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Inhibition of a major NAD(P)-linked oxidoreductase from rat liver cytosol by steroidal and nonsteroidal anti-inflammatory agents and by prostaglandins

(3α-hydroxysteroid dehydrogenase/dihydrodiol dehydrogenase/indomethacin/betamethasone/molecular modeling)

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ABSTRACT **A NAD(P)-linked 3α-hydroxysteroid dehydrogenase [3α-hydroxysteroid: NAD(P) oxidoreductase, EC 1.1.1.50], purified to homogeneity from male rat liver cytosol, accounts for most of the oxidative activity for 3α-hydroxysteroids and for benzenedihydrodiol (*trans*-1,2-dihydroxy-3,5-cyclohexadiene) of this tissue. This enzyme, which also promotes the reduction of quinones and certain aromatic aldehydes and ketones, is powerfully inhibited by the major types of nonsteroidal and steroidal anti-inflammatory drugs. The IC_{50} values for indomethacin and for betamethasone are in the low micromolar range. The rank order of the inhibitory potencies of a series of these agents paralleled closely that reported for the inhibition of cyclooxygenase by nonsteroidal anti-inflammatory agents or the indirect inhibition of phospholipase A_2 by anti-inflammatory steroids. A good correlation is found also between the logarithm of the concentrations of these drugs required to inhibit 3α-hydroxysteroid dehydrogenase and the human anti-inflammatory dose. The suggestion that this enzyme may play a role in mediating inflammation is further strengthened by the observation that the dehydrogenase binds arachidonic acid and is also potently inhibited by certain prostaglandins. This enzyme may be of use in the evaluation of anti-inflammatory activity.**

A dual nicotinamide nucleotide-dependent 3α-hydroxysteroid dehydrogenase [3α-hydroxysteroid: NAD(P) oxidoreductase, EC 1.1.1.50] was first described in rat liver cytosol by Tomkins (ref. 1; reviewed in ref. 2). This enzyme has attracted attention because it catalyzes an early step in the metabolism of steroid hormones, and because its activity is androgen-dependent in some tissues (3, 4). Recently, Vogel *et al.* (5) reported that dihydrodiol dehydrogenase of rat liver copurifies with 3α-hydroxysteroid dehydrogenase. Attention has been directed to the participation of this enzyme in the detoxification of metabolites of polycyclic aromatics. Thus, by oxidizing *trans*-dihydrodiols to catechols, this enzyme may suppress conversion of such diols to diol epoxides which are potent ultimate carcinogens (6).

This paper demonstrates that the purified 3α-hydroxysteroid dehydrogenase is potently inhibited by many nonsteroidal and steroidal anti-inflammatory drugs at concentrations comparable to those inhibiting their commonly considered target enzymes—i.e., cyclooxygenase and phospholipase A_2, respectively. Furthermore, the order of potency of inhibition of the dehydrogenase by these drugs parallels that observed for their presumed target enzymes.

MATERIALS AND METHODS

Anti-inflammatory compounds were donated by the manufacturers. Steroids were obtained from Steraloids (Wilton, NH). 1-Acenaphthenol, 1,4-benzoquinone, and 1,2-naphthoquinone (Aldrich) were recrystallized before use. 9,10-Phenanthrenequinone, 4-nitroacetophenone, and 4-carboxybenzaldehyde were products of Fluka-Tridom (Hauppauge, NY). Benzenedihydrodiol was synthesized (7).

Preparation of 3α-Hydroxysteroid Dehydrogenase. This enzyme was purified to homogeneity from the 40–75% ammonium sulfate fraction of liver cytosol of male Sprague–Dawley rats. The ammonium sulfate fraction was subjected to successive chromatography on DEAE-cellulose and hydroxylapatite followed by Chromatofocusing and gel filtration on Sephadex G-100. The homogeneous enzyme had the following specific activities: 3.5 μmol of androsterone oxidized per min per mg (50 μM androsterone, 2.3 mM NAD, pH 9.0); 5.7 μmol of 9,10-phenanthrenequinone reduced per min per mg (40 μM phenanthrenequinone, 180 μM NADPH, pH 6.0).

Inhibition of 3α-Hydroxysteroid Dehydrogenase. Initial velocities were determined spectrophotometrically in 1.0-ml systems at 340 nm and 25°C. The oxidation of androsterone was measured in systems containing 75 μM steroid in 20 μl of acetonitrile, 2.3 mM NAD, and 100 mM potassium phosphate at pH 7.0. The reduction of 9,10-phenanthrenequinone was measured in systems containing 40 μM phenanthrenequinone in 20 μl of acetonitrile, 180 μM NADPH, and 100 mM potassium phosphate (pH 6.0). Varying concentrations of inhibitors were added in 20 μl of methanol, ethanol, or acetonitrile. Control velocities were determined in the presence of appropriate quantities of organic solvent. The IC_{50} values were computed from median effect plots (8).

RESULTS

Purification of 3α-Hydroxysteroid Dehydrogenase. A 150-fold purification of the cytosolic activity for the oxidation of androsterone provided homogeneous preparations of the enzyme in 30% yield. During the purification, a constant ratio was maintained for the oxidation of androsterone (I) by NAD, of 1-acenaphthenol (II) by NADP, and of benzenedihydrodiol (III) by NADP and for the reduction of 4-nitroacetophenone (V) by NADPH, indicating that all of these activities are associated with the same protein. The purified enzyme comprises most of the 3α-hydroxysteroid dehydrogenase, dihydrodiol dehydrogenase, and aromatic ketone reductase of rat liver cytosol. The enzyme is a monomer with M_r = 33,000 and a pI of 5.7. In these and other respects it resembles the dihydrodiol dehydrogenase

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Abbreviation: PG, prostaglandin.

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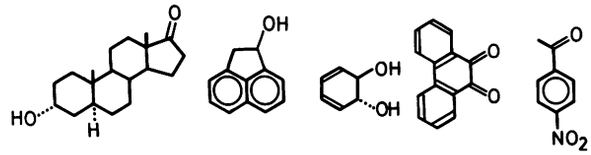
of Vogel *et al.* (5).



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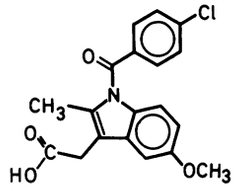
Substrate Specificity of 3α-Hydroxysteroid Dehydrogenase. The V_{max}/K_m ratio for androsterone is 50–100 times that for the reactive enantiomer of benzenedihydrodiol. The dehydrogenase reduces many quinones, including 9,10-phenanthrenequinone (**IV**) (K_m = 1.4 μM) and 1,4-benzoquinone but is not inhibited by dicoumarol and is clearly distinct from the NAD(P)H-dependent flavoprotein quinone reductase (9, 10). The enzyme also reduces 4-nitroacetophenone and 4-nitrobenzaldehyde but not 4-carboxybenzaldehyde. This finding and its dual nicotinamide nucleotide specificity distinguish the enzyme also from NADPH-linked "daunorubicin" reductase (11, 12).

Inhibition by Nonsteroidal Anti-Inflammatory Drugs. Indomethacin (**VI**) is a powerful inhibitor of the dehydrogenase. The IC_{50} values for indomethacin were 3.33 μM for the oxidation of androsterone by NAD at pH 7.0 and 0.735 μM for the reduction of 9,10-phenanthrenequinone by NADPH at pH 6.0 (Fig. 1; Table 1). Various other nonsteroidal anti-inflammatory



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agents also inhibited the enzyme, and the rank order of their inhibitory potencies correlated with their effectiveness in inhibiting cyclooxygenase (15–17)—i.e., indoleacetic acids (indomethacin) > some *N*-phenylanthranilic acids (mefenamic and flufenamic acids) > arylpropionic acids (ibuprofen, naproxen) > aspirin > acetaminophen. Furthermore, the IC_{50} values for the inhibition of 3α-hydroxysteroid dehydrogenase by these compounds were of the same magnitude as those reported for the inhibition of cyclooxygenase (Table 1). The IC_{50} values for the inhibition by indomethacin of bovine or sheep seminal vesicle cyclooxygenase (0.5–10 μM) are comparable to those for the inhibition of the dehydrogenase. An interesting feature is that salicylate was about 8 times more potent as an inhibitor of the dehydrogenase than aspirin. Aspirin is rapidly deacetylated *in vivo* and salicylate is responsible for many of the actions of aspirin, yet salicylate is not an inhibitor of cyclooxygenase.

Comparison of the inhibition of dehydrogenase with that of cyclooxygenase is complicated by the wide range of sensitivity of cyclooxygenase preparations to inhibition by indomethacin. The cyclooxygenase from human synovium is potently inhibited by indomethacin (IC_{50} = 3 nM) whereas the enzymes from rabbit brain or kidney or goat seminal vesicle require micromolar concentrations for inhibition (18). Even for cyclooxygenase preparations from a single source (e.g., bovine seminal vesicle), the IC_{50} values reported by several laboratories differ greatly, from a low of 0.07 μM to a high of 20 μM (18). In an attempt to relate our findings to anti-inflammatory potency, we have correlated the logarithm of the IC_{50} values for the inhibition of the dehydrogenase with the maximal daily human doses (in mmols) recommended for anti-inflammatory therapy (Table 1;

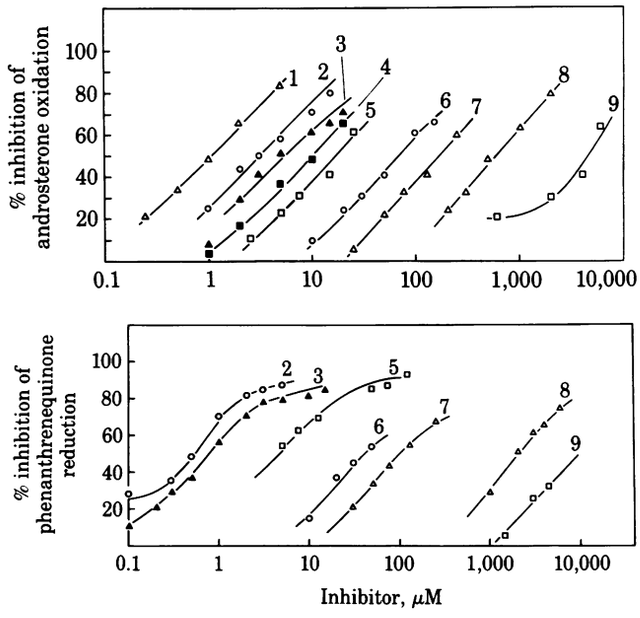

FIG. 1. Inhibition of 3α-hydroxysteroid dehydrogenase by nonsteroidal anti-inflammatory agents. The inhibition (%) is shown as a function of logarithm of inhibitor concentration. (*Upper*) Oxidation of androsterone by NAD at pH 7.0. (*Lower*) Reduction of 9,10-phenanthrenequinone by NADPH at pH 6.0. The compounds are: 1, *Z*-sulindac sulfide; 2, indomethacin; 3, sulindac; 4, *E*-sulindac sulfide; 5, mefenamic acid; 6, tolmetin; 7, ibuprofen; 8, aspirin; 9, acetaminophen.

Fig. 2). Although these doses are somewhat indirect measures of anti-inflammatory potency, the correlation of the logarithm of the IC_{50} values for androsterone inhibition and daily dose (13) gave a linear correlation coefficient of 0.890 for 12 compounds ranging in inhibitory potency from indomethacin (IC_{50} = 3.33 μM; dose = 0.42 mmol) to acetaminophen (IC_{50} = 4,920 μM; dose = 25.8 mmol). The linear correlation between the logarithm of IC_{50} values for 9,10-phenanthrenequinone reduction and daily human dose was equally high (r = 0.897). Comparisons between the logarithms of the IC_{50} values for the inhibition of sheep or bovine seminal vesicle cyclooxygenase with the daily dose gave correlation coefficients from 0.612 to 0.926. Thus, the inhibition of 3α-hydroxysteroid dehydrogenase by nonsteroidal anti-inflammatory drugs appears to provide a measure of their pharmacological potency that is at least as good as, if not better than, the inhibition of cyclooxygenase. Because such correlations between enzyme inhibition and human dose do not take into account differences in pharmacokinetic properties, the correlations are especially striking.

Our findings suggest that the 3α-hydroxysteroid dehydrogenase might be a useful screen for anti-inflammatory activity. We have found that, for a series of indomethacin analogues, the IC_{50} values for the inhibition of androsterone oxidation were 0.9, 10.0, and 7.0 μM for Z-sulindac sulfide, *E*-sulindac sulfide, and sulindac, respectively. This is in agreement with the enhancement of the activity of sulindac upon reduction of the sulfone to the sulfide and with the finding that the Z isomer is a much more potent inhibitor of inflammation than the *E* isomer (18). However, for at least one pair of enantiomers, (+)- and (−)-naproxen, both isomers were equipotent inhibitors of the dehydrogenase, whereas the *S* (+) enantiomer is more active both as a cyclooxygenase inhibitor and as an anti-inflammatory agent (19).

Most of the nonsteroidal anti-inflammatory compounds were competitive inhibitors of the dehydrogenase, whereas aspirin and sodium salicylate acted noncompetitively. The K_i values are considerably lower than the IC_{50} values (at near saturating sub-

Table 1. Inhibition of 3α-hydroxysteroid dehydrogenase and cyclooxygenase by nonsteroidal anti-inflammatory drugs

Drug	Maximal recommended daily dose*		IC_{50} value, μM			
			3α-Hydroxysteroid dehydrogenase		Cyclooxygenase	
	mg	mmol	9,10-Phenanthrenequinone reduction	Androsterone oxidation	Bovine seminal vesicle†	Sheep seminal vesicle‡
Indoleacetic acids						
Indomethacin	200	0.42	0.735	3.33	10.5	0.5
Sulindac	200	1.12	1.09	6.53	—	—
N-Phenylanthranilic acids						
Diclofenac	150	0.473	0.362	1.47	4.6	—
Meclofenamic acid	300	1.02	0.486	3.62	—	15.0
Flufenamic acid	600	2.14	3.18	5.52	6.0	2.5
Mefenamic acid	1,500	6.61	3.82	17.0	4.0	2.1
1-Methylpyrroleacetic acids						
Zomepirac	—	—	40.0	89.4	—	—
Tolmetin	1,200	5.07	44.0	71.1	11.7	—
Arylpropionic acids						
Naproxen	625	3.26	130	161	32.0	6.1
Fenoprofen	1,800	9.90	55.4	221	—	—
Ibuprofen	2,000	11.6	104	171	128	1.5
Salicylates						
Sodium salicylate	—	—	256	77.8	—	—
Aspirin	6,000	22.2	2,120	585	23,200	83.0
Acetaminophen	3,900	25.8	4,360	4,920	—	—
			Correlation between log IC_{50} and daily dose (mmol)			
No. of pairs of values (*n*)			12	12	8	7
Linear correlation coefficient (*r*)			0.897	0.890	0.926	0.612
Significance (*P*)			<0.0001	<0.0001	<0.001	<0.144

* Values from Shen (13), except for indomethacin and aspirin which are from standard texts.
† Values from Taylor and Salata (14).
‡ Values from Vane *et al.* (15).

strate concentrations). The K_i values for indomethacin in the inhibition of androsterone oxidation and 9,10-phenanthrenequinone reduction were 0.9 and 0.2 μM, respectively.

Inhibition by Steroidal Anti-Inflammatory Drugs. The oxidation of androsterone was also inhibited by anti-inflammatory steroids (Fig. 3). IC_{50} values for the inhibition by betamethasone of the reduction of 9,10-phenanthrenequinone and oxidation of androsterone were 1.20 and 3.97 μM, respectively. The order of potency for the inhibition of these reactions by anti-inflammatory steroids also correlated well with their pharmacological effects in the granuloma and glycogen deposition assays (Table 2) (22). Thus, betamethasone and dexamethasone were the most potent inhibitors of the dehydrogenase; cortisol and cortisone were no more than 1% as effective. 6α-Methylprednisolone, prednisolone, and prednisone were equipotent and were approximately 20–30 times more potent than cortisone. The inhibitory potency of steroidal anti-inflammatory drugs for 3α-hydroxysteroid dehydrogenase agreed well with that observed for their indirect inhibition of phospholipase A_2. Measurement of phospholipase A_2 inhibition after the exposure of guinea pig lung to rabbit aorta contracting substance releasing factor gave the following IC_{50} values for the inhibition of the release of prostaglandin H_2 (PGH_2): 3.5 μM for dexamethasone and betamethasone and 120 μM for cortisone (23). Furthermore, there is a close correlation between the logarithm of IC_{50} values of these drugs for their inhibition of 3α-hydroxysteroid dehydrogenase (9,10-phenanthrenequinone reduction) and the recommended daily dose to produce an anti-inflammatory effect ($r = 0.882$; $n = 7$).

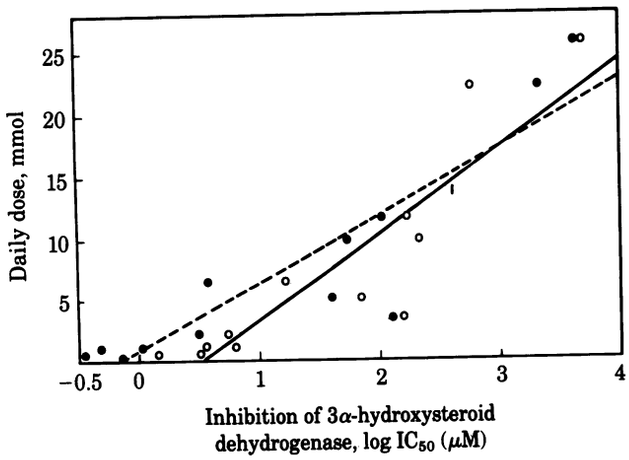

FIG. 2. Semilogarithmic plot of the relationship between the concentrations of anti-inflammatory agents producing 50% inhibition of 3α-hydroxysteroid dehydrogenase and the maximal recommended daily human dose required for anti-inflammatory effect. Measurements are shown for androsterone oxidation at pH 7.0 (○) and 9,10-phenanthrenequinone reduction at pH 6.0 (●). The IC_{50} values and doses were taken from Table 1. The lines are drawn by linear regression and the coefficients (*r*) were 0.890 for 9,10-phenanthrenequinone reduction and 0.890 for androsterone oxidation. The shift in slope between the lines is explained by the pH dependence of the IC_{50} values.

The inhibition of 3α-hydroxysteroid dehydrogenase by anti-inflammatory steroids is competitive (K_i, 18.0–300 μM) with respect to androsterone, except for the most potent compounds (betamethasone and dexamethasone, K_i = 3.0 and 10.0 μM, respectively) which are noncompetitive. Thus, the dehydrogenase binds nonsteroidal and steroidal inhibitors at two sites.

3α-Hydroxysteroid Dehydrogenase Binds Arachidonic Acid and PGs. The 3α-hydroxysteroid dehydrogenase also binds arachidonic acid (IC_{50}, 15 μM for oxidation of androsterone and

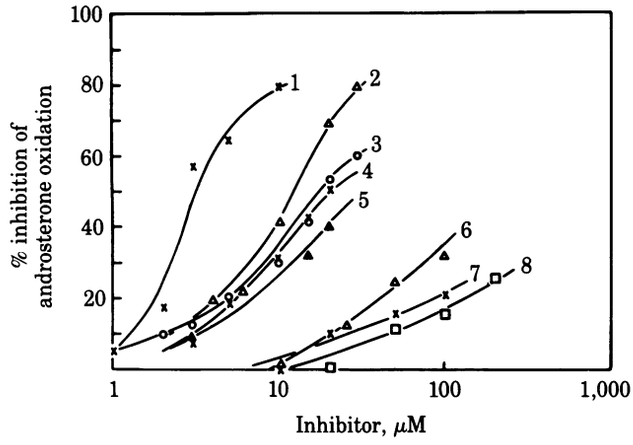

FIG. 3. Inhibition of 3α-hydroxysteroid dehydrogenase by anti-inflammatory steroids. The inhibition (%) is shown for the oxidation of androsterone by NAD at pH 7.0 as a function of the logarithm of inhibitor concentration. The compounds are: 1, betamethasone; 2, 6α-methylprednisolone; 3, dexamethasone; 4, prednisolone; 5, prednisone; 6, fludrocortisone; 7, triamcinolone; 8, cortisone.

9 μM for reduction of 9,10-phenanthrenequinone). The specificity of inhibition by other fatty acids has not yet been examined. Preliminary studies have revealed that primary PGs are potent competitive inhibitors of androsterone oxidation: PGB_1, $K_i = 0.8$ μM; PGA_1, $K_i = 3.1$ μM; PGE_1, $K_i = 7.5$ μM; and PGF_1, $K_i = 12$ μM. PGs of the E and F series are less potent. In contrast, thromboxane B_2 is a relatively weak inhibitor.

Although 3α-hydroxysteroid dehydrogenase binds certain PGs, it does not appear to possess 9-ketoreductase or 15-hydroxy-PG dehydrogenase activities. Large scale incubations of PGE_1 in the presence of NADH or NADPH (for 9-ketoreductase activity) and of PGE_2 with NAD or NADP (for 15-hydroxy-PG dehydrogenase activity) in the presence of the purified enzyme, followed by extraction at pH 4.0 and thin layer chromatography, led to the recovery of starting material only. Furthermore, none of the described PG dehydrogenases has dual nucleotide specificity (24).

Molecular Modeling of Androsterone and Indomethacin. Gund and Shen (25) have pointed out analogies between a specific conformation of arachidonic acid and the crystal structure of indomethacin. Alternative models emphasize conformational similarities between nonsteroidal anti-inflammatory drugs and a postulated transition state of the cyclooxygenase reaction (26). The substrate and inhibitor specificities of the 3α-hydroxysteroid dehydrogenase may be rationalized by substantial conformational similarities between indomethacin and androsterone when the docked molecules are viewed either from the front face of the steroid or from the side (Fig. 4). Two features are of interest. First, the C-10 carbonyl group of indomethacin overlies the C-11 position of the steroid, where an oxygen substituent is critical for anti-inflammatory activity. Second, the *p*-chlorophenyl ring of indomethacin is juxtaposed to the C-18 methyl group of the steroid, suggesting complementarity to a common hydrophobic region of the dehydrogenase.

DISCUSSION

Although several enzyme systems are inhibited by nonsteroidal anti-inflammatory agents, including some involved in PG metabolism (27–29), the view of Vane (15, 30)—that inhibition of cyclooxygenase is the primary site of action of these compounds—is widely accepted. The principal lines of evidence supporting this proposal are that: (*a*) the potency of inhibition for this enzyme is several orders of magnitude greater than that observed for other enzymes; (*b*) the rank order of cyclooxygenase inhibition correlates with the anti-inflammatory activity; (*c*) there is a concomitant inhibition of the synthesis of mediators of inflammation, pain, and pyresis; and (*d*) only those structural analogues that are anti-inflammatory inhibit cyclooxygenase. Several of these features are also characteristic of the inhibition of the 3α-hydroxysteroid dehydrogenase by nonsteroidal anti-inflammatory agents. The inhibition of the dehydrogenase and the cyclooxygenase occur at similar concentrations, and a good correlation exists between potency of inhibition of the dehydrogenase and therapeutic potency.

Anti-inflammatory steroids do not inhibit cyclooxygenase or prevent PG formation in cell-free systems, but they do reduce the levels of substrate available for the cyclooxygenase reaction (31). Other work supports this conclusion (23, 32, 33), and it has been shown that anti-inflammatory steroids induce the synthesis of a phospholipase A_2 inhibitor (21, 34).

Our finding that the inhibition of 3α-hydroxysteroid dehydrogenase by both steroidal and nonsteroidal anti-inflammatory drugs correlates well with their pharmacological activities raises the issue of whether this enzyme might play some role in modulating the inflammatory process. We have no direct experimental evidence, but several roles for the dehydrogenase may be suggested. Both cyclooxygenase and lipooxygenase reactions require reducing equivalents for the peroxidative conversions of hydroperoxy to hydroxy derivatives—i.e., conversion of PGG_2

Table 2. Effect of anti-inflammatory steroids on 3α-hydroxysteroid dehydrogenase and phospholipase A_2 and their anti-inflammatory and glucocorticoid activities

Steroid	Recommended daily dose*		IC_{50} value, μM			Relative activity†	
			3α-Hydroxysteroid dehydrogenase				
	mg	μmol	9,10-Phenanthrenequinone reduction	Androsterone oxidation	PGH_2 release‡	Granuloma test	Glycogen deposition test
Dexamethasone	0.75	1.90	3.62	25.3	3.5	104	181
Betamethasone	0.60	1.53	1.20	3.97	3.5	35.8	118
6α-Methylprednisolone	4.0	10.6	7.16	11.9	17.0	6.0	26.0
Prednisone	5.0	14.0	8.94	30.4	—	1.0	5.2
Prednisolone	5.0	13.9	6.28	22.6	—	3.1	9.9
Triamcinolone	4.0	10.1	20.4	>200	10.6	2.6	34.0
Cortisone	25	69.3	78.9	>200	120	0.5	1.0
Hydrocortisone	20	55.3	136	>400	—	1.0	2.0

* From Rodig (20).
† From Wolff (22). For granuloma test, hydrocortisone = 1; for glycogen deposition test, cortisone = 1.
‡ Inhibition of phospholipase A_2 as measured by decrease in prostaglandin H_2 (PGH_2) release; from Blackwell *et al.* (21).

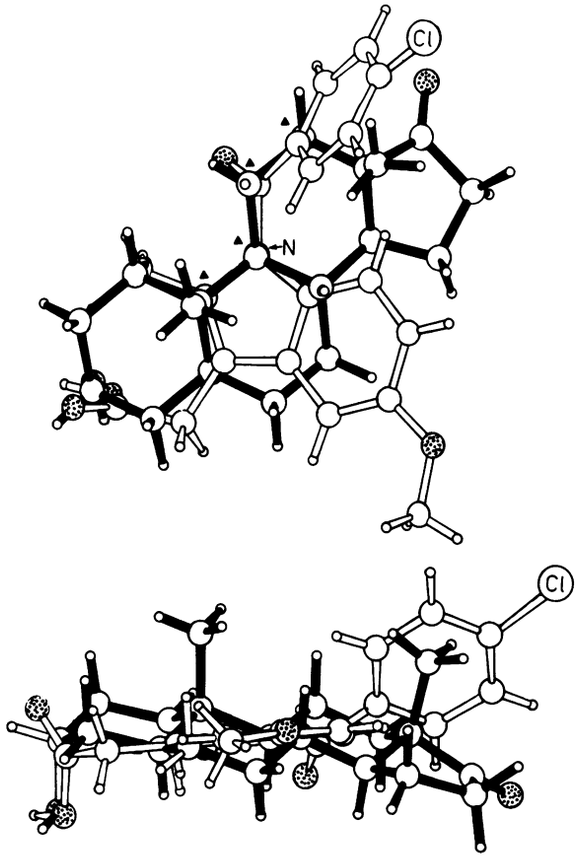

FIG. 4. Computer-generated drawings of androsterone (black bonds) and indomethacin (clear bonds) based on crystallographic coordinates. Oxygen atoms are stippled, carbon (large) and hydrogen (small) atoms are clear, the nitrogen (barely visible) is black, and the chlorine is marked Cl. The superimposition of the two molecules was optimized by least-squares minimizations of the positions of four pairs of atoms (▲). The top view shows the compounds in approximately the same orientation as their structural formulae in the text.

to PGH_2 and conversion of hydroperoxyicosatetraenoic acid to hydroxyicosatetraenoic acid. In fact, hydroquinone or other antioxidants are commonly added to promote conversion of PGG_2 to PGH_2 (35, 36). Because 3α-hydroxysteroid dehydrogenase reduces quinones efficiently, this enzyme may mediate an electron shuttle from reduced nicotinamide nucleotides to promote PGH_2 or hydroxyicosatetraenoic acid formation.

Although limited experiments so far have failed to reveal a direct role of the dehydrogenase in PG metabolism it is possible that, because this enzyme binds PGs, it may promote their interconversions. Inhibition of this process could cause feedback inhibition of cyclooxygenase.

More than 20 years ago, it was shown (37) that the NAD(P)-linked 3α-hydroxysteroid dehydrogenase in rat liver cytosol promotes an efficient hydrogen flow (transhydrogenation) between NADPH and NAD in the presence of catalytic levels of steroidal substrates. Transhydrogenation may serve a regulatory role by controlling hydrogen flow between NAD- and NADP-dependent reactions in the arachidonic acid cascade.

Finally, the presence on 3α-hydroxysteroid dehydrogenase of a common binding site for PGs and steroidal and nonsteroidal anti-inflammatory drugs suggests that these agents may regulate the capacity of the enzyme to metabolize steroids, including androgens (e.g., 5α-dihydrotestosterone) and to inactivate certain xenobiotics (e.g., *trans*-dihydrodiols).

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