. Thus, while the ordered binding of substrates is demonstrated to be preferred, if higher steroid concentrations could be attained, this association might be proven compulsory.

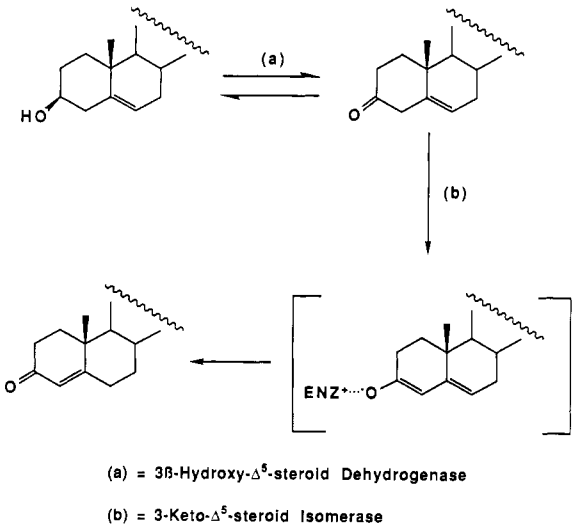
A comparison of exchange rates between steroid ($R$) and cofactor ($R'$) pairs at elevated substrate concentrations can be described by eq 6 (Silverstein & Boyer, 1964). When the

$$\frac{R'}{R} = \frac{1 + k'/k_{-7} + k/k_{-4}}{1 + k'/k_{-5} + k/k_{-2}} \qquad (6)$$

ratio of $R'/R$ is large, either $k_{-7}/k_{-5}$ or $k_{-4}/k_{-2}$ or both must be less than unity; hence, the dissociation of steroids must be faster than that of cofactors from their respective ternary complexes. The primary route for catalytic conversion thereby is suggested to be the upper pathways of Scheme I. This difference in exchange rates also implies that interconversion of the ternary complexes is not rate limiting but that binding or dissociation determines the reaction velocity. For chemical conversion to be the slow event, all exchanges would be limited by the same step (i.e., hydride transfer) such that $R$ and $R'$ would be equivalent. Similar results have been observed for rate-determining steps of other steroid dehydrogenases (Betz & Taylor, 1970; Schultz et al., 1977).

The potent inhibition of steroid 5$\alpha$-reductase by 3-oxo-4-aza steroids (**1**) as been rationalized to arise from the structural similarity of **1** to a $\Delta^3$-enolate intermediate generated during enzymatic catalysis (Rasmusson et al., 1983; Bertecs et al., 1984). Inhibition of 3-keto $\Delta^4$-steroid formation by members of the 3-oxo-4-aza steroid class might similarly result from **1** posing as the $\Delta^{3,5}$-dienolate intermediate proposed for the isomerization of 3-keto $\Delta^5$-steroids[4] (Scheme II) (Talalay &

Scheme II



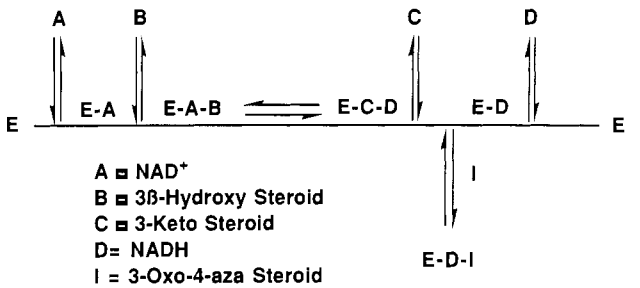

(a) = 3ß-Hydroxy-$\Delta^5$-steroid Dehydrogenase

(b) = 3-Keto-$\Delta^5$-steroid Isomerase

Benson, 1972; Smith & Brooks, 1977). The inhibition by 4-MA (**1c**) of progesterone biosynthesis from pregnenolone (Cooke & Robaire, 1986; Chan et al., 1987) and inhibition of reduction of the C-3 carbonyl of 4-androstene-3,6,17-trione by human placental microsomes (Numazawa et al., 1987) support this hypothesis. In addition, the observation that administration of several 3-oxo-4-aza steroids, including 4-MA, to dogs decreases prostatic testosterone concentrations (Brooks et al., 1982, 1986b) would be consistent with in vivo inhibition of pregnenolone biosynthesis. Such inhibition of steroid biosynthesis upstream from the divergence of paths to the steroid hormones could preclude therapeutic utility of compounds such as 4-MA. It might be expected that the physiological aberrations resulting from prolonged in vivo administration of a compound that inhibits the 3$\beta$-hydroxy-$\Delta^5$-steroid dehydrogenase or the 3-keto-$\Delta^5$-steroid isomerase would be similar to those characteristics for individuals with congenital adrenal hyperplasia originating from a deficiency of these enzyme activities (Bongiovanni, 1961).

Elucidation of the ordered addition of substrates to 3$\beta$-hydroxy-$\Delta^5$-steroid dehydrogenase enables interpretation of the observed dead-end inhibition patterns in Table III. Since **1a** and **1b** are competitive inhibitors of the 3-keto steroids, the kinetic patterns of the other three potential substrates, 3$\beta$-hydroxy steroid, $NAD^+$, and NADH, can be predicted; if **1** preferentially binds within a complex containing NADH, the patterns listed in Table IIIB would be expected. Equating the 3-keto steroid with substrate C in Table IIIB, it is observed that the experimental and theoretical patterns are the same. These results support the preferential binding of **1** to the 3$\beta$-hydroxy-$\Delta^5$-steroid dehydrogenase in the presence of NADH (Scheme III). The observation of a noncompetitive inhibition pattern of 4-MA versus pregnenolone with the 3-hydroxy-$\Delta^5$-steroid dehydrogenase/3-keto-$\Delta^5$-steroid isomerase from rat testis, in the absence of a cofactor regenerating system, also would be consistent with this proposed kinetic scheme (Cooke & Robaire, 1986).

Scheme III




The required protonation of the C-3 carbonyl in conversion of DHT to ADIOL (C to B in Scheme III) is, presumably, enzyme mediated. The dependence of catalysis upon an enzyme functionality with $pK_b$ = 8.2 (Figure 4B) could well represent protonation of the ketone by 3$\beta$-hydroxy-$\Delta^5$-steroid dehydrogenase. Coordination of the amide carbonyl of **1b** to this same functionality is supported by enhanced inhibitor binding upon protonation of a group with $pK_b$ = 8.5 (Figure 4C). An identical analysis can be made for inhibition of the 3-keto-$\Delta^5$-steroid isomerase by **1b**.

We have confirmed, herein, that compounds such as **1a–1c** are potent inhibitors of progesterone biosynthesis from pregnenolone. By decoupling the sequential activities of 3$\beta$-hydroxy-$\Delta^5$-steroid dehydrogenase and 3-keto-$\Delta^5$-steroid isomerase, both transformations (oxidation and isomerization) have been shown to be susceptible to inhibition by **1**, with a severalfold greater affinity of **1** for the dehydrogenase. Since conversion of the C-3 hydroxyl to the C-3 ketone is the rate-determining step for transformation of pregnenolone to progesterone (Marston et al., 1985), the 3$\beta$-hydroxy-$\Delta^5$-steroid dehydrogenase must be the primary site of inhibition by **1** within the coupled activities. We cannot, however, eliminate a scenario in which both catalytic activities originate from the same site. In fact, such a model could actually be supported both by the observed inhibition data demonstrating the preferential association of **1** to both 3$\beta$-hydroxy-$\Delta^5$-steroid dehydrogenase and 3-keto-$\Delta^5$-steroid isomerase in the presence of reduced nicotinamide cofactor and by the similarities in the pH profiles upon $V_m$, $V_m/K_m$, and $1/K_i$. It is interesting to note that an analogue of **1** [$R_1$ = $-C(O)CH_3$, $R_2$ = $-CH_3$], a potent inhibitor of rat pituitary steroid 5$\alpha$-reductase, is ineffective at inhibiting the pituitary 3$\alpha$-hydroxysteroid dehydrogenase (Bertics et al., 1984). Thus, not all 3-hydroxysteroid oxidoreductase activities are affected by **1**, and inhibition of 3$\beta$-hydroxy-$\Delta^5$-steroid dehydrogenase may result from its unique role of being coupled to the 3-keto-$\Delta^5$-steroid isomerase.

The binding to enzyme of amides, posing as substrate, product, or intermediate-state analogues, in the presence of reduced nicotinamide cofactor is not without precedent. For example, inhibition of alcohol dehydrogenase by fatty acid amides has been shown to require NADH (Winer & Theorell, 1960). In addition, 4-MA binds to steroid 5$\alpha$-reductase within an enzyme–inhibitor–NADPH ternary structure (Liang et al., 1984, 1985; Liang & Hess, 1981; Bertics et al., 1984). Such observations should prove important toward our ultimate goal of being able to design inhibitors which predictively interrupt catalysis by interacting with discrete enzyme forms. Work is currently in progress to expand our understanding of such interactions to other targents of interest.

ACKNOWLEDGMENTS

We thank Dr. Dennis Holt and Jill Erb for preparing compounds **1a–1g** and Dr. Frank Tobin for assisting in the analysis of data via SUPERFIT. We are grateful to Dr. Brian Metcalf

[4] Not all inhibitors of steroid 5$\alpha$-reductase which may pose as intermediate-state analogues interfere with catalysis by the 3$\beta$-hydroxy-$\Delta^5$-steroid dehydrogenase/3-keto-$\Delta^5$-steroid isomerase complex. A recently discovered new class of equally potent steroid 5$\alpha$-reductase inhibitors which could be envisioned as enolate mimics, $\Delta^3$-3-carboxy-17$\beta$-substituted androstenes, have been shown to interact only weakly ($K_{i,app}$ > 5 $\mu$M) with the 3$\beta$-hydroxy-$\Delta^5$-steroid dehydrogenase and the 3-keto-$\Delta^5$-steroid isomerase (M. A. Levy, M. Brandt, D. A. Holt, J. M. Erb, H.-J. Oh, J. I. Heaslip, and B. W. Metcalf, unpublished results).

for his critical review of the manuscript and for his continued support during this work.

**Registry No.** **1a**, 86283-92-3; **1b**, 73671-86-0; **1c**, 89631-78-7; **1d**, 86335-16-2; **1e**, 117773-66-7; **1f**, 117711-57-6; **1g**, 98319-26-7; DHEA, 53-43-0; ADIOL, 571-20-0; DHT, 521-18-6; NAD, 53-84-9; NADH, 58-68-4; pregnenolone, 145-13-1.

## References


Batzold, F. H., Benson, A. M., Covey, D. E., Robinson, C. H., & Talalay, P. (1976) *Adv. Enzyme Regul. 14*, 243–267.

Bertics, P. J., Edman, C. F., & Karavolas, H. J. (1984) *Endocrinology (Baltimore) 114*, 63–69.

Betz, G., & Taylor, P. (1970) *Arch. Biochem. Biophys. 137*, 109–114.

Bongiovanni, A. M. (1961) *J. Clin. Endocrinol. Metab. 21*, 860.

Boyer, P. D. (1959) *Arch. Biochem. Biophys. 82*, 387–410.

Boyer, P. D., & Silverstein, E. (1963) *Acta Chem. Scand. 17 (Suppl. 1)*, 5195–5202.

Bradford, M. (1976) *Anal. Biochem. 72*, 248–254.

Brooks, J. R., Berman, D., Glitzer, M. S., Gordon, L. R., Primka, R. L., Reynolds, G. F., & Rasmusson, G. H. (1982) *Prostate (N.Y.) 3*, 35–44.

Brooks, J. R., Berman, D., Primka, R. L., Reynolds, G. F., & Rasmusson, G. H. (1986a) *Steroids 47*, 1–19.

Brooks, J. R., Berman, C., Garnes, D., Giltinan, D., Gordon, L. R., Malatesta, P. F., Primka, R. L., Reynolds, G. F., & Rasmusson, G. H. (1986b) *Prostate (N.Y.) 9*, 65–75.

Chan, W. K., Fong, C. Y., Tiong, H. H., & Tan, C. H. (1987) *Biochem. Biophys. Res. Commun. 144*, 166–171.

Cheatum, S. G., & Warren, J. C. (1966) *Biochim. Biophys. Acta 122*, 1–13.

Cleland, W. W. (1970) *Enzymes (3rd Ed.) 2*, 1–65.

Cleland, W. W. (1977) *Adv. Enzymol. Related Areas Mol. Biol. 45*, 273–387.

Cleland, W. W. (1979) *Methods Enzymol. 63*, 103–138.

Cleland, W. W. (1982) *Methods Enzymol. 87*, 390–405.

Cooke, G. M., & Robaire, B. (1986) *J. Steroid Biochem. 24*, 877–886.

Dixon, M. (1953) *Biochem. J. 55*, 170–171.

Ellis, K. J., & Morrison, J. F. (1982) *Methods Enzymol. 87*, 405–426.

Ford, H. C., & Engel, L. L. (1974) *J. Biol. Chem. 249*, 1363–1368.

Ishii-Ohba, H., Inano, H., & Tamaoki, B.-I. (1986a) *J. Steroid Biochem. 25*, 555–560.

Ishii-Ohba, H., Saiki, N., Inano, H., & Tamaoki, B.-I. (1986b) *J. Steroid Biochem. 24*, 753–760.

Ishii-Ohba, H., Inano, H., & Tamaoki, B.-I. (1987) *J. Steroid Biochem. 27*, 775–779.

Jomain-Baum, M., & Schramm, V. L. (1978) *J. Biol. Chem. 253*, 3648–3659.

Levy, M. A., Holt, D. A., Brandt, M., & Metcalf, B. W. (1987) *Biochemistry 26*, 2270–2279.

Liang, T., & Heiss, C. E. (1981) *J. Biol. Chem. 256*, 7998–8005.

Liang, T., Heiss, C. E., Cheung, A. H., Reynolds, G. F., & Rasmusson, G. H. (1984) *J. Biol. Chem. 259*, 734–739.

Liang, T., Cascieri, M. A., Cheung, A. H., Reynolds, G. F., & Rasmusson, G. H. (1985) *Endocrinology (Baltimore) 117*, 571–579.

Marquardt, D. W. (1983) *J. Soc. Ind. Appl. Math. 11*, 431.

Marston, S. A., Waterman, M. R., & McCarthy, J. L. (1985) *J. Steroid Biochem. 22*, 789–794.

Neville, A. M., & Engel, L. L. (1968) *Endocrinology (Philadelphia) 83*, 864–872.

Northrop, D. B., & Cleland, W. W. (1974) *J. Biol. Chem. 249*, 2928–2931.

Numazawa, M., Tsuji, M., & Mutsumi, A. (1987) *J. Steroid Biochem. 28*, 337–344.

Oleinick, N. L., & Koritz, S. B. (1966) *Biochemistry 5*, 715–724.

Rasmusson, G. H., Liang, T., & Brooks, J. R. (1983) in *Gene Regulation by Steroid Hormones* (Roy, A. K., & Clark, J. H., Eds.) Vol. II, pp 331–334, Springer-Verlag, New York.

Rasmusson, G. H., Reynolds, G. F., Utme, T., Jobson, R. B., Primka, R. L., Berman, C., & Brooks, J. R. (1984) *J. Med. Chem. 27*, 1690–1701.

Rasmusson, G. H., Reynolds, G. F., Seinberg, N. G., Walton, E., Patel, G. F., Liang, T., Cascieu, M. A., Cheung, A. H., Brooks, J. R., & Berman, C. (1986) *J. Med. Chem. 29*, 2298–2315.

Schultz, R. M., Gromon, E. V., & Engel, L. L. (1977) *J. Biol. Chem. 252*, 3784–3790.

Segel, I. H. (1975) *Enzyme Kinetics*, Wiley, New York.

Silverstein, E., & Boyer, P. D. (1964) *J. Biol. Chem. 239*, 3908.

Smith, A. G., & Brooks, C. (1977) *Biochem. J. 167*, 121–129.

Talalay, P., & Wang, V. S. (1955) *Biochim. Biophys. Acta 18*, 300.

Talalay, P., & Benson, A. M. (1972) *Enzymes (3rd Ed.) 6*, 617–618.

Winer, A. D., & Theorell, H. (1960) *Acta Chem. Scand. 14*, 1729–1742.