Inhibition of 3(17)β-Hydroxysteroid Dehydrogenase from *Pseudomonas testosteroni* by Steroidal A Ring Fused Pyrazoles

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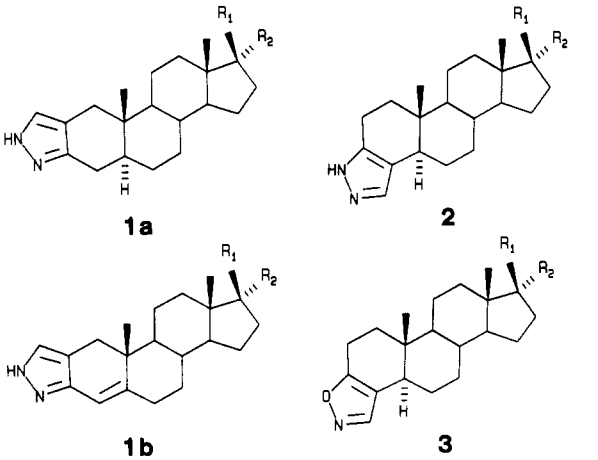
ABSTRACT: Several 2,3- and 3,4-steroidal fused pyrazoles have been investigated as potential inhibitors of NAD(P)H-dependent steroid oxidoreductases. These compounds are proven to be potent, specific inhibitors for 3(17)β-hydroxysteroid dehydrogenase from *Pseudomonas testosteroni* with K_i values of 6–100 nM. In contrast, the activities of 3α,20β-hydroxysteroid dehydrogenase from *Streptomyces hydrogenans*, steroid 5α-reductase from rat prostate, and 3α-hydroxysteroid dehydrogenase from rat liver were unaffected by micromolar concentrations of these compounds. Product and dead-end inhibition studies indicate an ordered association to the β-dehydrogenase with the cofactor binding prior to substrate or inhibitor. From the results of double inhibition experiments, it is proposed that inhibition occurs through formation of an enzyme–NAD^+–inhibitor ternate. On the basis of pH profiles of V_m/K_m, V_m, and $1/K_i$ and of absorbance difference spectra, a hypothetical mechanism of inhibition by the steroidal pyrazoles, drawn by analogy from the inhibition of liver alcohol dehydrogenase by alkylpyrazoles [Theorell, H., & Yonetani, T. (1963) *Biochem. Z. 338*, 537–553; Andersson, P., Kvassman, J. K., Lindström, A., Oldēn, B., & Pettersson, G. (1981) *Eur. J. Biochem. 113*, 549–554], is reconsidered. The pH studies and enzyme modification experiments by diethyl pyrocarbonate suggest the involvement of histidine in binding of the inhibitor. A modified proposal for the structure of the enzyme–NAD^+–steroidal pyrazole complex is proposed. In this ternate, the inhibitor bridges an enzymatic histidine and the nicotinamide ring of the NAD^+ through two hydrogen bonds: one formed between the lone pair of electrons of an imidazole nitrogen and the pyrazole N–H with the second between the lone pair of electrons of the second pyrazole nitrogen and the electropositive cofactor.

Thorough experimentation by Theorell and colleagues has found that the five-membered pyrazole ring is a potent inhibitor ($K_i = 2 \times 10^{-7}$ M) for the oxidation of alcohols by equine LADH[1] (Theorell et al., 1969) in which inhibition results from formation of a ternary complex with the oxidized cofactor, NAD^+, and the enzyme (Theorell & Yonetani, 1963). Pyrazoles substituted at the 4-position with a hydrophobic functionality are even more potent inhibitors than the parent compound (Theorell et al., 1969; Dahlbom et al., 1974; Tolf et al., 1979, 1982). Alcohol dehydrogenases from other sources such as rat liver (Reynier, 1969), human liver (Li & Theorell, 1969), and yeast (Singlevich & Barboriak, 1971) are inhibited less strongly by these compounds.

Spectrometric and titrimetric studies (Andersson et al., 1981; Eklund et al., 1982; Kvassman & Pettersson, 1980) indicate that formation of the ternary complex is associated with both the displacement of an active site, zinc-coordinated water molecule and the loss of 1 proton equiv. This evidence supports the original proposal by Theorell and Yonetani (1963) that the pyrazole coordinates to the active site zinc of LADH within a ternary complex. Deprotonation of this complex forms a delocalized pyrazole anion, stabilized through electrostatic interactions with the metal atom and the oxidized cofactor, with a characteristic difference absorbance at 290–310 nm (Shore & Gilleland, 1970). Crystallographic investigations of LADH binary and ternary complexes with pyrazole and cofactor at 2.9-Å resolution indicate that one pyrazole nitrogen (N-1) coordinates to the zinc, while the second (N-2) is in close proximity to C-4 of the nicotinamide. In this complex, the pyridine dinucleotide is twisted into a semiboat conformation (Eklund et al., 1982) reminiscent to that of the reduced cofactor, NADH. Using ^{15}N nuclear magnetic resonance, Becker and Roberts (1984) recently have proposed covalent attachment between the two positions with the pyridine ring having the dihydro structure resulting from addition of pyrazole to the 4-position of NAD^+.

Our interest in modulating specific pathways of steroid biosynthesis has led us to investigate an extension of this concept to NAD(P)H-dependent steroid oxidoreductases using steroidal pyrazoles as potential inhibitors. In particular, it was hoped that this concept could be extrapolated to target enzymes that are thought not to contain catalytically important metals such as the zinc in LADH. Saturated and unsaturated 2,3-steroidal fused pyrazoles such as **1a** (R_1 = β-OH, R_2 =



[1] Abbreviations: BSA, bovine serum albumin; T, testosterone; DHT, 5α-dihydrotestosterone; ADIOL, 3α,17β-androstanediol; ADIONE, androstenedione; LADH, liver alcohol dehydrogenase; DTT, dithiothreitol; DEPC, diethyl pyrocarbonate; TLC, thin-layer chromatography; SID, succinic acid, imidazole, and diethylamine ternary buffer system; μ, ionic strength of buffer; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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α-CH_3) have been reported to be inhibitors of 3(17)β-hydroxysteroid dehydrogenase from *Pseudomonas testosteroni* (Ferrari & Arnold, 1963a,b). In this paper, we report the specific inhibition of 3(17)β-hydroxysteroid dehydrogenase by 3,4-steroidal fused pyrazoles, **2**, and reinvestigate the specificity of inhibition induced by the 2,3-pyrazoles, **1**. Results of experiments designed to test a hypothetical mechanism of inhibition of 3(17)β-hydroxysteroid dehydrogenase of the most potent class of these inhibitors, the 3,4-fused pyrazoles (**2**), are described. These inhibitors, **2**, also are employed in studies to help characterize the mechanism of catalysis of the bacterial 3(17)β-hydroxysteroid dehydrogenase.

Experimental Procedures

Materials

[4-^{14}C]Testosterone (52 mCi/mmol), [1,2-^{3}H]-3α,17β-androstanediol (30 Ci/mmol), [9,11-^{3}H(N)]androsterone (53.5 Ci/mmol), [1,2-^{3}H(N)]androst-4-ene-3,17-dione (48.5 Ci/mmol), and Econosolve II were purchased from New England Nuclear. β-Hydroxysteroid dehydrogenase (EC 1.1.1.51) from *Pseudomonas testosteroni*, 3α,20β-hydroxysteroid dehydrogenase (EC 1.1.1.50) from *Streptomyces hydrogenans*, testosterone (T), androstenedione (ADIONE), 3α,17β-androstanediol (ADIOL), 5α-dihydrotestosterone (DHT), androsterone, androstanedione, dithiothreitol (DTT), bovine serum albumin (BSA), NAD^+, NADH, $NADP^+$, and NADPH were obtained from Sigma Chemical Co. Succinic acid, imidazole, diethanolamine, diethyl pyrocarbonate (DEPC), and all other chemicals were obtained from Aldrich Chemical Co. Protein concentrations were estimated by the method of Bradford (1976) with the Bio-Rad protein assay. Radioactivity was determined on either a Beckman LS-5801 scintillation counter, which was calibrated for correction to decompositions per minute (dpm) with Beckman ^{14}C and ^{3}H standards, or on a System 2000 BIOSCAN imaging scanner (BIOSCAN, Washington, DC). UV/vis spectra were recorded on a Hewlett-Packard 8450A spectrophotometer with diode array detector and disc drive for storage of data. 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, Baker). Livers and prostates of adult male Sprague-Dawley rats were removed, rinsed in cold 20 mM potassium phosphate buffer, pH 6.5, containing 330 mM sucrose and 1 mM DTT, and frozen at −80 °C until used for enzyme preparation.

Methods

Synthesis of Steroidal Pyrazoles. Syntheses of the 2,3-pyrazole (**1**) and the 3,4-pyrazole (**2**) fused steroids were carried out as described by Clinton et al. (1961) and Clinton (1969), respectively. The 3,4-isoxazole (**3**) was prepared by the method of Manson et al. (1963). All new compounds exhibited satisfactory NMR, IR, MS, and elemental analyses with melting points as follows: **1a**, 235–240 °C (lit. mp 229.8–242 °C); **1b**, 282–286 °C; **2a**, 254–260 °C; **2b**, 265–269 °C (lit. mp 259–269 °C); **2c**, 264–266 °C; **3**, 155–157 °C.

Enzyme Preparations and Assays. (A) 3(17)β-Hydroxysteroid Dehydrogenase. The commercially obtained 3(17)β-hydroxysteroid dehydrogenase from *P. testosteroni* was reconstituted in 20 mM potassium phosphate (pH 7.2) containing 20% glycerol and serially diluted in 10 mM phosphate, pH 7.2, containing 1–10 mg/mL BSA; the BSA was added to help maintain enzyme activity upon dilution. Diluted enzyme could be stored for several weeks at −20 °C.

Enzyme activity was determined by following the conversion of testosterone to androstenedione. [^{14}C]Testosterone in ethanol was deposited in test tubes and concentrated to dryness in a SAVANT Speed-Vac. NAD^+ and buffer were added to each tube. The reaction was initiated by introduction of the enzyme, to give a final reaction volume of 0.5 mL of 10 mM sodium pyrophosphate buffer, pH 8.9. After the tubes were incubated at 25 °C for 10 min, the reaction was terminated by the addition of 4 mL of ethyl acetate and 0.15 μmol each of testosterone and androstanedione as carrier. The organic layer was removed to a second test tube, and the solvent was evaporated in a Speed-Vac. The residue was redissolved in 20–30 μL of chloroform, spotted on 20 × 20 cm plastic-backed silica gel TLC plates, and developed with acetone–chloroform (1:9). The regions of the plates containing testosterone and androstanedione, as located by UV, were cut out, soaked in 2 mL of ethyl acetate, and counted in 10 mL of Econosolve II. The percent of recovered radiolabel converted to product was calculated, from which the enzyme activity was determined. All incubations were set up such that no more than 12% of the substrate was consumed. With this assay, the specific activity of the 3(17)β-hydroxysteroid dehydrogenase was 70 units/mg of protein.[2]

The reverse reaction was monitored by measuring the conversion of ADIONE to T in the presence of NADH. The procedure for this assay is the same as that described above for the forward reaction. Enzyme activity in the ADIONE to T direction at pH 8.9 was 5.0 units/mg of protein.

(B) 3α,20β-Hydroxysteroid Dehydrogenase. An aliquot of the *S. hydrogenans* 3α,20β-hydroxysteroid dehydrogenase solution provided as an ammonium sulfate suspension was pelleted by centrifugation. The residue was dissolved in 100 mM sodium phosphate, pH 7.5, containing 10 mg/mL BSA and was serially diluted with the same buffer immediately prior to use. Assays were performed as described above for the β-hydroxysteroid dehydrogenase except that [1,2-^{3}H]-3α,17β-androstanediol was used as the substrate and unlabeled ADIOL and 5α-dihydrotestosterone (product) were added as the carriers following the ethyl acetate quench. The residue from the reaction was spotted on prechanneled silica gel plates (Baker) and developed as above. Radiochemical content of the individual steroids was determined on a BIOSCAN imaging scanner. Enzyme activity was calculated from the percent of recovered radiolabel converted to DHT. The activity of this enzyme was determined to be 0.5 unit/mg of protein.

(C) 3α-Hydroxysteroid Dehydrogenase. 3α-Hydroxysteroid dehydrogenase from rat liver was prepared by the method of Penning et al. (1984) through the ammonium sulfate precipitation. The resulting precipitate was resuspended in 30 mL of buffer containing 1 mM EDTA, 1 mM DTT, and 10 mM Tris, pH 8.6. The solution was dialyzed 3 times against the suspension buffer and a fourth time against the same buffer containing 20% glycerol. Activity was determined as described for the 3(17)β-hydroxysteroid dehydrogenase, except that [9,11-^{3}H(N)]androsterone and $NADP^+$ were used as substrates and that unlabeled androsterone and androstanedione (product) were added during the quench. Activity of this enzyme preparation was 0.03 unit/mg of protein.

(D) Steroid 5α-Reductase. Rat prostatic microsomes were prepared as described by Blohm et al. (1980). Steroid 5α-reductase activity was determined as previously reported (Blohm et al., 1980) except that the incubations were run for 20 min at 37 °C and the reaction was quenched with 4 mL

[2] One unit of enzyme activity is defined as the conversion to product of 1 μmol of steroid/min.

of ethyl acetate. Steroids were separated by TLC in which the plates were developed twice under the same conditions as described above. This preparation had a specific activity of 54 pmol (mg of protein)$^{-1}$ h^{-1}.

Inhibition Experiments. Potential steroidal inhibitors in ethanol were added to test tubes at the same time as the steroid substrate, and the contents were evaporated to dryness. Cofactors were added following addition of the incubation buffer; all other procedures were as described above for the individual assays. Except as otherwise noted, the final concentration of NAD^+ or NADH in the assay solutions was kept constant at 400 μM. Initial estimates of inhibition constants were obtained by Dixon analyses (Dixon, 1953) with both substrate concentrations held constant. More complete analyses were performed as described below.

Dialysis of 3(17)β-Hydroxysteroid Dehydrogenase Inhibited by **2a**. A sample of 3(17)β-hydroxysteroid dehydrogenase (0.2 μg in 4 mL of 10 mM potassium phosphate, pH 7.2, containing 10 mg/mL BSA and 10 μM NAD^+) that had been incubated with 250 nM **2a** was dialyzed twice at 4 °C against 1 L of 10 mM potassium phosphate, pH 7.2, containing 1% glycerol and 10 μM NAD^+ over a 24-h period. A simultaneous control consisted of identical conditions with omission of the inhibitor **2a**. The dialyzed protein samples were diluted 1:5 with the same buffer used for dialysis and assayed for both protein content and enzyme activity. Enzyme activity relative to that prior to dialysis was determined.

UV/Vis Difference Spectrum of 3(17)β-Hydroxysteroid Dehydrogenase in the Presence of 3,4-Steroidal Pyrazole (**2a**) *and NAD$^+$.* Spectra of 3(17)β-hydroxysteroid dehydrogenase, NAD^+, and the 3,4-pyrazole, **2a**, were recorded both individually and after being mixed in the same solutions. The final enzyme concentration typically was 1 mg of protein (10 μM holoenzyme) in 1 mL of 10 mM potassium pyrophosphate buffer, pH 8.9. In a standard experiment, 10-μL aliquots of inhibitor in ethanol and/or cofactor in buffer were added to a cuvette containing the buffered enzyme to give the final incubation volume of 1 mL. The final concentrations of inhibitor and cofactor ranged from 1 to 50 μM. In no experiment did the concentration of added ethanol exceed 5% (50 μL) of the incubation volume. Recorded spectra were stored on disc, and difference spectra were obtained by computer-assisted subtractions.

Preparation of 3(17)β-Hydroxysteroid Dehydrogenase for the Determination of Zinc by Atomic Absorbance. A 3-mg sample of reconstituted enzyme in 0.2 mL was applied to a 1 × 50 cm column of Sephadex G-25 fine that had been equilibrated with 10 mM potassium pyrophosphate, pH 8.5. The column was washed with the same buffer; fractions of 1.0 mL were collected at rate of 0.25 mL/min. Protein elution was monitored at 260 nm. The fractions containing the protein were combined and determined to have a final concentration of 0.28 mg/mL, which corresponds to 2.8 μM tetrameric enzyme. A sample of this solution was analyzed for zinc by flame ionization atomic absorbance.

Dependence of Kinetic Parameters of 3(17)β-Hydroxysteroid Dehydrogenase on pH. A constant ionic strength three-buffer system (μ = 0.10 M) consisting of succinic acid, imidazole, and diethanolamine (SID) in molar ratios of 0.33:0.44:0.44, respectively (Ellis & Morrison, 1982), was prepared as a stock solution. Aliquots of this buffer were adjusted to a final pH with either HCl or NaOH and diluted to a working solution with μ = 0.01 M. Enzyme activities were determined with [^{14}C]testosterone varying from 0.36 to 1.83 μM at a constant concentration of NAD^+ (400 μM) in a final volume of 0.5 mL of SID buffer, μ = 0.01 M. The reaction was initiated by addition of the enzyme [(0.4–2.0) × 10^{-5} unit] to the otherwise complete solution, which had been preincubated at 27.5 °C. Following a 5-min incubation, the reaction was quenched and the activity determined as described above. Enzyme activities were calculated from the percentage of testosterone converted to androstenedione and were normalized to the amount of enzyme used in the assay. Initial velocity data were fitted to eq 1 with the HYPER program (Cleland, 1979). The pH dependence of V_m and V_m/K_m was analyzed by the BELL and HABELL programs as described by Cleland (1979, 1982).

The effects of pH upon the inhibition of the enzyme by the 3,4-pyrazole (**2a**) were performed as described above except that the concentration of [^{14}C]testosterone was held constant at 1.125 μM and inhibitor concentrations were varied from 2 to 100 nM. An apparent inhibition constant for the 3,4-pyrazole (**2a**) at each pH was determined by Dixon analysis (Dixon, 1953); the dependence of the values of $1/K_{i,app}$ upon pH was analyzed by the BELL and HABELL programs (Cleland, 1979, 1982).

Inhibition of 3(17)β-Hydroxysteroid Dehydrogenase by Diethyl Pyrocarbonate. DEPC (100 mM) in acetonitrile (CH_3CN) was prepared from a 6.9 M solution obtained commercially. These solutions were stored in a desiccator at 4 °C and were stable for at least 4 weeks. The concentration of DEPC was monitored during each experiment by diluting an aliquot to 1 mL with 10 mM imidazole or histidine, pH 7.5. The increase in absorbance at 242 nm, due to formation of *N*-carbethoxyimidazole (ϵ = 3200 cm^{-1} M^{-1}), was determined from the difference spectrum with a background solution containing an equal volume of CH_3CN. Dilutions of DEPC in CH_3CN were kept on ice prior to use.

The complete reaction solution (100 mM sodium phosphate, pH 6.7, containing 1 mg/mL BSA to stabilize the dehydrogenase plus cofactors, substrates and/or inhibitors) with enzyme and DEPC omitted was preincubated for 3–5 min at either 5.2 or 27.5 °C. Enzyme (0.57 μg), diluted in 10 mM potassium phosphate, pH 7.2, plus 10 mg/mL BSA, was added to bring the final volume to 300 μL. Following an additional 2–3-min incubation at the same temperature, 6 μL of CH_3CN or of 48 mM DEPC in CH_3CN was added to initiate the reaction. Total histidine concentration, as calculated from the amino acid compositions of BSA (MacGillivray et al., 1979) and of β-hydroxysteroid dehydrogenase (Schultz et al., 1977a), was 375 μM. At predetermined time intervals, a 20-μL aliquot was removed and immediately diluted into 500 μL of 10 mM histidine or imidazole, pH 7.5, containing 1 mg/mL BSA, at room temperature. An aliquot of the quenched mixture was assayed as described above in 10 mM sodium phosphate, pH 7.5, with 400 μM NAD^+ and 1.125 μM [^{14}C]testosterone at 27.5 °C. Enzyme activities relative to those at zero time (prior to the addition of either CH_3CN or DEPC) were calculated.

Changes in the UV Spectrum of 3(17)β-Hydroxysteroid Dehydrogenase upon Modification by DEPC. Several difference spectra (220–400 nm) of a sample of 3(17)β-hydroxysteroid dehydrogenase (0.125 mg/mL, 1.2 μM) in the presence of 300 μM DEPC at pH 7.2 (10 mM potassium phosphate) at 20 °C were recorded over 30 min with a diode array detector. No BSA was added to the solution upon which spectra were measured. All other procedures for this experiment were as described above.

Data Processing. Data from initial velocity, dead-end inhibition, product inhibition, and double inhibition experiments were fitted to the appropriate rate equation whenever possible

with the FORTRAN computer programs described by Cleland (1977, 1979). The points in the curves are the experimentally determined values; the curves are calculated fits of the data with the appropriate program. Data conforming to a straight line were fitted to the equation for a straight line ($y = mx + b$) with the LINE program. Kinetic studies involving only the variation of one substrate were computer fitted to eq 1 by the

$$v = V_m A/(K_a + A) \quad (1)$$

HYPER program. Initial velocity data obtained upon varying both structures were analyzed with eq 2 by the SEQUEN pro-

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

gram. Equation 3 (HYPRPLT program) was used for analysis

$$v = a(1 + V_m/K_{in})/(1 + V_m/K_{id}) \quad (3)$$

of hyperbolic initial velocity inhibition data. Velocities from inhibition experiments were fitted to eq 4–6 describing linear

$$v = V_m A/[K_a(1 + I/K_{is}) + A] \quad (4)$$

$$v = V_m A/[K_a(1 + I/K_{is}) + A(1 + I/K_{ii})] \quad (5)$$

$$v = V_m A/[K_a + A(1 + I/K_{ii})] \quad (6)$$

competitive (COMP), noncompetitive (NONCOMP), and uncompetitive (UNCOMP) inhibition, respectively. The patterns summarized under Results are those to which the data fit the best according to the criteria established by Cleland (1979). For eq 1–6, K_a and K_b are the apparent Michaelis constants for the individual substrates A and B, K_{ia} is the dissociation constant for substrate A, K_{is} and K_{ii} are the dissociation constants for the inhibitor derived from the slope and the intercept, respectively, of a double-reciprocal plot, K_{in} and K_{id} are the dissociation constants obtained from the two phases of the hyperbolic plot, A, B, and I are the reactant concentrations of substrates A and B and inhibitor I, respectively, and V_m is the maximum velocity. Data from double inhibition experiments at constant substrate concentrations were analyzed by eq 7 (Yonetani & Theorell, 1964; Northrop & Cleland,

$$v_i = v_0/[1 + I/K_i + J/K_j + IJ/(\beta K_i K_j)] \quad (7)$$

1974) in which v_0 and v_i are the velocities in the absence and the presence of the two inhibitors at concentrations of I and J whose respective dissociation contstants are K_i and K_j. The β term is an experimentally derived number that represents the degree of cooperativity between the two inhibitors. Data for pH profiles that decreased with a slope of 1 at low pH were fitted by eq 8 (HABELL) (Cleland, 1982). In eq 8, K_1 is the

$$\log y = \log [C/(1 + H/K_1)] \quad (8)$$

acid dissociation constant of an enzyme or substrate functional group that must be unprotonated for activity, H is the hydrogen ion concentration, and C is the pH-independent value of y, the parameter being fitted.

RESULTS

Enzyme Inhibition by Steroidal Fused Pyrazoles. The 2,3- and 3,4-steroidal fused pyrazoles (**1** and **2**) were found to be potent inhibitors of 3(17)β-hydroxysteroid dehydrogenase from *P. testosteroni* (Table I). The saturated 3,4-pyrazoles, **2a** and **2b**, were more potent inhibitors (K_i = 6–8 nM) than the 2,3-pyrazoles, **1** (K_i = 15–20 nM). Inhibition by compounds **1** and **2** was specific for the 3(17)β-hydrogenase; enzyme activity of neither steroid 5α-reductase from rat prostatic microsomes, nor cytosolic 3α-hydroxysteroid dehydrogenase from rat liver, nor 3α(20β)-hydroxysteroid dehydrogenase from

Table I: Inhibition of 3(17)β-Hydroxysteroid Dehydrogenase by Steroidal Ring Fused Pyrazoles

compound[a]	R_1[b]	R_2[b]	double bond	K_i (nM)[c]
1a	–OH	$-CH_3$		20 ± 4
1b	$-CH(CH_3)CH_2OH$	–H	Δ4,5	15 ± 3
2a	$-CH(CH_3)CH_2OH$	–H		7 ± 2
2b	–OH	$-CH_3$		6 ± 2
2c	$-CH(CH_3)CH_2OH$	–H	Δ1,2	100 ± 20
3	$-CH(CH_3)CH_2OH$	–H		190 ± 30

[a] Compounds **1** and **2** refer to the 2,3- and the 3,4-steroidal fused pyrazoles, respectively. [b] R_1 and R_2 represent the 17β- and the 17α-positions, respectively, of the steroidal system. [c] The K_i values were obtained, as described under Experimental Procedures, from either competitive inhibition or from Dixon analyses with testosterone as the variable substrate and with the concentration of NAD^+ held constant at 400 μM.

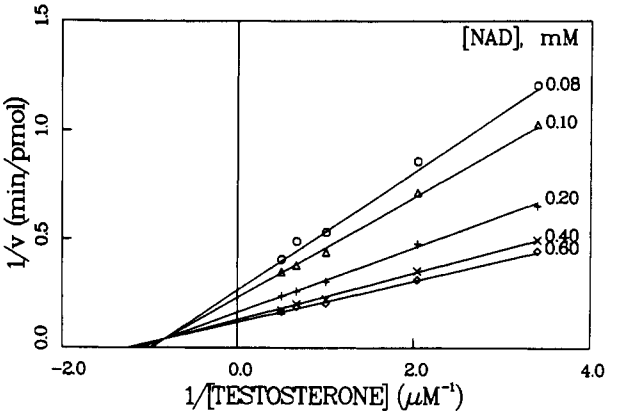


FIGURE 1: Initial velocity pattern for 3(17)β-hydroxysteroid dehydrogenase. The activity of 3(17)β-hydroxysteroid dehydrogenase (1×10^{-4} μg, 6×10^{-6} unit) was determined at varying concentrations of testosterone and NAD^+ at pH 8.9, and the data were fitted to the SEQUEN program as described in the text. The concentrations of NAD^+ are 80 (○), 100 (△), 200 (+), 400 (×), and 600 (◇) μM.

S. hydrogenans was affected at elevated concentrations (5–10 μM) approaching the solubility limits of the steroidal pyrazoles. All compounds investigated were found to be competitive inhibitors with respect to testosterone of 3(17)β-hydroxysteroid dehydrogenase. The isoxazole analogue of **2a**, compound **3**, was found to have a K_i of 190 ± 30 nM, 27 times that of the more potent 3,4-pyrazole inhibitor (**2a**).

Dialysis for 24 h of 3(17)β-hydroxysteroid dehydrogenase that had been preincubated with 250 nM **2a** (K_i = 7 nM) resulted in a return of 84% of the original enzyme activity. In contrast, the control sample, in which **2a** had been omitted, maintained 72% enzyme activity following dialysis. These results indicate that **2a** binds reversibly to the surface of the enzyme, and its presence may help stabilize the enzyme during an extended dialysis.

Initial Velocity Kinetic Pattern of 3(17)β-Hydroxysteroid Dehydrogenase. Initial velocity data, obtained by varying both NAD^+ and testosterone concentrations, were analyzed by the SEQUEN, PINGPONG, and EQORD programs described by Cleland (1979). The results of these analyses (Figure 1) indicate that the enzyme follows a sequential mechanism. The kinetic constants for testosterone, K_a and K_{ia}, and for NAD^+, K_b and K_{ib}, were found to be 0.7 ± 0.1 μM, 1.2 ± 0.3 μM, 140 ± 30 μM, and 250 ± 60 μM, respectively. Apparent kinetic constants also were obtained for ADIONE and NADH at fixed concentrations of the second substrate (data not shown). The $K_{m,app}$ for ADIONE at 400 μM NADH was 4.5 ± 0.3 μM and for NADH at 4 μM ADIONE was 12 ± 1 μM. Limited solubility of ADIONE prevented a more complete initial velocity analysis in the ADIONE to T direction.

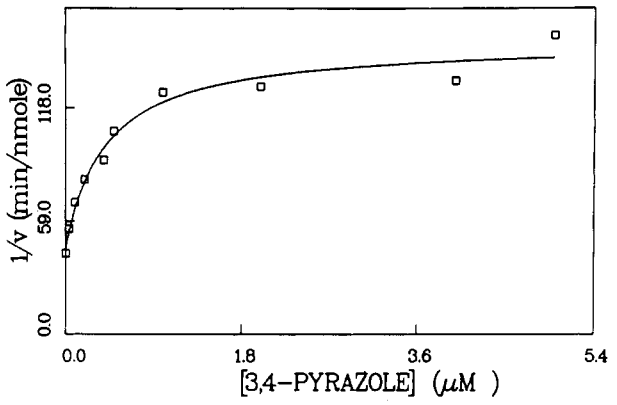


FIGURE 2: Inhibition of 3(17)β-hydroxysteroid dehydrogenase by the 3,4-steroidal fused pyrazole **2a** with ADIONE as substrate. 3(17)-β-Hydroxysteroid dehydrogenase (2×10^{-2} μg) was assayed in the ADIONE to T direction in the presence of varying concentrations of the steroidal pyrazole inhibitor (**2a**) at pH 8.9. The concentrations of ADIONE and NADH remained constant at 4.0 μM and 400 μM, respectively. The data was plotted by the method of Dixon (1953) and fitted to eq 3, as described in the text.

Product and Dead-End Inhibition Studies. The kinetic patterns obtained from product inhibition studies, as well as the predicted patterns (Segel, 1975) for ordered and random bi-bi kinetic mechanisms, are summarized in Table IIA. It should be noted that the observed patterns do not match either of the predicted sets. Unambiguous results were obtained from dead-end inhibition patterns in which NADH and the 3,4-pyrazole, **2a**, were used as analogues for NAD^+ and testosterone, respectively. These results, presented in Table IIB, indicate that the mechanism follows an ordered association of substrates with the adenine dinucleotide binding prior to the steroid.

Inhibition of the 3(17)β-hydroxysteroid dehydrogenase by **2a** also was monitored for the ADIONE and NADH to T and NAD^+ reaction, the reverse direction of that described above. Under these conditions, inhibition was induced only at concentrations of the steroidal pyrazole that were much higher (2 orders of magnitude) than those seen for the forward (T to ADIONE) direction. A Dixon plot (Dixon, 1953; Segel, 1975) of the data from one such experiment in which the concentration of **2a** was varied from 0 to 5 μM is shown in Figure 2. This nonlinear curve, which was fit to a hyperbola, shows maximum inhibition at the asymptote of 62%.

Double Inhibition Experiments Involving **2a** *and 3(17)β-Hydroxysteroid Dehydrogenase.* In order to elucidate if the steroidal pyrazoles bind preferentially to one form of the enzyme, E–NAD^+, double inhibition experiments employing both **2a** and NADH were conducted. In one set of experiments, the dead-end inhibition patterns of **2a** vs. testosterone were determined in the presence of increasing concentrations of the second inhibitor, NADH. As shown in Figure 3, the patterns remain competitive upon addition of exogenous NADH. The incremental concentrations of NADH were sufficient to ensure that a substantial concentration of enzyme–NADH complex was present in the steady state as demonstrated by a corresponding decrease in the values for V_{max} (Figure 3). A concomitant decrease in K_m values causes the calculated V_m/K_m

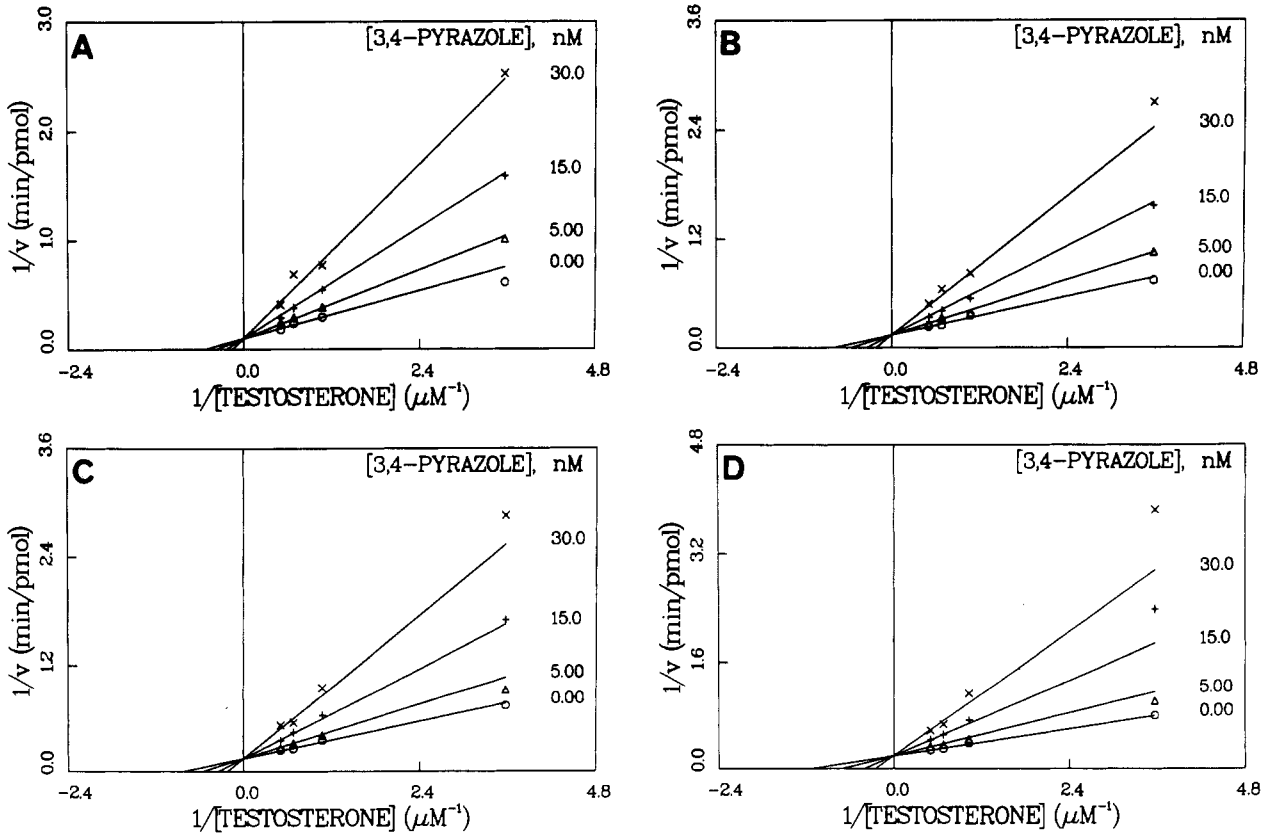


FIGURE 3: Inhibition of 3(17)β-hydroxysteroid dehydrogenase by the steroidal pyrazole **2a** in the presence of exogenous NADH. The kinetic patterns for 3(17)β-hydroxysteroid dehydrogenase (2×10^{-4} μg, 1.2×10^{-5} unit) upon variation of inhibitor (**2a**) and testosterone concentrations in the presence of added NADH were determined. The NAD^+ concentration was held constant at 400 μM. Comparative analyses of data were performed by fittings with the COMP, NONCOMP, and UNCOMP programs; all plots gave preferential fits to the competitive pattern (COMP). The concentrations of **2a** are 0 (○), 5 (△), 15 (+), and 30 (×) nM and of the exogenous NADH are (A) 0, (B) 6, (C) 12, and (D) 40 μM. Consistent with the increase in NADH concentration is the concomitant decrease in values for V_m and K_m: (A) V_m = 52 μmol/(min·mg), K_m = 1.9 μM, and V_m/K_m = 27.4 μmol/(min·mg·μM); (B) V_m = 36 μmol/(min·mg), K_m = 1.3 μM, and V_m/K_m = 27.7 μmol/(min·mg·μM); (C) V_m = 33 μmol/(min·mg), K_m = 1.2 μM, and V_m/K_m = 27.5 μmol/(min·mg·μM); (D) V_m = 25 μmol/(min·mg), K_m = 0.9 μM, and V_m/K_m = 27.7 μmol/(min·mg·μM).

Table II: Product and Dead-End Inhibition Patterns for 3(17)β-Hydroxysteroid Dehydrogenase

kinetic pattern	inhibitor[a]	variable substrate[b] A	B
(A) Product Inhibition			
ordered bi-bi	P	N[c]	N
	Q	C[c]	N
random bi-bi	P	N	N
	Q	N	N
observed	P	U[c]	C or N[d]
	Q	C	N
(B) Dead-End Inhibition			
ordered bi-bi	I	C	N
	J	U	C
random bi-bi	I	C	N
	J	N	C
observed	I	C	N
	J	U	C

[a] The inhibitors used were as follows: P = androstenedione, Q = NADH, I = NADH, and J = 3,4-steroidal pyrazole (**2a**). These abbreviations refer to those used in Scheme II. [b] All experiments were performed at subsaturating concentrations ($\leq K_m$) of the nonvariable substrate. The variable substrates are represented by A = NAD^+ and B = testosterone; these abbreviations refer to those used in Scheme II. [c] Kinetic patterns were determined as described under Experimental Procedures. The patterns are designated as competitive (C), noncompetitive (N), and uncompetitive (U) vs. the variable substrate. [d] An unequivocal assignment of the inhibition pattern for B (testosterone) vs. Q (androstenedione) could not be made on the basis of data from three separate experiments.

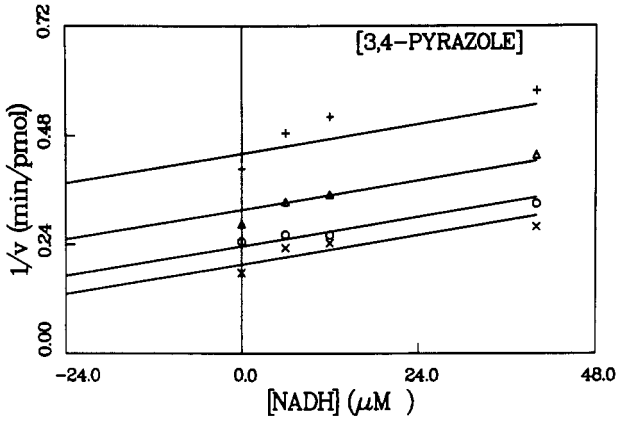


FIGURE 4: Double inhibition plot of 3(17)β-hydroxysteroid dehydrogenase by 3,4-steroidal pyrazole (**2a**) and NADH. Enzyme activity was determined as described in the text at pH 8.9 with 2×10^{-4} μg of enzyme at 2 μM testosterone and 400 μM NAD^+, and the data were fitted to eq 7. The concentrations of the pyrazole inhibitor were 0 (×), 5 (○), 15 (△), and 30 (+) nM.

to be invariant upon the incremental addition of NADH.

Double inhibition experiments at constant substrate concentrations can be described by eq 7, an expression that is algebraically equivalent to that derived by Yonetani and Theorell (1964) describing two inhibitors that are both competitive with the same substrate. The β term, an experimentally derived number, represents the degree of cooperativity between the two inhibitors I and J: if $\beta < 1$, the binding of I and J is synergistic; if $1 < \beta < \infty$, there is negative cooperativity upon the binding of one inhibitor in the presence of the second; and if $\beta = \infty$, the binding of the two inhibitors is not cooperative. For those cases in which $\beta < \infty$, a plot of $1/v_i$ vs. I or J at different constant concentrations of J or I, respectively, will yield a set of intersecting lines. If $\beta = \infty$, the lines of such a plot will be parallel. As seen with one such set of curves in Figure 4, parallel lines are observed, indicating

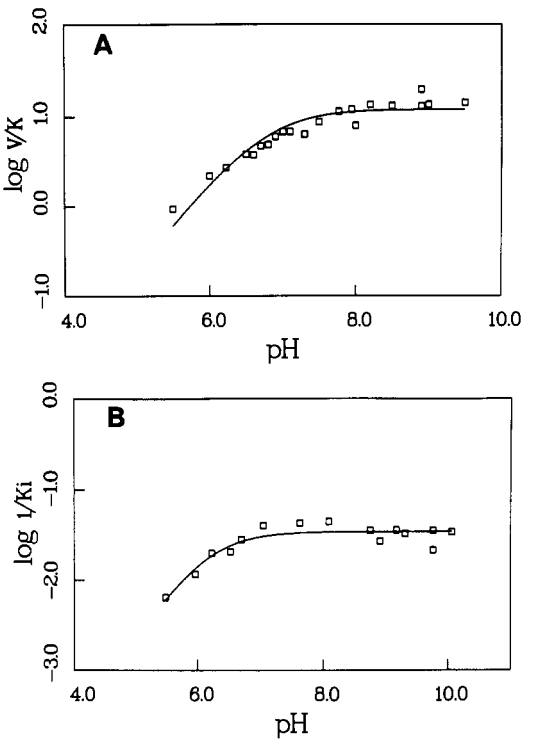


FIGURE 5: Variation of kinetic parameters upon pH. Apparent values of V_m/K_m for testosterone and of $1/K_i$ for the 3,4-pyrazole (**2a**) were determined throughout the indicated pH ranges at constant ionic strength in the SID buffer. Both sets of data were fitted to eq 8 (HABELL). The curves presented are (A) log V_m/K_m [μmol·(min·mg·μM)$^{-1}$] vs. pH and (B) log $1/K_i$ (nM^{-1}) vs. pH. The inflections in (A) and (B) were at pH 6.7 and 6.3, respectively.

that the binding of the 3,4-pyrazole (**2a**) and NADH is mutually exclusive.

Metal Dependence of 3(17)β-Hydroxysteroid Dehydrogenase. When a 2.8 μM solution of the active tetrameric holoenzyme (M_r 105 000) was analyzed for zinc by flame ionization atomic absorbance, less than 0.01 mg/L zinc was detected. This concentration corresponds to an upper limit of 0.15 μM zinc in the solution, or less than 0.05 mol equiv of zinc/mole of holoenzyme and 0.015 mol of zinc/equiv of enzyme monomer. In addition, no enzyme activation was observed upon the addition of micromolar concentrations of Zn^{2+}, Fe^{2+}, Fe^{3+}, Cu^{2+}, Mn^{2+}, or Mg^{2+} to complete enzyme assays. Higher concentrations of metal ions inhibited enzymatic catalysis.

Dependence of Kinetic Constants on pH. The pH dependence upon V_m and/or V_m/K_m has been investigated to determine the importance of ionizable groups in catalysis and/or testosterone binding. While no effect is observed on V_m (data not shown), a single deprotonation with pK_a of 6.7 ± 0.2 enhances the binding of steroid to the enzyme (Figure 5A). Similarly, a pH profile for the inhibition of the 3(17)β-hydroxysteroid dehydrogenase by the 3,4-pyrazole (**2a**) as depicted in Figure 5B consists of a single deprotonation event with a pK_a of 6.3 ± 0.2.

UV/Visible Difference Spectra upon Inhibition of 3(17)β-Hydroxysteroid Dehydrogenase by the 3,4-Pyrazole (**2a**). Subtraction of individual solution spectra of enzyme, NAD^+, and inhibitor from spectra of a solution with all the components needed to induce enzyme inhibition resulted in only a small residual absorbance (less than 0.010 AU) of variable wavelength between 290 and 315 nm. Incremental additions of inhibitor in ethanol to a solution of excess enzyme resulted in no absorbance increase upon appropriate spectral subtraction of the individual components. In separate control experiments,

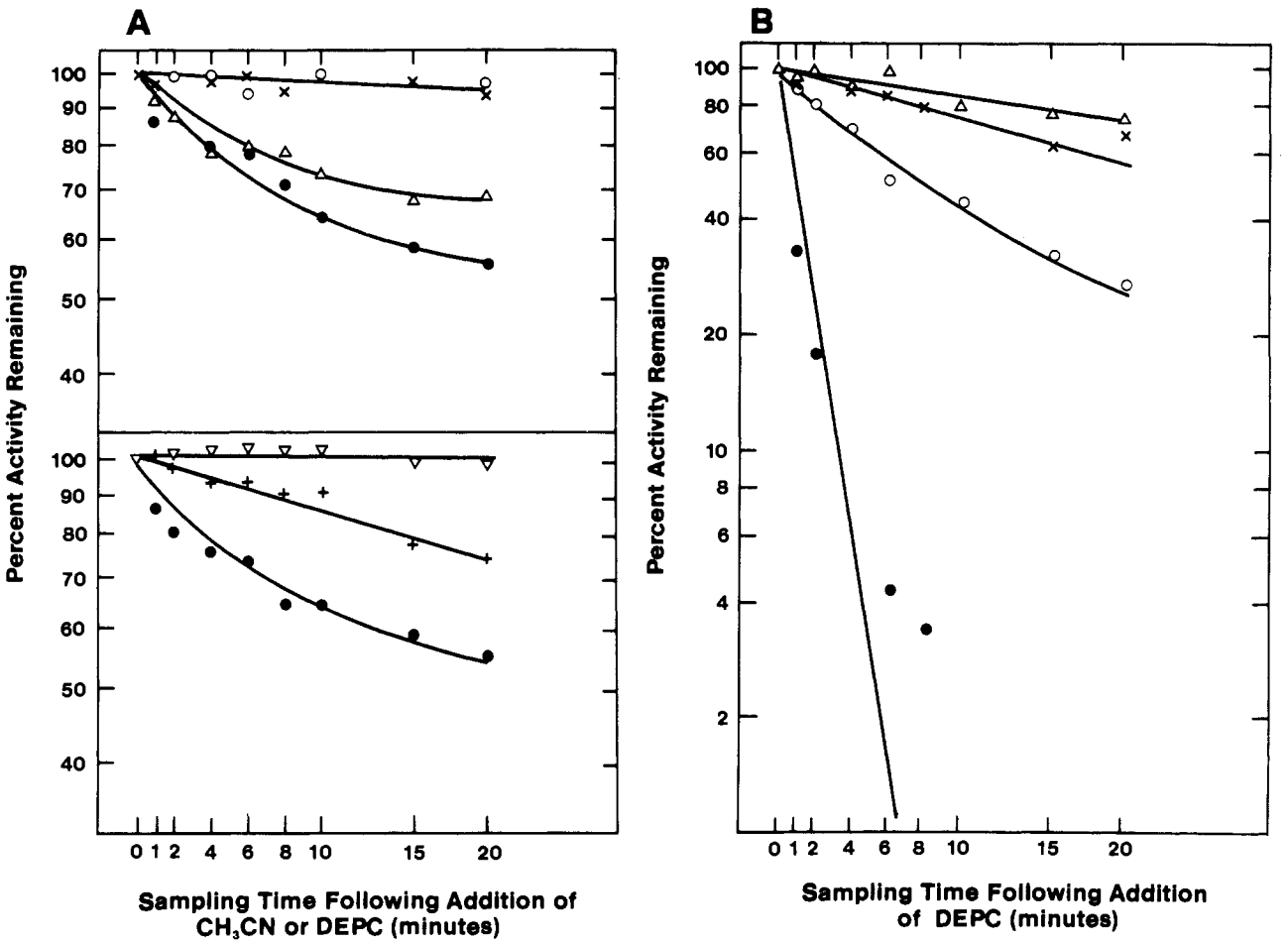


FIGURE 6: Inhibition of 3(17)β-hydroxysteroid dehydrogenase by diethyl pyrocarbonate. Samples of 3(17)β-hydroxysteroid dehydrogenase (0.57 μg) were preincubated at pH 7.2 in the presence of 960 μM DEPC either alone or with the addition of potential protecting agents. Aliquots were removed, quenched, and assayed as described under Experimental Procedures. Enzyme activites relative to those at zero time were plotted on a semilog scale. The temperatures for the preincubations were (A) 5.2 °C and (B) 27.5 °C. The preincubation conditions represented by the curves are as follows: (A) 960 μM DEPC (●), 960 μM DEPC and 1 mM NAD^+ (△), 960 μM DEPC, 100 nM **2a**, and 1 mM NAD^+ (×), 960 μM DEPC and 1 mM NADH (○), 960 μM DEPC and 100 nM **2a** (+), and CH_3CN control (▽); (B) CH_3CN control (×), 960 μM DEPC (●), 960 μM DEPC, 1 mM NAD^+, and 100 nM **2a** (○), and 960 μM DEPC and 1 mM NADH (△).

it was demonstrated that ethanol, up to 5% total volume, was neither a substrate nor an inhibitor of enzyme activity. Conversely, incremental additions of enzyme to a solution of molar excess inhibitor and cofactor showed no absorbance increase in the difference spectra. Thus, any residual difference spectrum can be attributed to small concentration differences in the solutions from which the spectra were taken and subsequently subtracted. These results indicate that no new chromophore is being formed upon inhibition of the 3(17)β-hydroxysteroid dehydrogenase by the 3,4-pyrazole (**2a**).

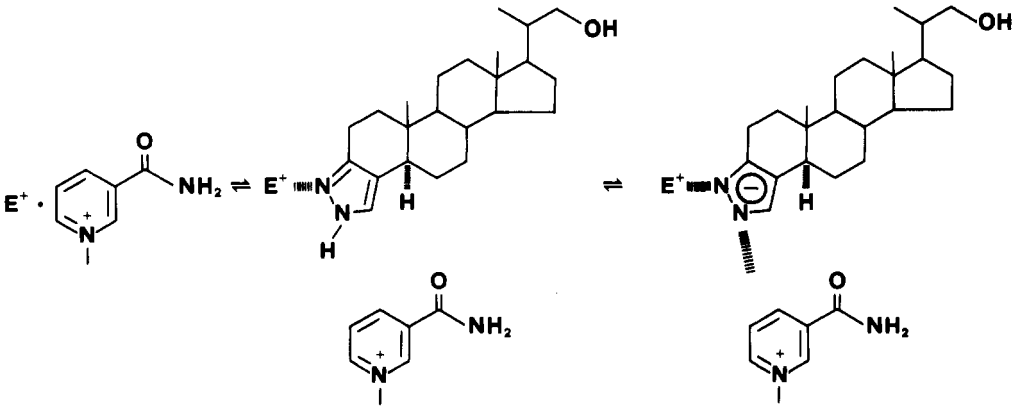
Inactivation and UV Spectral Characterization of 3(17)β-Hydroxysteroid Dehydrogenase in the Presence of DEPC. The enzyme 3(17)β-hydroxysteroid dehydrogenase was inhibited upon addition of DEPC, a reagent that has been shown to modify histidines preferentially to other amino acids (Miles, 1977). This time-dependent inhibition was characterized by a half-life of inactivation at 5.2 °C of 25 min and at 27.5 °C of 2.5 min. Greater than 98% of enzyme catalysis was inactivated at the higher temperature after 15 min. Activity did not return upon dialysis of the enzyme solution. As shown in Figure 6, protection from inactivation was achieved by the introduction of NAD^+ or the 3,4-pyrazole (**2a**); more complete protection was afforded by NAD^+ and the inhibitor (**2a**) together or with NADH alone. In a separate experiment, it was determined that the 3,4-pyrazole does not react with DEPC.

Difference spectra of the enzyme in the presence of excess DEPC demonstrated a time-dependent increase in absorbance at 238–240 nm, characteristic of *N*-carbethoxyhistidine formation (Dickenson & Dickinson, 1974). Maximal absorbance difference (0.029 AU) was reached after 13 min of incubation. This increase corresponds to an average modification of between one and two histidines per enzyme subunit[3] when calculated with known extinction coefficients [$\Delta\epsilon_{340nm}$ = 2900–3600 M^{-1} cm^{-1}; see Miles (1977)]. In addition, no significant absorbance change at 278 nm (an increase of less than 0.003 AU) was observed. O-Carbethoxylation of tyrosyl residues would result in a decrease of the difference absorbance at 278 nm (Burstein et al., 1974).

These data suggest that the active site of the dehydrogenase contains a functionality important histidine, which probably is involved in stabilization of the complex formed upon binding of substrate and inhibitor to the enzyme. The enhanced protection afforded by NADH results from its lower binding constant (K_m = 12 μM) as compared to that for NAD^+ (K_m = 140 μM). The inhibition and binding data may reflect the existence of enzyme forms that differ in their protonation states.

[3] The 3(17)β-hydroxysteroid dehydrogenase from *P. testosteroni* catalyzes the reversible oxidation of 3β- and 17β-hydroxyl groups upon the steroid nucleus (Marcus & Talalay, 1955, 1956). Each subunit of the 105 000-dalton $\alpha_3\beta$ holoenzyme is capable of catalyzing both 3β and 17β activities (Schultz et al., 1977a) and of independently binding 1 equiv of cofactor (Schultz et al., 1977b).

Scheme I



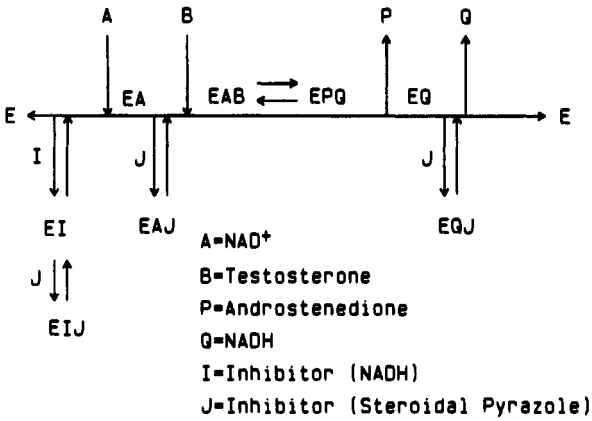
Discussion

In our attempts to develop new approaches to the inhibition of adenine dinucleotide dependent enzymes, several A ring fused pyrazoles have been investigated as potential inhibitors of four enzymes that can perform reductions of functionalities in the steroidal A ring. Thus, several 2,3- and 3,4-steroidal fused pyrazoles (**1** and **2**, respectively) have been investigated as inhibitors of steroid 5α-reductase from rat prostate, 3-(17)β-hydroxysteroid dehydrogenase from *P. testosteroni*,[3] 3α,20β-hydroxysteroid dehydrogenase from *S. hydrogenans*, and 3α-hydroxysteroid dehydrogenase from rat liver cytosol. Of these four enzymes, only the 3(17)β-hydroxysteroid dehydrogenase is inhibited by **1** and **2**. As summarized in Table I, these compounds are potent inhibitors of the 3(17)β-hydroxysteroid dehydrogenase, characterized by tightly associated enzyme–inhibitor complexes (K_i = 6–120 nM).

The syntheses of steroidal pyrazoles such as **1** and **2** were first reported in the early 1960s (Clinton et al., 1961; Clinton, 1969). One such compound, **1b** (stanozolol, USP), currently is marketed as an anabolic/nonandrogenic steroid. However, except for an isolated report (Ferrari & Arnold, 1963a) concerning the inhibition of 3(17)β-hydroxysteroid dehydrogenase by 2,3-steroidal fused pyrazoles, no information about the effects of such compounds on isolated enzyme activities has been published. The inhibition data, IC_{50} values of 0.83–4.8 μM, presented by Ferrari and Arnold (1963a) would suggest that these compounds are less potent than shown in Table I. Our results suggest that these steroidal pyrazoles are a unique, potent, and selective class of reversible inhibitors for the 3(17)β-hydroxysteroid dehydrogenase.

Prior to addressing the mechanism of enzyme inhibition by these compounds, it was necessary to better understand the kinetic order of the 3(17)β-dehydrogenase as determined by steady-state analysis. The sequential nature of the reaction was confirmed through initial velocity studies (Figure 1). To determine the order of substrate binding in the formation of this ternate, product inhibition patterns were determined. The divergence of these results from the predicted patterns (Table IIA) suggests that ADIONE-induced inhibition is occurring by a mixed process involving both product and dead-end pathways. In the latter, ADIONE acts as a competitive inhibitor of the steroid substrate thereby binding to the same enzyme form as does T. Subsequent dead-end inhibition experiments using the 3,4-steroidal fused pyrazole **2a** as a competitive inhibitor of T were consistent with an ordered binding to the enzyme in which the cofactor binds prior to the steroid substrate or inhibitor. These conclusions are similar to those previously obtained by isotope exchange techniques where the binding order was shown to be preferentially ordered (Schultz et al., 1977b).

Scheme II



We initially hypothesized that in the mechanism of 3-(17)β-hydroxysteroid dehydrogenase inhibition (Scheme I) the steroidal pyrazole would bind preferentially with one enzyme form resulting in a ternary complex composed of NAD^+ and inhibitor within the active site of the enzyme. Coordination of one of the nitrogens of the pyrazole inhibitor to an enzyme-associated electrophile, analogous to the zinc in LADH, could lead to facile deprotonation of the steroidal pyrazole functionality through stabilization of the resulting anion. Further stabilization would be afforded by the electropositive nature of the cofactor, NAD^+.

The proposal that the steroidal inhibitor (J in Scheme II) binds to the enzyme–NAD^+ complex (E–A) was tested with double inhibition experiments. Under initial velocity conditions, a double-reciprocal plot of velocity vs. steroid substrate ($1/v$ vs. $1/B$) should be competitive; as noted above, this pattern was observed (Table IIB). However, in the presence of exogenous NADH (Q), such a plot would remain competitive only if the pyrazole inhibitor, J, preferentially binds to E–A. The formation of a tight complex of enzyme–NADH–steroidal pyrazole (E–Q–J) would contribute to an intercept effect, resulting in a noncompetitive pattern. Added NADH (Figure 3) did not alter the pattern from competitive, thereby suggesting that the inhibitor binds selectively to E–A.

Similarly, treatment of data from double inhibition experiments using two inhibitors not both competitive against the same substrate can be analyzed according to eq 7 as demonstrated by Northrup and Cleland (1974). The resulting parallel lines in Figure 4 (I = NADH, J = **2a**) indicate that the binding of NADH to the enzyme has no cooperative effect, positive or negative, on the binding of the steroidal pyrazole. If the presence of the reduced cofactor (I) would promote binding of the inhibitor (J) , as would be expected for a tightly associated ternary complex, intersecting lines would result with

a value of β that is less than one. These deductions are not intended to suggest that no enzyme–NADH–pyrazole (EIJ or EQJ) can form but rather that the components within such a complex are less tightly associated than in the enzyme–NAD^+–inhibitor (EAJ) alternative.

The involvement of EAJ in the observed inhibition is underscored by the higher concentrations of **2a** required to decrease activity in the reaction direction of ADIONE to T. The absence of a component required to induce the previously observed potent inhibition hereby is suggested. The data from this experiment (Figure 2) are consistent with a product–(NAD^+) induced inhibition. Since the amount of NAD^+ produced in the reaction is well below that required for saturation, increasing concentrations of **2a** will trap proportionally less enzyme in the ternary complex (E–NAD^+–**2a**), resulting in the observed hyperbolic dependence upon the inhibitor concentration.

It was important to impugn the proposed involvement of pyrazole deprotonation yielding the stabilized ternary structure. Loss of the proton might occur only if the pK_a of the pyrazole functionality were shifted down from its typical value of 14 (Schofield et al., 1976; Katritzky & Lagowski, 1984) to within a range where deprotonation would be facile. In the case of LADH, this drop is proposed to be over 10 orders of magnitude (Andersson et al., 1981). Thus, a pH effect on the inhibition constant (K_i) of the inhibitor **2a** should be observed if the pK_a of the pyrazolo moiety of **2a** is shifted into the functional pH range of the enzyme (pH 6–10). The dependences of V_m/K_m and $1/K_i$ upon pH both indicate a single deprotonation event is involved in steroid binding. Since there is no ionizable group in either testosterone or the inhibitor within the region of the observed inflections (6.7 and 6.2, respectively), ionization of an enzyme-bound functionality must be involved in binding of substrate (Cleland, 1982). The lack of an additional inflection in the pH profile of $1/K_i$ vs. the profile of V_m/K_m indicates that formation of the tightly associated complex is not dependent on any additional proton loss within the accessible pH range.

This experiment, however, does not eliminate the possibility that the pK_a of the pyrazolo functionality in **2a** has not been shifted so acidic as to be deprotonated at the lowest pH studied, that is, below the lower limit for maintenance of enzyme activity. Therefore, difference spectra between solutions of enzyme, inhibitor, and cofactor and the individual components were recorded. Deprotonation of the pyrazole functionality would introduce a new, aromatic chromophore that would be detectable in the ultraviolet range. No significant absorbance in the difference spectra, however, was observed. These data, together with that from the pH profiles on the kinetic constants, precludes formation of a pyrazole anion within the tightly associated ternary complex.

The effects of pH on V_m/K_m and on $1/K_i$ for compound **2a** indicate that an enzyme-associated ionizable residue with pK_a near 6.5 is involved in substrate binding. While this value could be ascribed to shifting of the pK_a of any one of several amino acid functionalities, the more direct comparison with an imidazole side chain of histidine (pK_a = 6.1) is compelling. The observed time-dependent loss of enzyme activity and increase at 238 nm in the difference spectrum upon incubation of 3(17)β-hydroxysteroid dehydrogenase with diethyl pyrocarbonate are further support for this assignment. Protection from DEPC-induced inactivation by substrates and the inhibitor **2a** supports the involvement of histidine in binding interactions. By analogy, crystallographic analyses of both malate dehydrogenase (Birktoft & Banaszak, 1983) and lactate dehydrogenase (Holbrook et al., 1975) indicate binding of an active site histidine to the redox active oxygen.

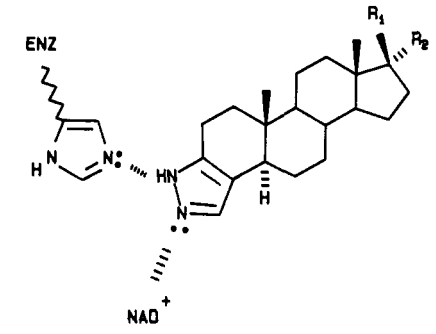


FIGURE 7: Proposed structure for the enzyme–inhibitor–NAD^+ ternary complex.

Furthermore, several steroid dehydrogenases are known to have histidine involvement upon binding. Three histidines and two arginines are thought to be juxtaposed to the steroid binding site of human placental estradiol 17β-dehydrogenase (Inano & Tamaoki, 1983). Kim et al. (1986) have suggested an active site functional role for histidine and cysteine in 3α-hydroxysteroid dehydrogenase. Affinity modification of 3α,20β-hydroxysteroid dehydrogenase from *S. hydrogenans* demonstrated that a common histidine was involved in both the 3α and the 20β activities and that alkylation of the imidazole impaired hydrogen transfer within the active site (Sweet & Samant, 1980; Tanimoto et al. 1982).

The absence of evidence supporting pyrazole deprotonation requires a reevaluation of the proposed ternary structure which results in tight binding inhibition. In consideration of the pH dependence of the inhibition constant of the most potent of the inhibitors reported in this paper (compound **2a**), the DEPC modification experiments, and the lack of metal dependence on enzyme activity, we propose that the tightly associated enzyme–NAD^+–inhibitor complex be represented by Figure 7. In this structure,[4] a lone pair of electrons of the imidazole nitrogen forms a hydrogen bond to the hydrogen of the pyrazole N–H. The second pyrazole nitrogen could coordinate through its lone pair of electrons to the electropositive nicotinamide. Conceivably, the nicotinamide could be deformed into a semiboat conformation, placing a larger positive charge on C-4 of the ring as proposed by Cook et al. (1981); such deformation would increase the coulombic potential between the nitrogen and the cofactor. In contrast, the isoxazole, **3**, is a less tightly associated inhibitor (Table I), presumably due to its inability to form such favorable hydrogen bonds.

The steroidal pyrazoles (**1** and **2**) are potent inhibitors of only one of four potential NAD(P)H-dependent targets examined. Such specificity emphasizes the importance of geometric constraints in order to gain proper juxtaposition of functionalities to induce tight binding. Hence, spacial considerations will determine if favorable binding energetics between enzyme and pyrazole result in a strong association. The design of highly specific inhibitors of nicotinamide-dependent enzymes by substrate or product pyrazole analogues should prove to be applicable to other targets of interest. Further work in this area is in progress.

[4] Alteration of the proposed structure of the tightly associated ternary complex from that depicted in Scheme I to that in Figure 7 necessarily requires a tautomeric change of the pyrazolo functionality of **2**. The movement of a proton from N-2 to N-1 reverses the role of the nitrogens such that the formerly nucleophilic N-1 now becomes electrophilic. Such annular tautomerization of pyrazoles is known to be rapid (Katritzky & Lagowski, 1984). In the case of 3,4-dialkylpyrazoles, it is expected that the two tautomeric forms would be of similar energy.

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