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OXIDATION OF THE 17-ALDOL (20β HYDROXY-21-ALDEHYDE) INTERMEDIATE OF CORTICOSTEROID METABOLISM TO HYDROXY ACIDS BY HOMOGENEOUS HUMAN LIVER ALDEHYDE DEHYDROGENASES*

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SUMMARY

In human liver, the oxidation of corticosteroids to 20-hydroxy-21-oic acids proceeds via the formation and oxidation of aldol (20-hydroxy-21-aldehyde) intermediates. Human liver aldehyde dehydrogenases E_1 and E_2, which we have previously purified to homogeneity, catalyzed the oxidation of steroid aldols by NAD^+ to 20-hydroxy-21-oic acids. The hydroxy acids formed after oxidation of the aldol isomer of cortisol (isocortisol) or of 11-deoxycorticosterone (isoDOC) by E_1 and E_2 respectively, were identified by the criteria of chromatographic mobility, derivatization, and reverse isotope dilution of 4-^{14}C labeled acid end products. Both enzymes showed broad substrate specificity and oxidized both 17-hydroxy and 17-deoxy steroids, though at widely varying rates. Kinetic analysis of the course of oxidation of isocortisol and isoDOC by NAD^+ gave intersecting initial velocity plots that conform with a sequential mechanism. The inhibition patterns for both enzymes with thionicotinamide adenine dinucleotide or chloral hydrate were consistent with random sequential behavior.

INTRODUCTION

A significant fraction of cortisol is metabolized in humans to a class of metabolites in which the ketol side chain is replaced by a 20-hydroxy-21-oic acid side chain [1, 2]. The formation of acid results from a sequence of reactions which includes rearrangement of the ketol to an aldol (20-hydroxy-21-aldehyde) that is directly oxidized to the hydroxy acid. All the enzymes required for this transformation are found in the liver. The isomerase that catalyzes the interconversion of ketol and aldol side chain has been isolated [3] but the aldehyde dehydrogenase responsible for the subsequent oxidation has not yet been identified. We have found that the well characterized aldehyde dehydrogenases of horse liver catalyze the final oxidation to hydroxy acid [4]. Isoenzyme F_1 oxidized 17α-hydroxy steroids better than 17-deoxysteroids; 17-deoxysteroids were better substrates for isoenzyme F_2. While it is not at all clear that these enzymes participate in the oxidative metabolism of the corticosteroid side-chain themselves, they have provided a valuable model system which is consistent with the overall oxidative scheme we have proposed.

Recently, one of us succeeded in purifying human liver aldehyde dehydrogenase isoenzymes E_1 and E_2 to homogeneity [5]. As a result, we have been able to examine if these enzymes oxidize steroid aldols to hydroxy acids. In this paper we show that steroid aldols are substrates for the dehydrogenases of human origin. The end-products are hydroxy acids. The kinetic properties of these enzymes with steroid substrates are reported.

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Abbreviations. F, Cortisol, 11β,17,21 trihydroxy-4-pregnen-3,20-dione; THF, tetrahydrocortisol, 3β,11β,17,21-tetrahydroxy-5β-pregnan-20-one; DOC, 11-deoxy corticosterone, 21-hydroxy-4-pregnen-3, 20-dione; B, corticosterone, 11β, 21-dihydroxy-4-pregnen-3, 20-dione; E, cortisone, 17,21-dihydroxy-4-pregnen-3,11,20-trione; prednisolone, 11β,17, 21-trihydroxy-1, 4-pregnadiene-3, 20-dione; S, 11-deoxycortisol, 17, 21-dihydroxy-4-pregnen-3,20-dione; isoDOC, 20β-hydroxy-3-keto-4-pregnen-21-al: Other isocorticosteroids are named in a similar way; 21-dehydro DOC, 3,20-diketo-4-pregnen-21-al: Other 21-dehydrocorticosteroids are named in a similar way.

MATERIALS AND METHODS

Materials

β-Nicotinamide adenine dinucleotide (β-NAD^+), β-NADH and thionicotinamide adenine dinucleotide (thio-NAD^+) were purchased from Sigma Chemical Co. Cortisol (F), tetrahydrocortisol (THF), 11-deoxycorticosterone (DOC), corticosterone (B), cortisone (E), prednisolone, and 11-deoxycortisol (S) were

obtained from Steraloids Co. (Wilson, New Hampshire). Chloral hydrate was purchased from Matheson, Coleman & Bell. Acetaldehyde (Analytical Reagent grade) was from Baker Chemical Co. Analytical grade solvents were used for enzyme assay. All other chemicals were also of Analytical Reagent grade. [4-^{14}C]-cortisol (55.0 mCi/mmol) and [4-^{14}C]-DOC (58.3 mCi/mmol) were purchased from New England Nuclear Co. Plastic backed precoated silica gel thin layer plates with fluorescent indicator (GF_{254}, 0.25 mm) were bought from Brinkman Instruments, Inc., POPOP was bought from Amersham/Searle (Chicago, IL).

Enzyme purification

Two isoenzymes of human liver aldehyde dehydrogenase were isolated as described earlier [5] *via* classical chromatography combined with affinity chromatography on 5'AMP Sepharose 4B. To ensure complete homogeneity both enzymes were rechromatographed on DE-BioGel columns. Homogeneity was tested by electrophoresis and by peptide mapping. The nomenclature E1 and E2 (for enzymes 1 and 2) corresponds to that of F1 and F2 (for enzyme fraction 1 and 2) of horse liver aldehyde dehydrogenases described previously by Eckfeldt *et al.*[6]. By employing an assay system described by Feldman and Weiner[7], the specific activity of E1 isozyme was determined to be 0.55 μmol/mg/min while that of E2 isozyme was 1.5 μmol/mg/min. The enzymes were free of alcohol dehydrogenase activity.

Assay of aldehyde dehydrogenase

Enzyme activity was determined spectrophotometrically with a Gilford Model 2000 automatic recorder attached to a Beckman DU monochromator. The change of absorption at 340 cm caused by formation of NADH as a result of the oxidation of substrate by NAD^+ was measured. One ml of reaction mixture contained 0.088 M tricine buffer, pH 8.5, 0.5 mM NAD^+, and 0.3 mM steroid substrate added in 20 μl of 50% methanol. Controls were run with 20 μl of 50% methanol instead of steroid. Methanol at this concentration (1%) did not affect the rate of oxidation. Enzymes were dialyzed before use as described previously [8].

Data processing

Initial velocities are expressed as nmol/min/mg protein. The appropriate equations to use for calculation of kinetic constants according to Cleland[9] were determined after preliminary plots were made of reciprocal initial velocity versus reciprocal substrate concentration. Kinetic constants were calculated by least square fitting of triplicate data with the aid of Wang 600 calculator using the assumption of equal variances for experimental velocities as described by Wilkinson[10].

Thin layer chromatography

Analyses were performed on glass-backed precoated silica plates (GF_{254}, 0.25 mm, Brinkman). The following solvent systems (v/v) were used: I, chloroform–methanol (98:2, v/v); II, chloroform–methanol–formic acid (98:2:1, by vol.); III, chloroform–methanol (90:10, v/v); IV, chloroform–methanol–formic acid (90:10:1, by vol.); V, chloroform–methanol–formic acid (95:5:1, by vol.); VI, dichloromethane–acetone (80:2, by vol.); VII, dichloromethane–acetone–formic acid (80:20:1, by vol.); VIII, ethyl acetate.

Preparation of isocorticosteroids

Corticosteroid derivatives containing the 17-aldol side chain (i.e. isocorticosteroids) were synthesized by either the chemical method of Oh and Monder[11] or enzymatic method of Lippman and Monder[12]. The 21-dehydrocorticosteroids used in the synthesis were prepared by oxidation of corticosteroids with cupric acetate [13]. 4-^{14}C labeled isocorticosteroids, prepared by modifications of the same procedures [11, 12] were purified when necessary by thin layer chromatography in solvent system I just before use. The purity of [4-^{14}C]-isocortisol and [4-^{14}C]-isoDOC as determined in solvent system III were 85 and 93% respectively. Because isocortisol is labile, attempts to obtain higher purity were not successful.

Preparation of steroidal 20-hydroxy acids

The synthesis of steroidal 20-hydroxy-21-oic acids was achieved by the method of Lewbart and Mattox[14]. Methyl esters were prepared by treating the acids with diazomethane in diethyl ether. They were purified by thin layer chromatography in solvent system IV.

Product identification

Product of E_1 isozyme: [4-^{14}C]-isocortisol (1.05 μCi, 690 nmol) dissolved in 50 μl of methanol was incubated with 2 μmol of NAD^+ and 0.2 mg of human liver aldehyde dehydrogenase E_1 in 0.088 M tricine buffer, pH 8.5, in a total vol. of 1 ml at room temperature for 3.75 hr. A control was run with no enzyme. The reaction was stopped by adding ethyl acetate (0.5 ml) and an excess of solid sodium chloride. A single extraction of the aqueous phase with ethyl acetate to remove neutral steroids was performed. The aqueous layer was then acidified to pH 2 with 3 N HCl and extracted 4 times with 0.5 ml ethyl acetate each time. The combined extracts were washed with distilled water followed by saturated sodium chloride and dried with anhydrous sodium sulfate. The solvent was reduced to a small volume under a stream of nitrogen and the product was purified by preparative thin layer chromatography in solvent system IV. Radioactivity on the plate was localized with a Packard Recording Radiochromatogram Scanner, model 7201. To confirm the identity of the metabolite, the radioactive polar product was cochro-

matographed as the free acid and as the methyl ester with authentic synthetic reference compounds in different solvent systems. Product of E_2 isozyme: [4-^{14}C]-isoDOC (0.5 μci, 750 nmol) dissolved in 50 μl of methanol was incubated with 2 μmol of NAD^+ and 0.3 mg of enzyme E_2 and 0.1 M tricine buffer, pH 8.5, in a total volume of 1 ml for 1.5 h. Products were extracted and analysed as above for the product of E_1 enzyme.

Reverse isotope dilution of methylated products. Reverse isotope dilution was carried out on the acid methyl ester prepared by reacting the free acids with excess of diazomethane in ether, for the methyl esters could be crystallized, and the free acids could not. To a solution of pure methyl ester of 11β,17,20β trihydroxy-3-oxo-4-pregnen-21-oic acid (7 mg/ml) or 20β-hydroxy-3 oxo-4-pregnen-21-oic acid (15 mg/ml) in dichloromethane was added respectively 0.12 μCi and 0.008 μCi of the methyl esters of the corresponding labeled incubation product of enzyme action. Hexane was added until turbidity developed. The mixture was cooled in ice. After 30 min the supernatant liquid was removed, the crystals dried under a stream of nitrogen and aliquots transferred to tared aluminium trays. The remaining crystals were redissolved in dichloromethane and recrystallized as before. Three crops were collected in this way. They and an aliquot of the final mother liquor were weighed after drying overnight in a desiccator.

Determination of Radioactivity

^{14}C was counted in 4 ml of ACS scintillation fluid fortified with 0.02% POPOP in a Packard 3380 Scintillation Counter. The counts were corrected for quenching, and expressed as disintegrations per minute.

RESULTS

pH Dependence

Figure 1 shows how pH affects the activities of enzyme E_1 with the 17β-hydroxylated isosteroids, isocortisol and isoTHF, and of enzyme E_2 with isoDOC. The curves were obtained in 0.09 M tricine buffer. E_1 and E_2 each show a single pH optimum at approximately pH 9. These values differ somewhat from those obtained with propionaldehyde as substrate. They are generally consistent with the high optima shown by liver aldehyde dehydrogenases from humans and other species reported by us [5] and others [15–18].

Substrate specificity

The substrate specificities of the aldehyde dehydrogenase E_1 and E_2 are shown in Table 1. The values were all determined at a fixed concentration of steroid (3×10^{-4} M) and NAD^+ (5×10^{-3} M). Each substrate was oxidized by both enzymes, but the rates varied over a wide range. There appeared to be no clear cut preference of the enzymes for any particular structural characteristic of the steroid. The substrate most rapidly oxidized by E_1 was isoTHF, and isoDOC was the best substrate for E_2. A carbonyl replacing a hydroxy group at C-20 (i.e. 21-dehydro steroid) greatly decreased the rate of oxidation of the aldehyde at C-21 when E_2 was the enzyme. E_1 also oxidized most ketoaldehydes more slowly than the corresponding isosteroids. However, isoDOC (20-hydroxy-3-oxo-4-pregnen-21-al) was not as good a substrate as 21 dehydro DOC; isoB (11β, 20-dihydroxy-3-oxo-4-pregnen-21-al) and 3,20 dihydroxy-5-pregnen-21-al were oxidized at about the same rate as the corresponding ketoaldehydes. E_2 oxidized acetaldehyde 2.5 times faster that E_1. The evidence

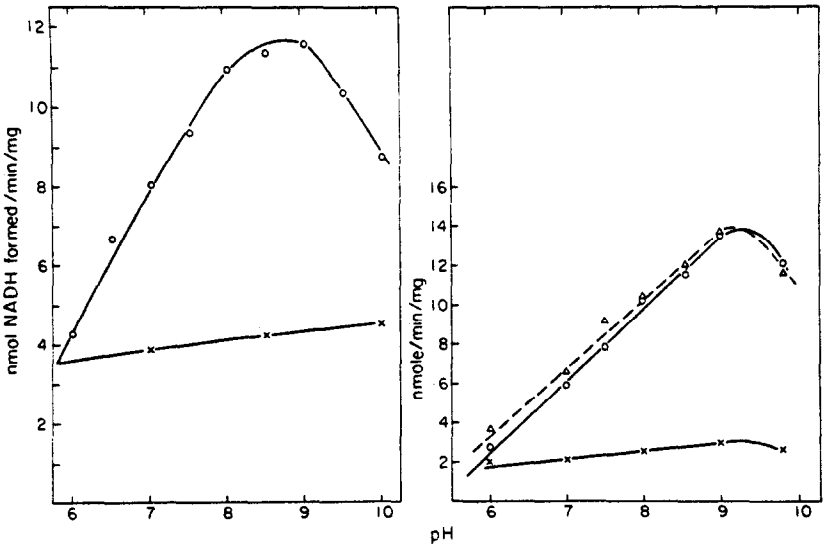


Fig. 1. Oxidation of isocorticosteroids by human liver aldehyde dehydrogenases E_1 and E_2 as a function of pH. The assay mixtures contained *Left panel*: 0.1 M tricine buffer, 0.5 mM NAD^+, 60 μg of E_2, 60 μM isoDOC in 20 μl of methanol (○) or 20 μl of methanol with no steroid (×). *Right panel*: 0.1 M tricine buffer, 0.5 mM NAD^+, 30 μg of E_1, 60 μM isocortisol (△), 60 μM isotetrahydrocortisol (3α, 11β,17, 20β tetrahydroxy-5β-pregnan-21-aldehyde) (○), or no steroid (×), and 20 μl methanol.

Table 1. Substrate specificity of two aldehyde dehydrogenase isoenzymes on corticosteroid derivatives*

Parent steroid	Oxidation rate with isoenzyme E1		Oxidation rate with isoenzyme E2	
	Isosteroid	21-dehydro steroid	Isosteroid	21-dehydro steroid
	nmol NADH formed $(min)^{-1}$ (mg protein)-1			
Cortisol	5.9 ± 1.3	0.5 ± 0.4	0.93 ± 0.27	0.15 ± 0.11
Tetrahydrocortisol	8.1 ± 4.2	0.13 ± 0.18	1.3 ± 0.44	0.19 ± 0.02
Cortisone	3.2	0.0 ± 0.0	0.4 ± 0.2	0.2 ± 0.02
Tetrahydrocortisone	4.2 ± 0.72	0.0 ± 0.0	1.07 ± 0.15	0.2 ± 0.01
11-deoxycortisol	4.1 ± 0.61	0.7 ± 0.13	1.2 ± 0.46	0.2 ± 0.0
Prednisolone	2.2 ± 0.21	0.0 ± 0.0	0.45	0.0 ± 0.0
11-deoxycorticosterone	0.0	3.7 ± 1.8	1.88 ± 0.49	0.46 ± 0.26
Corticosterone	3.3 ± 1.1	2.89 ± 1.07	0.87 ± 0.32	0.72 ± 0.28
21-hydroxy pregnenolone	0.8 ± 0.28	1.5 ± 0.17	0.3 ± 0.07	0.21 ± 0.14

* Incubation was performed with 0.3 μmol of steroid dissolved in 50 μl of methanol, 5 μmoles of NAD^+, 80 μg of enzyme E_1 or 100 μg of E_2 in a final vol. of 1 ml in Tricine, pH 8.5. Values are means and standard deviations for three determinations, except where single values occur.

presented in Table 1 indicates that, with the exception of isoDOC, isosteroids were generally less effectively oxidized by E_2 than E_1.

Identification of steroid oxidation products

The product of oxidation of isocortisol with E_1 was determined by thin layer chromatography. Development was in the neutral solvent system III. Of the total radioactivity on the plate, 63% remained at the origin. The corresponding amount at the origin in the absence of enzyme was 7%. The highly polar material became mobile in solvent systems containing formic acid. The mobility corresponded to that of 11β, 17, 20β-trihydroxy-3-oxo-4-pregnen-21 oic acid in solvent systems III ($R_F = 0.02$), IV ($R_F = 0.11$) and VII ($R_F = 0.06$). Chromatographic mobility of the methyl ester derivative of the polar product was identical to the derivative of the authentic reference compound. In three solvents, the values were $R_F = 0.45$, (III); $R_F = 0.10$, (VI); $R_F = 0.26$, (VIII). More than 85% of the polar material moved with the expected hydroxy acid.

The product of the oxidation of DOC by E_2 was analyzed in a similar way. The extent of conversion of isoDOC to polar metabolite was 23% after development with solvent system V. Polar products in the absence of enzyme were 2% of the total counts on the plate. The polar product of enzyme action was 90% homogeneous and corresponded in mobility to 20β-hydroxy-3-oxo-4-pregnen-21-oic acid in solvent system I ($R_F = 0.03$); II ($R_F = 0.08$); VII ($R_F = 0.28$). Methyl esters of known and unknown steroids coincided in solvent systems I ($R_F = 0.25$); VI ($R_F = 0.42$); VIII ($R_F = 0.45$).

Identification by reverse isotope dilution. Specific activities of the methyl ester of the product of E_1 isozyme action on 4-^{14}C labeled isocortisol mixed with authentic steroid acid ester remained constant in three successive cycles of crystallization. Values were 39,000 ± 1200; 39,200 ± 3100 and 37,800 ± 1900 dpm per mg. The final supernatant had a specific activity of 34,000 ± 1900 dpm per mg. The corresponding figures for the methyl ester of the product of E_2 isozyme action on isoDOC were 1340 ± 30; 1300 ± 15; 1300 ± 20 and 1280 ± 30 dpm/mg.

Kinetic analysis

Analyses of the kinetic properties of the system were performed in order to establish if the behavior of the enzymes with steroid aldols were the same as those found with aliphatic aldehydes. Oxidation of the isosteroids to hydroxy acids by aldehyde dehydrogenases E_1 and E_2 were irreversible. The acids up to a level of 600 μM did not inhibit either enzyme. Solubility of the steroids in aqueous medium was limited and consequently measurements of initial velocities could only be obtained over a restricted range of steroid concentrations. Steroids were introduced into the enzyme mixture in methanol. The presence of 1% methanol (300 mM) in the final incubation mixture had no detectable effect on the initial velocities.

Analyses of the kinetic behavior of the enzymes E_1 and E_2 were undertaken, using steroidal aldols as substrates. With enzyme E_1, double reciprocal plots of initial velocity at pH 8.6 using isocortisol at different constant concentrations of NAD^+ gave straight lines converging on the abscissa. Secondary plots of slopes and intercepts against reciprocal nonvaried substrate concentration were linear (Fig. 2). An identical pattern was obtained with dehydrogenase E_2, when isoDOC was the steroid substrate. Corresponding initial velocity data for NAD^+ as variable substrate and isosteroid as fixed substrate also gave converging patterns for both enzymes. The initial velocity data for each enzyme was fitted to equation 1 de-

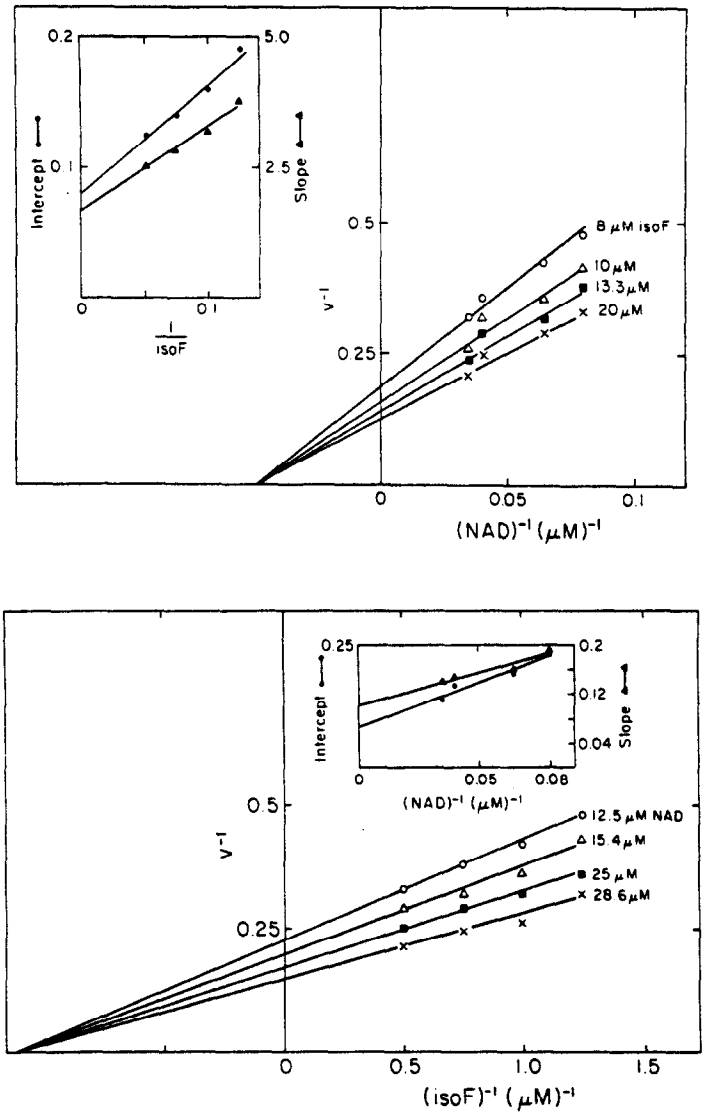


Fig. 2. Kinetics of the enzyme catalyzed oxidation of isocortisol by NAD^+. Concentrations of NAD^+ and isocortisol are indicated in the figures. The secondary plots determined from intercepts and slopes are shown in the insets. Slope (▲) and intercept (●) replots. Solid lines of primary plots were drawn from calculated fit to equation 1. Reaction mixture contained in 1 ml of 0.088 M Tricine, pH 8.5, steroid and NAD^+ as indicated; 20 μl of methanol, and 30 μg of human aldehyde dehydrogenase E_1. *Upper panel*: Double reciprocal plot with NAD^+ as the variable substrate; *Lower panel*: double reciprocal plot with isocortisol as the variable substrate.

scribing a sequential mechanism [19]

$$v = \frac{VAB}{K_{ia}K_b + K_aB + K_bA + AB}. \quad (1)$$

In equation 1, K_a and K_b are Michaelis constants of substrates A and B, NAD and steroid, respectively. K_{ia} is the dissociation constant for substrate A. V and v are maximal velocity and initial velocity, respectively. Values for the kinetic constants obtained from this plot are listed in Table 2. Thus, the behavior of the human enzyme E_1 and E_2 with respect to the isosteroids is consistent with a sequential mechanism, and resembles that for the simple homologue glyceraldehyde reported by Sidhu and Blair[20] using an enzyme preparation corresponding to E_2. It is concluded that both substrates must bind to the enzyme before any product is released.

Inhibition studies

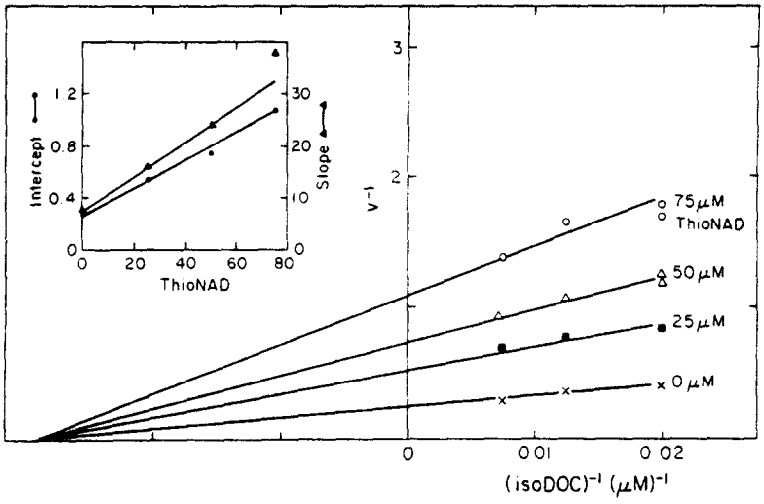
It is not possible to distinguish ordered and random sequential mechanisms using initial velocity data. An indication of the mode of substrate addition can be made by the use of inhibitors. Because of the limited solubility of the steroids in aqueous medium and the irreversibility of the reaction, we utilized an approach based in the following considerations. In an ordered BiBi mechanism, an inhibitor will give com-

Table 2. Kinetic constants for aldehyde dehydrogenase activity

Substrate	Enzyme	K_a μM	K_b μM	K_{ia} μM	V^*_{max} nmol/min/mg P
Isocortisol	E_1	21.5 ± 0.67	9.0 ± 3.0	21.5 ± 0.67	18.8 ± 4.8
isoDOC	E_2	67.5 ± 0.7	63.9 ± 0.4	67.5 ± 0.7	11.6 ± 3.2

* Values are corrected to compensate for loss of activity of enzymes with time during storage.

petitive inhibition with respect to the first substrate (A) if it interacts with the binding site for that substrate only. Non-competitive inhibition will result with respect to the second substrate (B). An inhibitor interacting at the binding site for substrate (B) forms a dead end inhibitor with the binary complex formed from enzyme and substrate (A). Consequently, the inhibition pattern is competitive with respect to substrate B and uncompetitive with substrate A. When substrates A and B are kinetically equivalent, and both can form binary complexes prior to the formation of the ternary AB complex (random sequential



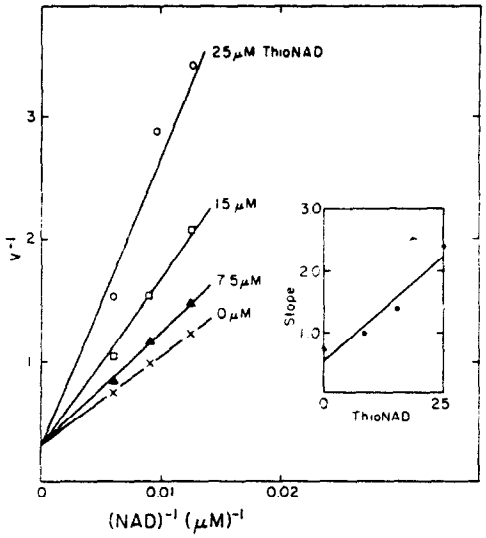


Fig. 3. Kinetics of inhibition of isoDOC oxidation by Thionicotinamide adenine dinucleotide. *Upper panel*: Double reciprocal plot with isoDOC as the variable substrate. Reaction mixtures contained 0.088 M Tricine, pH 8.5, 300 μM NAD, 60 μg of enzyme E_2, 20 μl of methanol, and inhibitor as shown. Inset, slope (▲) and intercept (●) replots. *Lower panel*: Double reciprocal plot with NAD^+ as the variable substrate. Reaction mixtures contained 0.88 M Tricine pH 8.5, 200 μM isoDOC, 60 μg of enzyme E_2, 20 μl of methanol and inhibitor as shown. Inset, slope (●) replot.

Table 3. Inhibition of human liver aldehyde dehydrogenases E_1 and E_2

Enzyme	Inhibitor (range, μM)	Variable substrate (range, μM)	Fixed substrate (μM)	Pattern	K_i slope (μM)	K_i intercept (μM)
E_1	Chloral hydrate (0–10)	isoF (8–50)	NAD^+ (100)	Competitive	1.7 ± 0.17	
		NAD^+ (6.7–50)	isoF (125)	Non competitive	16 ± 2.1	18 ± 2.0
E_1	Thio NAD^+ (0–25)	isoF (10–50)	NAD^+ (150)	Non competitive	22.3 ± 5.7	53.3 ± 13.8
		NAD^+ (13–50)	isoF (125)	Competitive	7 ± 0.8	—
E_2	Chloral Hydrate (0–20)	isoDOC (33–133)	NAD^+ (200)	Competitive	4.2 ± 0.66	—
		NAD^+ (40–182)	isoDOC (200)	Non competitive	152	380
E_2	Thio NAD^+ (0–70)	isoDOC (33–133)	NAD^+ (300)	Non competitive	24.7 ± 2.1	20.3 ± 1.8
		NAD^+ (33–167)	isoDOC (200)	Competitive	22.6 ± 5.3	—

BiBi mechanism) an inhibitor binding at one substrate site will inhibit competitively with that substrate and non-competitively with the other substrate.

In these studies, thionicotinamide adenine dinucleotide, an analogue of NAD^+, was employed as a dead end inhibitor specific for the NAD^+ site. It gave linear competitive inhibition with respect to NAD^+ and linear non-competitive inhibition with respect to isoDOC with E_2 enzyme as shown in Fig. 3. An identical pattern was found for isocortisol and enzyme E_1. A summary of the kinetic constants are presented in Table 3. This pattern is consistent with a random addition mechanism. It could also be interpreted as indicating an ordered mechanism in which NAD^+ adds first. A mechanism with isosteroid adding first is ruled out.

An analogue of acetaldehyde, chloral hydrate*, gave linear competitive inhibition with either steroid and the respective enzymes maintaining NAD^+ as the fixed substrate. Chloral hydrate gave linear non-competitive inhibition with respect to NAD when either isoDOC or isocortisol was used with the corresponding enzyme. The pattern obtained with E_1, NAD^+, and isocortisol is shown in Fig. 4. The kinetic inhibitor constants are summarized in Table 3. The patterns observed again suggest a random mechanism. A compulsory ordered mechanism in which isosteroid adds first is inconsistent with the findings of the previous paragraph. A compulsory ordered sequence with NAD^+ adding first is not possible, and we conclude therefore that the reaction proceeds by a random sequential mechanism [19].

Inhibition of enzymes E_1 and E_2 by the products of their respective reactions was examined. With enzyme E_1, the steroid acid 11β,17,20β-trihydroxy-3-oxo-4-pregnen-21-oic acid did not inhibit the oxidation of isocortisol when added in amounts up to 400 μM. The reaction conditions were: isocortisol, 50 μM; NAD, 50 μM; E_1, 23 μg in 1 ml of 0.088 M Tricine, pH 8.5. Enzyme E_2 was not inhibited by 20β-hydroxy-3-oxo-4-pregnen-21-oic acid in amounts of steroid up to 400 μM in a system containing isoDOC, 150 μM; NAD^+, 200 μM and E_2, 60 μg. With both E_1 and E_2 inhibition by NADH wus determined up to 600 μg per ml incubation mixture. Inhibition of activity was seen, but because of the strong absorption at 340 nm of the reduced nucleotide which taxed the limits of our spectophotometer, it was not possible to get reproducible measurements of the inhibition and consequently kinetic analysis by end product inhibition could not be used as a diagnostic criterion. Crude estimates of K_i were 240 μM for E_1 and 420 μM for E_2.

DISCUSSION

The aldehyde dehydrogenases that have been isolated from the livers of various species show broad substrate specificity [5–7, 15, 16, 21, 24]. Their physiological functions have never been precisely defined. *In vitro*, they metabolize any aldehyde that has the required structural characteristics. Included among these are the 26-aldehyde sterol metabolites, precursors of the bile acids [22, 23]. In this paper we show that an important class of intermediates formed from the corticosteroids and containing a 17-aldol side chain is oxidized to α-hydroxy acid with NAD^+ dependent aldehyde dehydrogenases E_1 and E_2 from human liver. These enzymes, which have been purified to homogeneity, show no qualitative differences in their behavior with steroid aldols as substrates when compared with simple aliphatic aldehydes and glyceraldehyde. The end product with steroid aldol in each case is the hydroxy acid, demonstrating that the

* Since chloral hydrate dissociates reversibly to a small extent into its free aldehyde form, chloral (Cl_3CHO), we have tested chloral hydrate as substrate for both isozymes E_1 and E_2. Under the conditions used in this paper, it was entirely inactive.

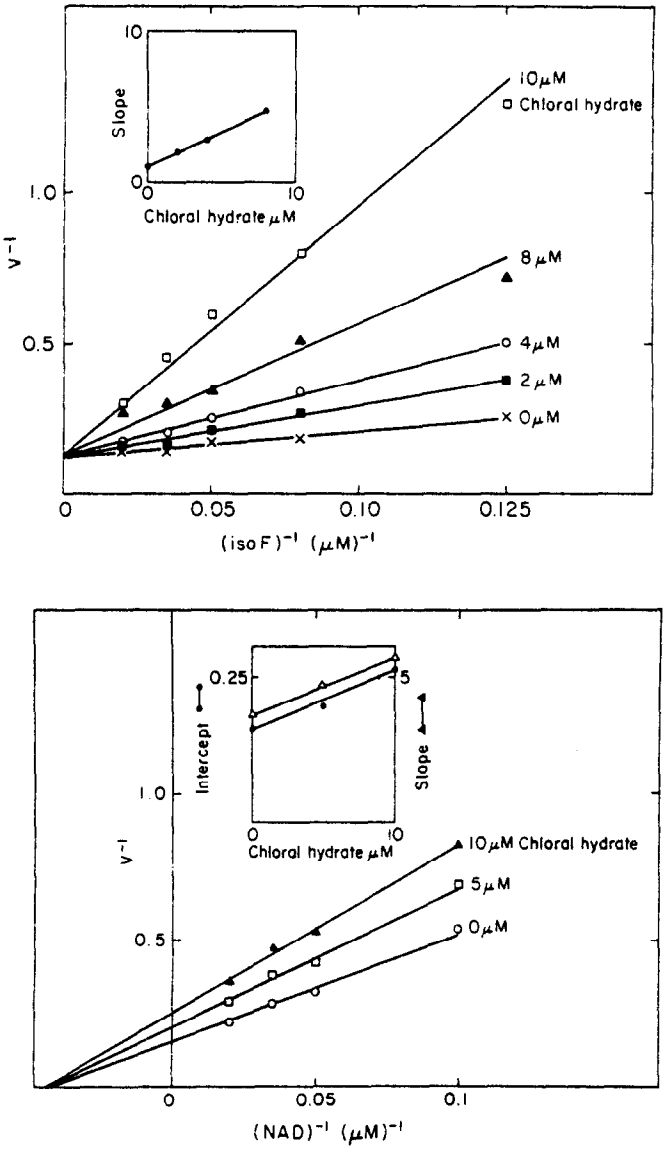


Fig. 4. Kinetics of inhibition of isocortisol oxidation by chloral hydrate. *Upper panel*: Double reciprocal plot with isocortisol as the variable substrate. Reaction mixtures contained 0.088 M Tricine, pH 8.5, 100 μM NAD^+, 30 μg of enzyme E_1, 20 μl of methanol, and inhibitor as shown. Inset, slope (●) replot. Lower panel: Double reciprocal plot with NAD^+ as the variable substrate. Reaction mixtures contained 0.088 M Tricine, pH 8.5. 125 μM isocortisol, 30 μg enzyme E_1, 20 μl of methanol and inhibitor as shown. Inset slope (▲) and intercept (●) replot.

action of the enzyme is limited to oxidation of accessible aldehydes. The two enzymes show quantitative differences in their ability to oxidize steroid. E_1 is a better enzyme than E_2 for steroids. This is opposite to their effects on aliphatic aldehydes [5]. The data on Table 1 suggests that E_1 shows a preference for 17α-hydroxy steroids and E_2 prefers 17-deoxy substrates. The distinction is not clear cut; both enzymes oxidized all substrates but at distinctly different relative rates. These properties are similar to those which we found earlier for the horse liver enzyme F_1 and F_2 [4].

The kinetic behavior of the two enzymes with these steroids appears consistent with a random BiBi mechanism. This is in agreement with the conclusion of Sidhu and Blair[20] who studied the E_2 enzyme with aliphatic aldehydes as substrates. Our evidence shows that the same mechanism can be attributed to E_1 as well. However, while investigating the kinetic mechanism of E_1 isozyme with acetaldehyde [8] a biphasic effect of chloral hydrate with varied NAD was observed. In the paper of Vallari and Pietruszko[8] intercept effects occurred at all chloral hydrate concentrations used, while a small but reproducible slope effect occurred only at low concentrations of chloral hydrate. Their intercept replot was linear while the slope replot was hyperbolic. They suggest that, in addition to being a dead end inhibitor, chloral hydrate

can also alter the reaction pathway to a slower one. In the present investigation, the concentrations of chloral hydrate used are low but higher concentrations of this inhibitor blocked steroid oxidation almost completely. For this reason, although we have concluded that the mechanism with steroids is random, the possibility of a sequential ordered mechanism with NAD binding first cannot be entirely excluded. We and Vallari and Pietruszko[8] find some differences in kinetic and inhibition constants. These may be attributed to differences in the pH of measurement as well as differences due to the nature of the substrates used.

In addition to the steroid aldols the steroidal ketoaldehydes, i.e. the 21-dehydro-corticosteroids, are also substrates but, with some exceptions, are more poorly oxidized. The 17-deoxyketoaldehydes are good substrates for the E_1 enzyme, but not for the E_2 enzyme. Kraemer and Deitrich[16] found that the human enzyme they purified, which probably corresponds to E_2, oxidized glyoxal and methylglyoxal. The steroidal ketoaldehydes can be viewed as substituted glyoxals; thus bulky substituents in this series do not block enzyme action. However, glyoxals in general are poor substrates for aldehyde dehydrogenase, and they are more likely oxidized to keto acids by ketoaldehyde dehydrogenase [25].

These studies support our proposal that the metabolic conversions of steroid ketols to hydroxy acids occur by a sequence of reactions in which the terminal step is oxidation of an aldol intermediate by aldehyde dehydrogenase. Although our results affirm that the steroid aldol intermediates may be substrates, we have yet to present direct evidence that aldehyde dehydrogenases E_1 or E_2 are in fact acting in this capacity in the intact liver.

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