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OXIDATION OF STEROIDS WITH THE 20β-HYDROXY-21-OXO SIDE CHAIN TO 20β-HYDROXY-21-OIC ACIDS BY HORSE LIVER ALDEHYDE DEHYDROGENASES

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SUMMARY

In this paper we show that C_{21} steroids with the 20β-hydroxy-21-aldehyde side chain (isocorticosteroids) are oxidized by horse liver aldehyde dehydrogenases to 20β-hydroxy-21-oic acids. The aldehyde dehydrogenases F1 and F2, purified as described by Eckfeldt *et al.*[10] had different substrate specificities. Isoenzyme F1 oxidized 17α-hydroxylated steroids; isoenzyme F2 oxidized 17-deoxysteroids. Steroids with the 20-oxo-21-aldehyde side chain were poor substrates. With isoenzymes F1 and F2, enzyme activity increased between pH 6 and 9.5 with no optimum. The K_M values of the F1 enzyme for isocortisol was 3×10^{-5} M and of F2 for isoDOC was 9×10^{-5} M with NAD^+ as cofactor. Low concentrations of methanol, ethanol and isopropanol inhibited the oxidations. The acids resulting from the oxidation of 11β,17,20β-trihydroxy-3-oxo-4-[1,2-^{3}H]-pregnen-21-al and 20β-hydroxy-3-oxo-4-[4-^{14}C]-pregnene 21-al were isolated and identified. The results support our postulate that the oxidation of corticosteroids to 20-hydroxy-21-oic acids may proceed through 20-hydroxy-21-aldehyde intermediates.

INTRODUCTION

We have demonstrated that a significant proportion of corticosteroids are metabolized to steroidal 21-oic acids *in vivo* by human subjects and *in vitro* by mammalian liver [1–5]. Both 20-oxo and 20-hydroxy acids have been isolated and identified unambiguously [2, 3].

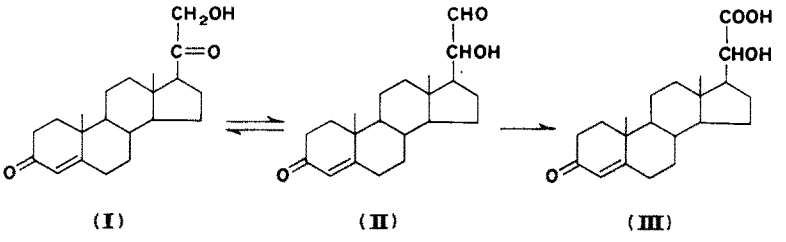
Recently, we isolated an isomerase from hamster liver [6] which catalyzed the reversible interconversion of the ketol (**I**) and aldol (**II**) forms of the steroid side chain (illustrated with 11-deoxycorticosterone, Scheme 1). It appeared to us that a straightforward mechanism for the conversion of the corticosteroid (**I**) to hydroxy acid (**III**) could be achieved by oxidation of the C_{21} aldehyde of the intermediate aldol (**II**) to an acid group. This transformation could be mediated by mammalian liver aldehyde dehydrogenases, since aldehyde dehydrogenases convert steroid aldehydes [4] and sterol aldehydes [7] to acidic products. In order to explore this possibility, substrate amounts of the aldol were needed. We recently synthesized the suspected steroidal 20β-hydroxy-21-aldehyde intermediates (isocorticosteroids) [8, 9] and have taken advantage of this achievement to study the oxidation step **II** → **III** of Scheme 1. In this paper, we show that aldehyde dehydrogenases of horse liver oxidize isocorticosteroids to 20-hydroxy acids and demonstrate significant differences in steroid specificity between two isozymic forms of the dehydrogenase.

The following trivial names have been used: tetrahydrocortisol, 3α,11β,17,21tetrahydroxypregnan-20-one; 17-deoxycortisol, 11β, 21 dihydroxy-4-pregnen-3,20-dione; prednisolone, 11β,17,21-trihydroxy-1,4-pregnadiene-3,20-dione; 21-dehydrocortisol, 11β,17-dihydroxy-3,20-dioxo-4-pregnen-21-al; 21-dehydroDOC, 3,20-dioxo-4-pregnen-21-al; Other 21 dehydro steroids are named in a similar manner. Isocortisol, 11β,17,20β-trihydroxy-3-oxo-4-pregnen-21-al; isoDOC, 20β-hydroxy-3-oxo-4-pregnen-21-al; other isocorticosteroids are named in a similar manner; EDTA, ethylene diamine tetraacetic acid; tricine, N-tris-(hydroxymethyl)-methylglycine.

MATERIALS AND METHODS

Materials

Nicotinamide adenine dinucleotide was purchased from Sigma Biochemicals Co. CM-cellulose (CM52) and DEAE-cellulose (DE52) were obtained from Reeve Angel Co. Bio Gel A-1.5 m was a product of Bio-Rad. Acetaldehyde (Analytical Reagent grade) was used as received from Baker Chemical Co. Cortisol, 11-deoxycorticosterone, tetrahydrocortisol, corticosterone, 17-deoxycortisol and prednisolone were obtained from Steraloids Co. All analytical grade solvents were used as received from the manufacturer. Spectrograde solvents were used for enzyme assay. [1,2-^{3}H]-cortisol (45.9 Ci/mmol) and [4-^{14}C]-11-deoxycorticosterone (59.8 mCi/mmol) were purchased from New England Nuclear Co. Plastic backed precoated silica gel thin layer plates (GF_{254} 0.25 mm) were bought from Brinkmann Instruments, Inc. When the plates were to be used for purification of steroids or quantitative analysis, they were first washed with methanol and air-dried.



(I) (II) (III)

Scheme 1. Proposed pathway for the conversion of steroidal ketols of type (I) to hydroxy acids of type (III).

Preparation of F1 and F2 isozymes of aldehyde dehydrogenase

Horse liver was obtained from a local slaughterhouse and kept frozen until use. Aldehyde dehydrogenases were purified by the method of Eckfeldt *et al.*[10] with modifications to meet smaller scale preparation.

Approximately 80 g of horse liver were partially thawed, minced, and homogenized in 100 ml of 2 mM EDTA in 0.25% thioglycerol in a Waring blendor. All procedures were performed at 3°C. The homogenate was centrifuged at 13,000 *g* for 60 min. The supernatant was subjected to ammonium sulfate fractionation. The fraction sedimenting between 1.4 M and 2.3 M ammonium sulfate was collected and resuspended in 40 ml of 10 mM sodium phosphate buffer, pH 6.3, containing 0.25% thioglycerol and 1 mM EDTA. Before placing it on a CM-cellulose column (Whatman CM 52, 2.5 cm × 20 cm), the suspension was desalted by passing through a Sephadex G-25 column (2.5 cm × 40 cm). The protein fractions from the column were concentrated to approximately 10 ml by ultrafiltration using Diaflo PM-10 membrane. Enzyme active fractions (measured with acetaldehyde as substrate) from the CM-cellulose column eluted with the same phosphate buffer were pooled and the buffer was exchanged with 5 mM imidazole-HCl, pH 7.2, containing 0.25% thioglycerol by passing the enzyme solution through a Sephadex G-25 column (2.5 cm × 40 cm) equilibrated with the same imidazole buffer.

DEAE-cellulose column (Whatman DE 52, 2.5 cm × 22 cm) chromatography was performed on this fraction. After enzyme was adsorbed, the column was washed with 50 ml of imidazole buffer and a linear gradient of sodium chloride (0–0.13 M) was carried out by mixing 250 ml of 0.2 M sodium chloride dissolved in the imidazole buffer into 250 ml of the imidazole buffer.

Two separate peaks of aldehyde dehydrogenase were eluted. Aldehyde dehydrogenase fractions which eluted from DEAE-cellulose column first with lower ionic strength solution was designated as F1 isozyme. This was purified further on a Bio Gel A-1.5 m Column (2.6 × 52 cm). F2 isozyme was eluted with increased concentration of sodium chloride and was also passed through Bio Gel A-1.5 m column. All experiments were done with these fractions unless otherwise stated. Proteins were measured by Kalckar's method [11].

Assay of aldehyde dehydrogenase activity

Enzyme activity was determined spectrophotometrically by following NADH formation at 340 nm using a Gilford Model 2000 automatic recorder attached to a Beckman DU monochrometer. During purification, enzyme activity was monitored in 0.1 M tricine buffer, pH 8.4, containing 0.5 μmol of NAD^+ and 1 μmol of acetaldehyde at 25°C. Total volume of assay medium was 1.0 ml. For kinetic studies, steroid substrate dissolved in 50 μl of methanol was added in place of acetaldehyde. Controls were run with 50 μl of methanol instead of steroid. K_M values were calculated by least squares fitting of data with the aid of a Wang 600 computer.

Preparation of isocorticosteroids

21-Dehydro-20-dihydro-corticosteroids (isocorticosteroids) were synthesized chemically from 21-dehydrocorticosteroids by the method of Oh and Monder[8]. All 21-dehydrocorticosteroids were synthesized by oxidation of corticosteroid with cupric acetate [12]. [4-^{14}C]-IsoDOC (7.3 mCi/mmol) was prepared by enzymic reduction of [4-^{14}C]-21-dehydroDOC as described by Lippman and Monder[9], and was purified by thin layer chromatography with chloroform–methanol (98:2, v/v) just before use. Purity was over 95%. [1,2-^{3}H]-Isocortisol (6.6 mCi/mmol) was synthesized chemically [8] and used immediately. Purity of [1,2-^{3}H]-isocortisol was 88% as determined by thin layer chromatography in chloroform–isopropanol (90:10, v/v). Isocortisol is so much more labile than isoDOC that purification by thin layer or column chromatography cannot be applied to it as with isoDOC.

Preparation of steroidal 20 hydroxy acids

The synthesis of steroidal-20-hydroxy-21-oic acids was achieved by the method of Lewbart and Mattox[13]. Their properties are described in our earlier publications [1, 3, 14].

Isolation of steroid products

(*1*) *Product of F1 isozyme* [1,2-^{3}H]-Isocortisol (0.5 μCi, 75 nmol) dissolved in 50 μl of methanol was incubated with 1 μmol of NAD^+, and 0.3 mg of aldehyde dehydrogenase F1 in 0.1 M tricine, pH 8.4, at room temperature for 1 h. Total volume was 1.0 ml. The reaction was stopped by adding ethyl acetate and solid sodium chloride to saturation. Following a single extraction of steroid with 0.3 ml of ethyl acetate, the resulting aqueous layer was acidified to pH 2 with 3N hydrochloric acid and reextracted with ethyl acetate three times more to ensure the extraction of acidic metabolites. All organic layers were combined, washed with saturated sodium chloride solution twice and dried with anhydrous sodium sulfate. The solvent was reduced to a small volume under a stream of nitrogen. The extract was chromatographed on a thin layer plate with benzene–ethanol (90:10, v/v) as developing solvent in the cold room. Controls were run under the same condition without enzyme. The band corresponding to polar product was scraped off the plate and steroids were eluted from silica gel with methanol.

(*2*) *Product of F2 isozyme.* [4-^{14}C]-IsoDOC (0.33 μCi, 45 nmol) dissolved in 50 μl of methanol was incubated with 1 μmol of NAD^+ and 0.3 mg of aldehyde dehydrogenase F2 in 0.1 M tricine, pH 8.4, for 1 h at room temperature. Steroids were extracted as described above.

Reverse isotope dilution of methylated products

Reverse isotope dilution was carried out on the acid methyl esters prepared by reacting the free acids with diazomethane in ether, for the methyl esters were less labile and easier to crystallize than free acids. Methyl esters of radioactive products were purified by thin layer chromatography, then cocrystallized with the corresponding synthetic steroids. The solvent pairs acetone–hexane and dichloromethane–hexane were used for the cortisol and DOC metabolites, respectively.

Determination of radioactivity

Tritium and ^{14}C were counted in 10 ml of Aquaflour (New England Nuclear Corp.) with a Packard 3380 Scintillation Spectrophotometer. All values were corrected by the method of external standardization, and are expressed as d.p.m.

RESULTS

Horse liver aldehyde dehydrogenase activity

A typical separation of F1 and F2 isozyme of horse liver aldehyde dehydrogenase with DEAE-cellulose column is shown in Fig. 1. The enzymes were each purified further by passing them through Bio Gel A-1.5 m column as described in Methods. Approximately 13 mg and 15 mg of the respective purified isozymes were obtained from 80 g of frozen horse liver.

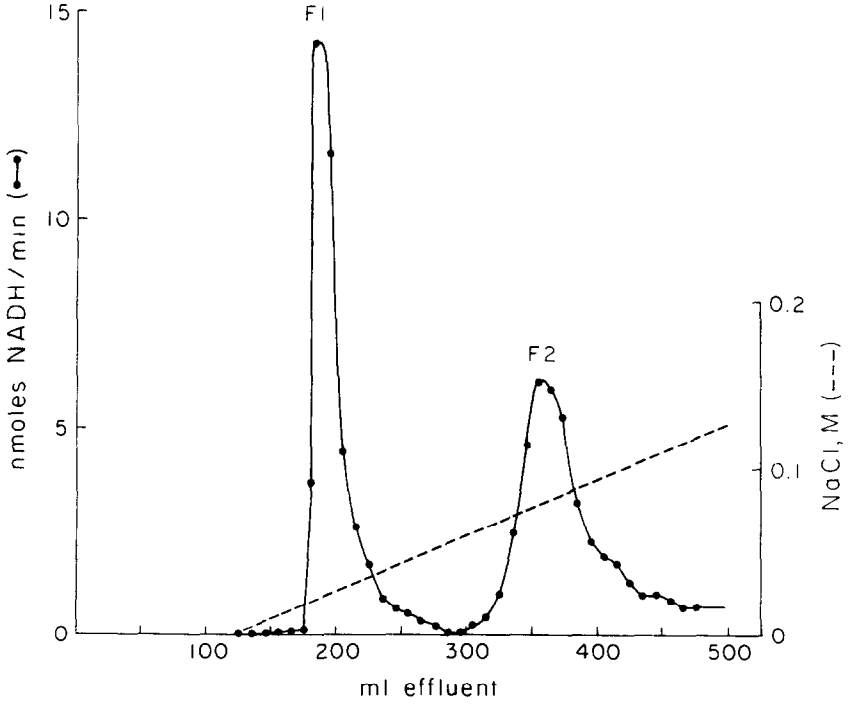


Fig. 1. Elution profiles of horse liver aldehyde dehydrogenase F1 and F2 isozymes from DEAE-cellulose. The column (2.5 cm × 22 cm) was developed with 5 mM imidazole-HCl, pH 7.2, with a gradient of sodium chloride. Buffer contained 0.25% thioglycerol. (——) enzyme activity (– – –) sodium chloride gradient.

Table 1. Substrate specificity of two aldehyde dehydrogenase isozymes on corticosteroid aldehydes*

		F1 isozyme		F2 isozyme	
	17α-OH	iso-corticosteroids	21-dehydro-corticosteroids	iso-corticosteroids	21-dehydro-corticosteroids
		(μmol NADH formed/min/g. protein)			
11-deoxycortico-sterone	−	0.4 ± 0.6	6.2 ± 0.9	17.3 ± 1.8	2.0 ± 0.4
Corticosterone	−	5.7 ± 1.2	11.7 ± 0.4	6.7 ± 0.9	1.9 ± 0.2
11-deoxycortisol	+	40.2 ± 1.7	7.1 ± 0.4	2.9 ± 0.4	1.0 ± 0.4
Cortisol	+	44.2 ± 1.4	10.5 ± 1.1	1.2 ± 1.4	0.0 ± 0.3
Cortisone	+	38.5 ± 1.2	4.9 ± 0.9	2.8 ± 1.6	1.6 ± 0.4
Tetrahydrocortisol	+	42.7 ± 1.4	0.0 ± 0.0	2.3 ± 1.5	0.0 ± 0.0
Prednisolone	+	48.2 ± 2.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

* Incubation was carried out with 0.3 μmol of steroid dissolved in 50 μl of methanol, 0.5 μmol of NAD^+, approximately 50 μg of enzyme and 88 μmol of Tricine buffer, pH 8.4, at room temperature. Total volume was 1.0 ml. Values are expressed as Mean ± S.D. (at least 3 determinations).

Specific activities for our most active preparation obtained with acetaldehyde were 135 nmol/min/mg and 277 nmol/min/mg protein for F1 and F2, respectively. These values are about one half of those reported by Eckfeldt *et al.*[10] with propionaldehyde as substrate.

Substrate specificity

Distinctive differences in substrate specificity between the two isozymes were observed. Effects of the dehydrogenases on steroids containing an aldehyde group at C-21 are shown in Table 1. The F1 isozyme oxidized isocorticosteroids with a 17α-hydroxy group, including isocortisol, iso-11-deoxycortisol, isocortisone, isotetrahydrocortisol and isoprednisolone. These were oxidized at almost equal rates (approx. 40 nmol/min/mg protein). Substituents in the fused ring system did not affect the rate of oxidation. IsoDOC and isocorticosterone, which do not have the 17α-hydroxy group, were poor substrates for F1 isozyme. The 17-deoxy steroids were preferred substrates for the F2 enzyme. Oxidation of isocorticosteroids with the 17α-hydroxy group by F2 isozyme was negligible. The 20-oxo-21-dehydro steroids were also very poor substrates for F2. F1 oxidized them at a slow rate, ranging from 5 to 12 nmol/min/mg protein. There was not clear relationship between structure of steroid and susceptibility to action in the case of the 21-dehydrocorticosteroids.

pH Optimum

Enzyme activities increased between pH 6 and pH 9.5 with no optimum. As shown in Fig. 2, the patterns of the pH activity curves were similar for both F1 isozyme with isocortisol as substrate and F2 with isoDOC or acetaldehyde as substrate. Potassium ions did not activate the reaction.

Kinetics

Both enzymes used NAD^+ as cofactor for steroid aldehyde oxidation. $NADP^+$ was inactive at all concentrations tested, up to 5 mM. Apparent K_M for NAD^+ with F1 isozyme measured with 0.28 mM isocortisol was 3.5×10^{-5} M. With 9 mM acetaldehyde the K_M value of NAD was 1.5×10^{-5} M. A value of 3.9×10^{-5} M was obtained for the F2 isozyme with 0.30 mM isoDOC. Apparent K_M values of F1 and F2 isozymes were 3.1×10^{-5} M and 9.2×10^{-5} M for isocortisol and isoDOC, respectively when NAD^+ was added at 5×10^{-4} M.

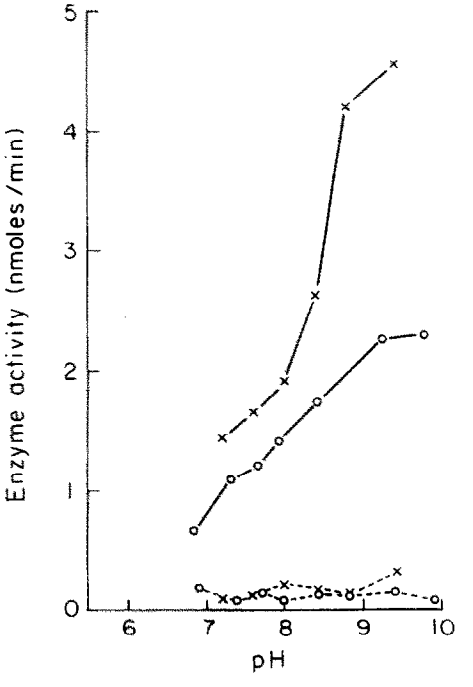


Fig. 2 pH-Activity curves of aldehyde dehydrogenase F1 and F2 isozymes. Enzyme activity at indicated pH values was determined with isocortisol (×——×) using F1 isozyme and with isoDOC (O——O) using F2 isozyme. Controls for F1 enzyme (×·····×) and F2 (O·····O) contained methanol in place of steroid. Analyses were performed in Tricine with 0.5 mM NAD^+.

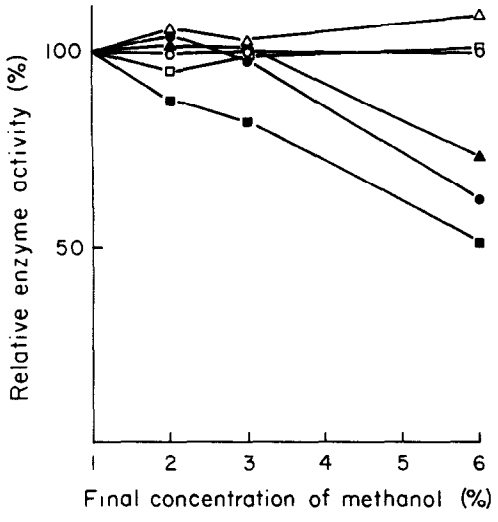


Fig. 3 Effect of methanol on steroid oxidation. Substrate steroids dissolved in 10 μl of methanol were added to the reaction mixture (total volume 1.0 ml). Activities at this alcohol concentration (1%) were considered 100%. Concentrations of isocortisol with F1 enzyme were △—△, 0.28 μM; ○—○, 0.14 μM; □—□, 0.07 μM. Concentrations of isoDOC with F2 enzyme were: ▲—▲, 0.30 μM; ●—●, 0.15 μM; ■—■, 0.08 μM.

Effect of alcohol

Neither F1 nor F2 isozymes were affected by added alcohol at 6% of final concentration when acetaldehyde was substrate. However, in 6% methanol, oxidation of isoDOC (0.3 mM) by F2 isozyme was decreased by 30%. Figure 3 shows that the relative inhibitory effect was enhanced when substrate concentration was lower; It was 40% at 0.15 mM and 50% at 0.08 mM. At the lowest concentration of steroid tested, 0.08 mM, addition of alcohol to a final concentration of 2% already resulted in 10–25% inhibition. Methanol, ethanol and isopropanol had similar effects. With F1 isozyme, the effect of alcohol on isocortisol oxidation was less noticeable. At 0.3 mM isocortisol no inhibitory effect was observed up to 6% of alcohol concentration.

Identification of steroid products

(a) *From isocortisol.* Following the incubation of [1,2-^{3}H]-isocortisol with F1 isozyme and NAD^+, the medium was acidified, extracted with ethyl acetate, and chromatographed on a thin layer silica gel plate. About 90% of the radioactivity remained at the origin. Controls were run under the same conditions except that no F1 enzyme was added. Isocortisol is labile and was partially degraded in the controls during the procedures. Most of these degradation products migrated from the origin. Only a few percent of tritium was located at the origin in the controls, in contrast with the incubations containing enzyme. The polar steroid remaining at the origin was extracted from the chromatographic plate and co-chromatographed with authentic 11β,17,20β trihydroxy-4-pregnen-21-oic acid. The major radio-active peak coincided with authentic acid in three different solvent systems: (a) chloroform–isopropanol (90:10, v/v), R_F = 0.00, (b) water saturated ethyl acetate, R_F = 0.01 (c) benzene–ethanol–formic acid (90:25:2, by vol.), R_F = 0.24. These correspond to 97, 99 and 81% respectively of tritium on the plate. Chromatographic mobilities of methyl derivatives of the radioactive polar enzyme products were identical to the methyl ester of authentic synthetic steroid in three solvent systems: chloroform–isopropanol (90:10, v/v) R_F = 0.31; water saturated ethylacetate, R_F = 0.13; benzene–ethanol (90:10, v/v) R_F = 0.20.

(b) *Product of F2 isozyme.* When [4-^{14}C]-isoDOC was incubated with F2 isozyme net conversion to acid was 58% of the added steroid. After chromatographic separation of the most polar metabolites on thin layer plates, single major peak of ^{14}C containing steroid coincided with authentic 20β hydroxy-4-pregnen-21-oic acid in three solvent systems: (a) chloroform–methanol (98:2, v/v), R_F = 0.00; (b) upper phase of toluene–acetic acid–water (50:50:10, by vol.), R_F = 0.23; (c) chloroform–methanol–formic acid (90:10:1, by vol.), R_F = 0.28. The methyl derivative of the product had the same chromatographic mobilities as 20-hydroxy-4-pregnen-21-oic acid-21 methyl ester in chloroform–methanol (98:2, v/v), R_F = 0.30; methylene chloride–acetone (80:20, v/v), R_F = 0.38; ethylacetate, R_F = 0.43. Analysis of distribution of radioactivity on the chromatograms indicated that more than 90% of polar product coincided with 20-hydroxy-4-pregnen-21-oic acid.

Identification by reverse isotope dilution

Specific activities of the methyl esters of the products cocrystallized with methyl esters of 11β,17,20β-trihydroxy-4-pregnen-21-oic acid or 20β-hydroxy-4-pregnen-21-oic acid were constant in three successive cycles as shown on Table 2. These results confirm that isocortisol and isoDOC were converted to their corresponding carboxylic acids by isozymes F1 and F2 of horse liver aldehyde dehydrogenase.

Stoichiometry

In order to establish the relationship between the reduction of NAD^+ and the oxidation of steroid, the formation of NADH, measured spectrophotometrically, was compared with the extent of acid formation. Table 3 shows that both enzymes catalyzed equivalent transformations of steroid and pyridine nucleotide. Stoichiometry persisted over the entire range of transformation, from 14 to 72% conversion. It was not possible to measure the decrease in substrate because of its lability.

Table 2. Reverse isotope dilution of products of horse liver aldehyde dehydrogenases

F1 isozyme product cocrystallized with 11β,17,20β-trihydroxy-3-oxo-4-pregnen-21-oic acid 21 methyl ester			
		Specific activity*	
Solvent pair	Sequence	Precipitate	Mother liquor
		(^{3}H d.p.m./mg steroid)	
acetone/hexane	1	8,739 ± 643	
	2	8,201 ± 512	
	3	8,298 ± 132	7,995 ± 238

F2 isozyme product cocrystallized with 20β-hydroxy-3-oxo-4-pregnen-21-oic acid 21-methyl ester			
		Specific activity	
Solvent pair	Sequence	Precipitate	Mother liquor
		(^{14}C d.p.m./mg steroid)	
CH_2Cl_2/hexane	1	3,843 ± 290	
	2	3,896 ± 91	
	3	3,846 ± 38	3,914 ± 68

*Mean ± S.D. of 3 determinations.

DISCUSSION

The oxidation of the ketol or dihydroxyacetone side chains of corticosteroids to the 20-hydroxy-21-oic acid configuration is catalyzed by enzymes in mammalian liver. We have postulated that the immediate precursor of the acid is a class of steroid with a 20-hydroxy-21-aldehyde side chain to which we have given the trivial name "isocorticosteroids". These are formed from corticosteroids by the action of an isomerase present in liver [6]. We have shown in this paper that the terminal step in this process, the oxidation of the side chain, is catalyzed by liver aldehyde dehydrogenases F1 and F2. There are sharp differences in the steroid specificities of the two enzymes. The F1 enzyme oxidizes 17-hydroxy steroids, such as isocortisol and isocortisone, while the specificity of the F2 enzyme appears to be directed to the 17-deoxysteroids, exemplified by iso-11-deoxycorticosterone and isocorticosterone. There is, consequently, a functional division into compartments with respect to the metabolism of these two classes of steroid. Not only do the two aldehyde dehydrogenases have different substrate specificities, but they are, in addition, physically separated in the cell. This partition appears to occur in human liver [15], and possibly in the livers of other species [16–21]. Its metabolic significance remains to be determined.

The oxidation of isocortisol and other 17α hydroxylated steroids by the cytosolic F1 enzyme is, according to our current view, coupled to its formation from cortisol by the cytosolic isomerase. The overall flow of intermediates to hydroxy acid therefore proceeds in a single compartment. The utilization of isoDOC generated in the cytosol by the mitochondrial aldehyde dehydrogenase F2 introduces a problem. The labile 17-deoxy aldol intermediate must survive passage to the mitochondria unaltered if it is to be oxi-

Table 3. Stoichiometry of oxidation of steroids*

Initial conditions			Final conditions			
[4-^{14}C]-isocortisol (μmol)	NAD (μmol)	F1 enzyme (mg)	Incubation time (min)	NADH formed (μmol)	Acid formed (μmol)	Acid NADH
118.6	1.0	0.05	20	50.6	53.9	1.06
118.6	1.0	0.10	20	71.2	66.5	0.93
113.6	1.0	0.51	5	82.4	81.7	0.99
113.6	1.0	0.51	60	75.2	78.9	1.05
[4-^{14}C]-isoDOC (μmol)	NAD (μmol)	F2 isoenzyme (mg)				
331.4	0.5	0.40	30	49.8	45.4	0.91
127.1	1.0	0.80	30	55.8	54.9	0.98
147.6	1.0	0.80	60	92.8	84.7	0.91

* NADH formation was determined by absorbance increase at 340 nm. Steroid acid was measured by ^{14}C after chromatographic separation. [4-^{14}C]-isocortisol, 4.7 × 10^4 d.p.m./μmol; [4-^{14}C]-isoDOC, 3.6 × 10^4 d.p.m./μmol; 0.1 M Tricine, pH 8.4. Total volume was 1.0 ml.

dized. The hydroxy aldehydes are susceptible to reduction by a NADPH dependent 21-oxo-20-hydroxy-steroid reductase to a 20,21 diol [22]. If the isomerase is proximal to the mitochondria, aldol may be passed into the mitochondria without traversing any significant distance. It appears to us that the geographic relationship of the isomerase and the cell organelles must be established, and the action of intact mitochondria on isoDOC be evaluated, before speculation on the mechanism would be profitable. The possibility that other aldehyde dehydrogenases and aldehyde oxidases of the hepatocyte also participate in the oxidation of steriodal aldols must also be considered. The reaction is however, not a general property of aldehyde dehydrogenases, for yeast aldehyde dehydrogenase is inert. (Martin, unpublished observation.)

The general properties of the F1 and F2 enzymes as described by Eckhardt *et al.*[10] with low molecular weight aldehydes are retained when steroid aldehydes are substrates. The effects of alcohols on activity provided a major distinction between steroid and acetaldehyde as substrate. The addition of ethanol, methanol, or isopropanol all decreased the oxidation of isoDOC by the F2 enzyme. The alcohols did not denature the enzyme, for there was no measureable change in the oxidation of acetaldehyde. This observation also implies that alcohols are not competitive inhibitors of aldehyde oxidation. Alcohol did not decrease the pH of the incubation system. The pH was 8.39 with no alcohol, and 8.41 with 5% methanol. It is possible that alcohols, by affecting the dielectric constant of the medium altered the equilibrium between the free and solvated form of the 17-deoxy aldehyde. If the free aldehyde is the reactive species, then an increase in the solvated form of the substrate would decrease the rate of reduction. This would be more pronounced at lower concentrations of total steroid where the concentration of free aldehyde becomes rate limiting. This proposed mechanism is consistent with our observations. In the context of this hypothesis, we propose that there was probably no effect of alcohol on the ratio of free and hydrated 17α-hydroxylated isosteroids, for the oxidation of isocortisol by F1 isozyme was not affected by the presence of alcohol.

The ability of the enzymes described in this paper to oxidize steroidal aldols to hydroxy acids lends further support to the importance of these new steroidal hydroxyaldehyde intermediates in corticosteroid metabolism. The available evidence suggests that the pathway outlined in Scheme 1 is the major, if not exclusive, route leading from the steroidal ketol to the hydroxy acid side chain.

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