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OXIDATION OF 21-DEHYDROCORTICOSTEROIDS TO STEROIDAL 20-OXO-21-OIC ACIDS BY KETOALDEHYDE DEHYDROGENASE OF SHEEP LIVER

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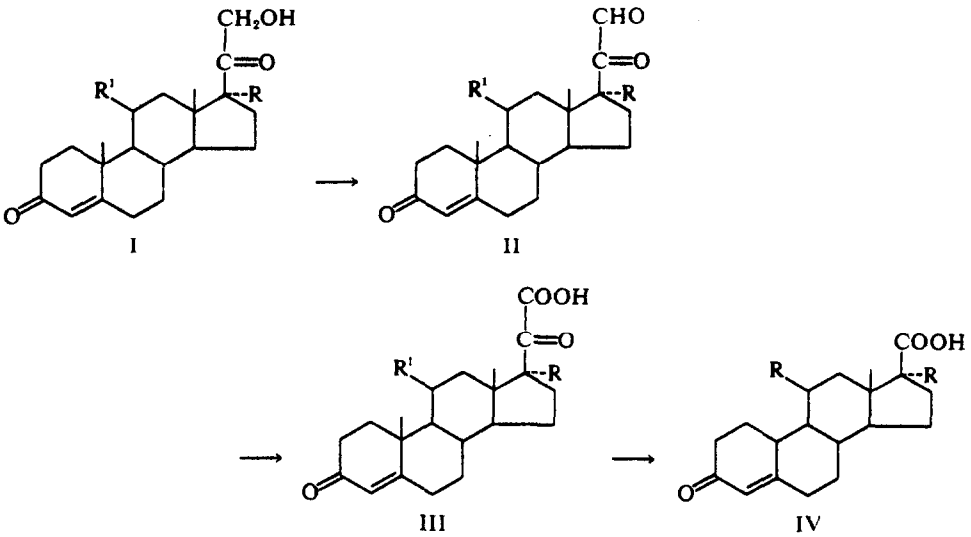
SUMMARY

21-Dehydrocorticosteroids were converted to steroidal 20-oxo-21-oic acids by α-ketoaldehyde dehydrogenase of sheep liver. The NAD^+-dependent reaction oxidized only 17α-deoxycorticosteroids; $NADP^+$ also oxidized some 17α-hydroxycorticosteroids. Prior addition of 21-dehydrocorticosterone but not NAD^+ partially protected the enzyme from inactivation by p-hydroxymercuribenzoate. The conversion did not involve an intermediate hydroxy acid.

EVIDENCE, direct and circumstantial, has accumulated to indicate that mammals are able to oxidize corticosteroids to 3-oxo-4-androstan-17β-carboxylic acids (IV) [1–6]. The mechanism of transformation is obscure, but undoubtedly involves a series of oxidations resulting in the removal of a single carbon from the 17β-ketol side-chain. The following general sequence may be proposed to account for the formation of the acid product:



The observation that keto acid intermediate III (R=R¹=H) was formed when 11-deoxycorticosterone (I, R=R¹=H) was incubated with guinea pig liver slices [5] is in accord with the pathway. Further support is lent by the isolation of a steroidal keto acid from the urine of healthy human subjects given a synthetic corticosteroid [7].

We propose that the class of keto acids III is derived from the enzymic oxidation of the keto aldehyde II. In this paper we present evidence that the oxidation of steroidal 20-oxo-21-al to 20-oxo-21-oic acid is catalyzed by α-ketoaldehyde dehydrogenase, an enzyme found in mammalian liver[8].

METHODS

The α-ketoaldehyde dehydrogenase of sheep liver was purified as described earlier[8]. Enzymic oxidation of the steroid substrate was measured as the initial change in absorbance at 340 mμ in a system consisting of 0·1 ml of a solution containing 0·2 μmoles of 21-dehydrocorticosteroid[9] dissolved in 50% aqueous dimethylsulfoxide, 0·75 μmoles of pyridine nucleotide, 70 μmoles of N-tris-(hydroxymethyl)-methylglycine (Tricine), pH 8·5, and enzyme in a volume of 1·0 ml at 30°C in a Gilford model 2000 multiple absorbance recording spectrophotometer. Thin layer chromatography of steroids was performed on 0·3 mm thick silica gel supports (GF_{254}, Brinkmann) activated by heating at 110°C for 1 h. Synthesis of 11β-hydroxy-3-oxo-4-androstene-17β-carboxylic acid was achieved by oxidizing corticosterone with periodic acid as described by Mason *et al.*[10].

The 21 methyl ester of 11β,20ξ-dihydroxy-3-oxo-4-pregnen-21-oic acid was prepared by rearrangement of 11β-hydroxy-3,20-dioxo-4-pregnen-21-al catalyzed by cupric ion[11]. The product contained both 20α and 20β isomers. The mixture was acetylated with pyridine: acetic anhydride (1 : 1 v/v) and the isomers were separated on thin layer plates of silica gel with ethyl acetate: chloroform (1 : 3 v/v). The two components were not completely resolved.

λ_{max} (ethanol) 242 nm (15,600); t.l.c. in ethyl acetate chloroform, $R_F = 0{\cdot}29$, 0·35; IR, cm^{-1}, (KBr) 1740 (C=O); 1238 (C(=O)—O), 1655, 870 (O=C—C=C—).

NMR analysis showed less polar and more polar components in a 3 : 1 ratio. NMR analysis (100 MHz, $CDCl_3$) of the two isomers showed (δ less polar; δ more polar) C-18 CH_3 (0·973; 1·040, s) C-19 CH_3 (1·436; 1·436, s), C-20 O—$COCH_3$ (2·113, 2·078, s), C-21 CO_2CH_3 (3·696; 3·723, s), C-4 H (5·65; 5·65, s), C-11 αH (4·33; 4·33, s) C-20H (4·75, dt, J = 10·2; not detected). The isomers have been tentatively assigned the 20R and 20S configurations, respectively. The diagnostic criterion is the downfield shift in the $18H_3$ resonance in the more polar isomer (J. C. Orr and C. Monder, in preparation).

Anal. Calculated for $C_{24}H_{34}O_6$: C, 68·9; H, 8·2. Found C, 69·0; H, 8·3.

Twenty mg of the steroid ester was dissolved in 0·5 ml methanol. After adding 1·5 ml of water and 0·5 ml 2 N NaOH, the milky solution was stirred for 60 min. When clear, it was acidified with 10% HCl, extracted into ethyl acetate, back into 4% $KHCO_3$, and acidified. White needles of free acid formed slowly. Yield 8 mg (40%).

M.p., 111°, λ_{max} (ethanol) 241 nm (16,100); IR cm^{-1} (KBr) 2700 to 2400 (shoulder) (bonded OH); 1710 (C=O), 1655 (C—C=C—); 948; 868.

Anal. Calculated for $C_{21}H_{30}O_5 \cdot H_2O$; C, 66·3; H, 8·5. Found C, 66·4; H, 8·3.

Synthesis of 11β-hydroxy-3,20-dioxo-4-pregnen-21-oic acid was achieved by oxidation of 21-dehydrocorticosterone with chromium trioxide under neutral conditions[12]. To a solution of 100 mg (1·0 mmole) of chromium trioxide and 5 mg (0·075 mmoles) of sodium cyanide in 250 ml of 0·05 M N-tris-(hydroxy-

methyl)-methylglycine (Tricine) at pH 7·2 was added dropwise over a 20 min period with vigorous stirring 250 mg (0·72 mmol) of 11β-hydroxy-3,20-dioxo-4-pregnen-21-al dissolved in a minimum amount of methanol. After 30 min, 0·1 ml of formaldehyde was added, and 10 min later the solution was acidified to Congo Red paper with concentrated hydrochloric acid. The steroids were extracted into ethyl acetate. The steroid acid was then transferred from the organic solvent into sodium bicarbonate (0·1 N) and crystallized from aqueous solution after acidification. To remove a small amount of 11β-hydroxy-3-oxo-4-androsten-17β-carboxylic acid, the steroid acid dissolved in ethyl acetate was transferred to a column of silica gel, and eluted with ethyl acetate: formic acid (99 : 1, v/v). Yield was 177 mg, m.p. 212–216° (dec); λ_{max}(ethanol) 237 mμ (14,600); $[\alpha]_D^{27°} = +208$ (c, 0·5 ethanol). Spectrum in sulfuric acid after 2 h; 580 (shoulder), 444 (max), 380 (shoulder), 358 (min), 280 mμ (max).

Anal. Calculated for $C_{21}H_{28}O_5$: C, 69·9; H, 7·8. Found C, 69·6; H, 8·0.

RESULTS

Course of the reaction

α-Ketoaldehyde dehydrogenase of sheep liver (specific activity 0·25 μmol methylglyoxal oxidized/min/mg protein) catalyzed the reduction of pyridine nucleotides by 21-dehydrocorticosterone. No reaction occurred if any one of the components was omitted. The rate of the reaction was linear with respect to enzyme concentration in the range 0·5 mg protein/ml to 5·0 mg protein/ml. Under the conditions described in Methods, $NADP^+$ was reduced more rapidly than NAD^+ (12·8 vs. 6·4 $\mu mol \times 10^{-3}$/min). In either case, the rate of the reaction remained linear for at least 30 min.

Effect of cations

Unlike other aldehyde dehydrogenases from mammalian liver[13, 14], there was no stimulation of activity by potassium ions, nor was there any effect of magnesium ions, which has been shown to influence the activity of some aldehyde dehydrogenases[13, 15]. Addition of EDTA had no effect on the activity of the purified enzyme.

Identification of steroid product

In order to identify the product derived from the enzymic oxidation of 11β-hydroxy-3,20-dioxo-4-pregnen-21-al, all components were increased tenfold, and the reaction was permitted to proceed for 8 h. The mixture was then acidified to pH 1 with hydrochloric acid, and the steroids were extracted into ethyl acetate. The extract was washed with dilute hydrochloric acid and extracted with 2% aqueous sodium bicarbonate. The precipitate which formed after acidifying the bicarbonate solution was centrifuged down, washed with water and dried *in vacuo* over phosphorous pentoxide. The properties of the acid coincided with authentic 11β-hydroxy-3,20-dioxo-4-pregnen-21-oic acid with respect to chromatographic mobility in ethyl acetate: formic acid, 99 : 1 v/v ($R_F = 0·42$) and water saturated ethyl acetate, ($R_F = 0·00$), infra-red spectrum, spectrum in sulfuric acid, and chromatographic mobility of the methyl ester in chloroform: ethanol 95 : 5 v/v ($R_F = 0·70$) and benzene: ethanol, 9 : 1 v/v ($R_F = 0·54$). The chromatographic and spectral properties of three other possible products, 11β,20α-dihydroxy-3-oxo-4-pregnen-21-oic acid, 11β,20β-dihydroxy-3-oxo-4-pregnen-21-oic acid and 11β-

hydroxy-3-oxo-4-androsten-17β-carboxylic acid differed from that of the isolated steroid acid. Figure 1 illustrates the relative mobilities of the several derivatives of corticosterone on thin layer plates with silica gel support and ethyl acetate: formic acid (99 : 1 v/v) as developing solvent. The steroid acids were differentiated as well by the colors which developed after the chromatogram was sprayed with 50% sulfuric acid and heated. Figure 1 also shows that control incubations containing no enzyme or no NAD^+ yielded no keto acid.

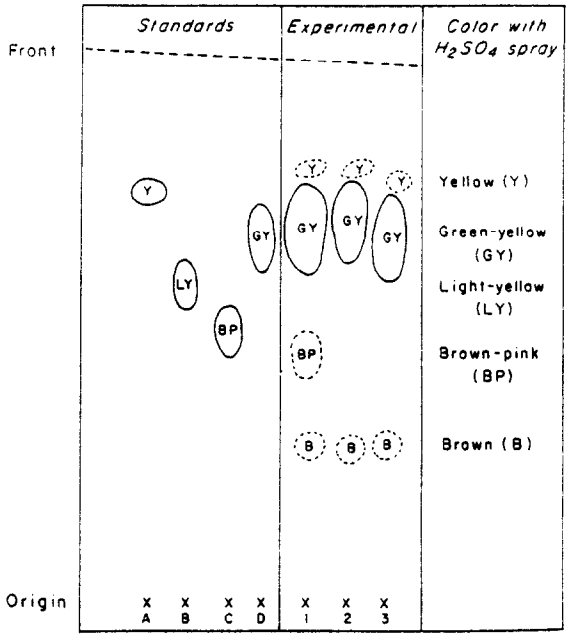


Fig. 1. Thin layer chromatograph of incubation mixtures. Steroids were resolved on silica gel support with ethyl acetate: formic acid (99 : 1, v/v). Standards: A, 11β-hydroxy-3-oxo-4-androsten-17β-carboxylic acid; B, 11β,20-dihydroxy-3-oxo-4-pregnen-21-oic acid; C, 11β-hydroxy-3,20-dioxo-4-pregnen-21-oic acid; D, 11β-hydroxy-3,20-dioxo-4-pregnen-21-al. Incubation mixtures contained (1) 1·6 μmoles of 21-dehydrocorticosterone, 6 μmoles of NAD^+, 560 μmoles of Tricine, pH 8·5, 6 mg of enzyme in 8 ml final volume; (2) enzyme omitted; (3) NAD^+ omitted. Steroids were extracted after 8 h at 30°C.

pH Dependence

The oxidation of 21-dehydrocorticosterone in Tricine buffer in the range pH 7·2–9·6 is shown in Fig. 2. No oxidation of 21-dehydrocorticosterone with NAD^+ occurred at pH 7·6. With increasing pH values, the rate gradually increased, reaching a plateau of activity at pH 8·4 which persisted to pH 9·5.

Kinetic constants and substrate specificity

Reduction of NAD^+ was linear with respect to 21-dehydrocorticosterone at low concentrations of steroid substrate. Levels of steroid above $1·7 \times 10^{-4}$ M led to inhibition as shown in Fig. 3. Double reciprocal plots of the 21-dehydrocorticosterone concentration vs velocity at several NAD^+ concentrations resulted in a series of parallel lines (Fig. 4a). A similar relationship was seen when NAD^+ was the variable at several 21-dehydrocorticosterone concentrations (Fig. 5a).

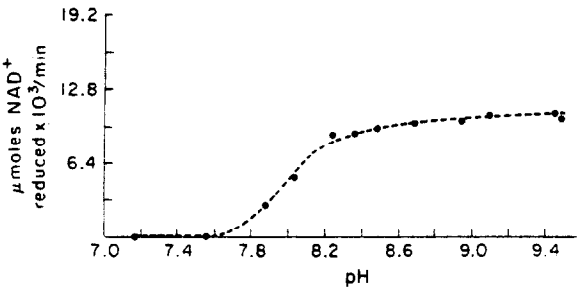


Fig. 2. pH-activity curve of 21-dehydrocorticosterone oxidation. Tricine buffer was used over the entire pH range.

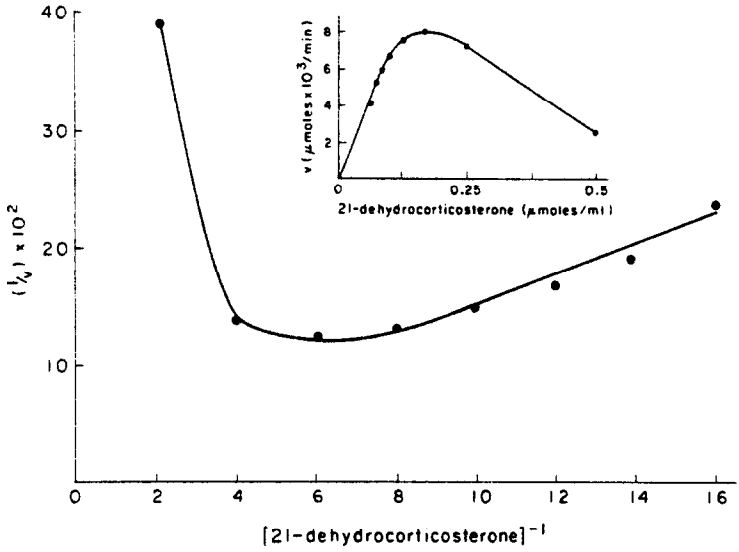


Fig. 3. Relationship between rate of steroid oxidation and 21-dehydrocorticosterone concentration. NAD^+ was maintained at $7{\cdot}5 \times 10^{-4}$ M.

From these data secondary plots were made from which the Michaelis constants (K_m) and maximum velocities for 21-dehydrocorticosterone and NAD^+ were determined (Figs. 4b, 5b). Primary double reciprocal plots of other substrates showed a similar pattern of parallel lines.

Substrate specificity of the enzyme with respect to steroids is summarized on Table 1. With NAD^+ only 17α-deoxy steroids were oxidized. With $NADP^+$, however, 3α,17α-dihydroxy-11,20-dioxo-5β-pregnan-21-al was also oxidized. K_m values for all active steroids were about an order of magnitude smaller for $NADP^+$ than for NAD^+. All active steroids showed substrate dependent inhibition at high concentrations.

The K_m values for NAD^+ and $NADP^+$ with 21-dehydrocorticosterone as substrate were $1{\cdot}42 \times 10^{-4}$ M and $1{\cdot}0 \times 10^{-4}$ M, respectively. These values are similar to those obtained with methylglyoxal[8].

In order to determine if the same enzyme was oxidizing both methylglyoxal and 21-dehydrocorticosterone, the oxidation of mixtures of substrates was examined.

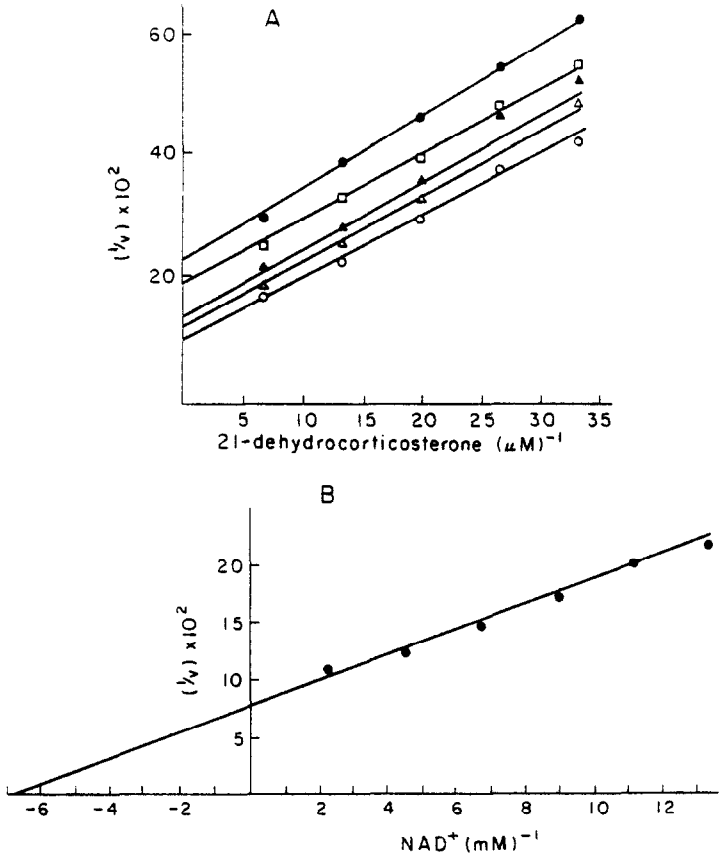


Fig. 4. (a) Double reciprocal plot of 21-dehydrocorticosterone concentration vs. velocity. ●, 0·075 mM NAD^+; □, 0·090 mM NAD^+; ▲ 0·150 mM NAD^+; △, 0·226 mM NAD^+; ○, 0·452 mM NAD^+. (b) plot of intercept against the reciprocal of NAD^+ concentration.

Table 1. Effect of the sequence of addition of components on the inhibition of 21-dehydrocorticosterone (21DB) oxidation by p-HMB

First addition	Second addition	Relative velocity
NAD^+ + 21-DB	p-HMB	0·46
p-HMB + 21-DB	NAD^+	0·60
p-HMB + NAD^+	21-DB	0·12
p-HMB	NAD^+ + 21-DB	0·18
—	NAD^+ + 21-DB	1·00

To 0·7 ml of 0·2 M Tricine, pH 8·4, in a 1·0 ml cuvette was added 0·1 ml of enzyme and the components in column 1. After 2·5 min the components in the second column were added. The values in the last column represent the ratio of the rate of increase in absorbance at 340 mμ of the mixtures containing inhibitor to that of the uninhibited rate. Steroid was added in 0·1 ml 50% aqueous DMSO. Final concentrations were 0·19 mM 21-dehydrocorticosterone; 0·753 mM NAD^+; 0·10 mM p-hydroxymercuribenzoate.

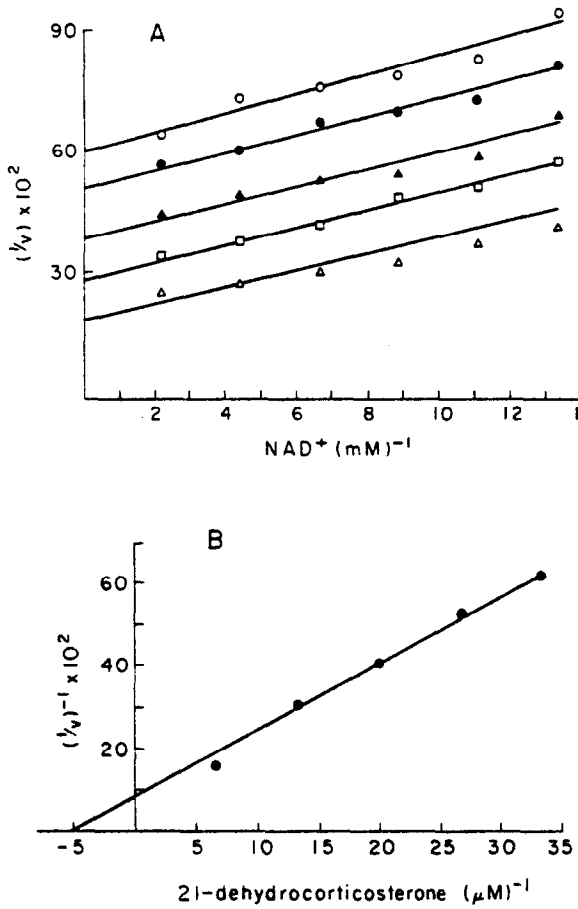


Fig. 5. (a) Double reciprocal plot of NAD^+ concentration vs velocity at ○ 29 μM; ● 36 μM; ▲ 48 μM; □ 72 μM; △ 145 μM 21-dehydrocorticosterone concentration. (b) plot of intercept against the reciprocal of 21-dehydrocorticosterone concentration.

Table 2 shows that simultaneous addition of 21-dehydrocorticosterone and methylglyoxal resulted in a rate of oxidation intermediate between that of either substrate, when NAD^+ was the cofactor. With 0·145 mM 21-dehydrocorticosterone or 4·511 mM methylglyoxal, rates were $0{\cdot}77 \times 10^{-3}$ μmol/min and $3{\cdot}91 \times 10^{-3}$ μmol/min, respectively. Both added together at these concentrations gave a

Table 2. Reduction of pyridine nucleotides by combinations of 21-dehydrocorticosterone and methyglyoxal

Addition	Concentration	NAD^+ (mμ mol/min)	$NADP^+$ (mμ mol/min)
21-dehydrocorticosterone	0·145	0·77	0·39
Methyglyoxal	4·5	3·91	1·08
21-dehydrocorticosterone + methylglyoxal		2·36	1·08

rate of $2{\cdot}36 \times 10^{-3}$ μmol/min. With $NADP^+$, the mixture of steroid and methylglyoxal was not oxidized faster than methylglyoxal alone. The data indicate that the steroid substrate competed with methylglyoxal. It is concluded, therefore, that a single enzyme was responsible for oxidation of both substrates.

Effect of glutathione

Glutathione is essential for the conversion methylglyoxal to D-lactate via the glyoxalase reaction[17]. The possibility was considered that a glutathione requiring enzyme in our preparation was required to convert the 21-dehydrocorticosteroid to a 20-hydroxy acid by a mechanism similar to that of glyoxalase. The resulting 20-hydroxy steroid could then be oxidized to the 20-keto acid by a 20 hydroxysteroid dehydrogenase. Glutathione had no effect on any of the steroid substrates. The 17-deoxy steroids were not oxidized faster, and the 17α-hydroxy steroids remained unresponsive to the enzyme. The nature of the product from 21-dehydrocorticosterone as assessed by thin layer chromatography and sulfuric acid chromogen formation was not changed. In addition, neither 20α- nor 20β-hydroxy-21-oic acids were oxidized. They were also not seen on thin layer chromatograms when the reaction was monitored during its progress. It was therefore concluded that 21-dehydrosteroids, like methylglyoxal, are oxidized to keto acids directly.

Effects of reagents which react with —SH groups

Aldehyde dehydrogenases in general are inhibited by reagents that block sulfhydryl groups. α-Ketoaldehyde dehydrogenase is also inhibited by these reagents [8]. Of particular interest is the inhibition by p-hydroxymercuribenzoate (pHMB).

Extent of inhibition by p-hydroxymercuribenzoate depended on the order of addition of components. Table 3 indicates that 21-dehydrocorticosterone, but not NAD^+, partially protected the enzyme from inhibition by p-HMB. When p-HMB was added before 21-dehydrocorticosterone, inhibition was 80–90%; when added after the steroid, inhibition was 40–50%. Sodium arsenite did not inhibit the steroid aldehyde oxidation, whether or not 3·8 mM β-mercaptoethanol was present. These observations may be interpreted to mean that the enzyme contained mercaptyl groups essential for enzyme activity, but did not contain contiguous mercaptyl groups accessible to arsenite at the active site[18].

DISCUSSION

In recent years, a number of laboratories have presented evidence for the presence in the urine of several species of acids derived from the metabolism of corticosteroid. It has been shown that isolated liver[2, 5], adrenal[1, 3, 4, 19, 20] or placenta[21] may effect this transformation as well. The metabolic pathways leading from a 21 carbon corticosteroid to a 20 carbon acid have not been determined. Our demonstration that 21-dehydrocorticosteroids are oxidized to α-keto acids by a reasonably well characterized enzyme of liver, α-ketoaldehyde dehydrogenase, provides support for a pathway involving a sequence of oxidations at position 21 culminating in an oxidative decarboxylation step to yield the etienic acid. It is striking that only 17α-deoxy-steroids were susceptible to oxidation with NAD^+, though the physiological meaning of this selectivity remains to be deter-

Table 3. Comparison of K_m and V_{max} for several 21-dehydrocorticosteroids*

Substrate	Apparent K_m		Apparent V_m†	
	NAD^+	$NADP^+$	NAD	$NADP^+$
3,20-dioxo-4-pregnen-21-al	$3{\cdot}0 \times 10^{-5}$	$8{\cdot}1 \times 10^{-6}$	0·63	0·36
11β-hydroxy-3,20-dioxo-4-pregnen-21-al	$2{\cdot}4 \times 10^{-4}$	$6{\cdot}8 \times 10^{-6}$	1·92	1·00
3β-hydroxy-20-oxo-5-pregnen-21-al	$3{\cdot}6 \times 10^{-4}$	$2{\cdot}9 \times 10^{-6}$	2·98	0·71
3α-17α-dihydroxy-11,20-dioxo-5β-pregnan-21-al	—	$1{\cdot}3 \times 10^{-5}$	—	0·56
17α-hydroxy-3,11-20-trioxo-4-pregnen-21-al	—	—	—	—
11β,17α-dihydroxy-3,20-dioxo-4-pregnen-21-al	—	—	—	—
17α-hydroxy-3,11-20-trioxo-1,4-pregnadien-21-al	—	—	—	—

*Estimates were made from double reciprocal plots, varying steroid, and maintaining the concentration of pyridine nucleotide at 0·35 mM. Dashed lines indicate no detectable activity.

†Values for V_m with respect to each nucleotide are expressed as the ratio of the maximum velocity for a given steroid to that of 21-dehydrocorticosterone (11β-hydroxy-3,20-dioxo-pregnen-21-al) oxidized with $NADP^+$.

mined. The pathway supported by the evidence in this paper requires a sequence of oxidations at carbon 21, leading from alcohol to aldehyde to acid. The final step, oxidative decarboxylation to the etienate, remains to be explored. A mechanism in which internal dismutation of ketoaldehydes to α-hydroxy carboxylic acids would lead, by oxidation at position 20 to the steroidal keto acid does not appear to be likely. The expected 20 hydroxylated intermediates were not detected, nor were the synthetic 20-hydroxy acids converted to keto acids.

Apparent K_m values for the steroid substrates were about tenfold lower with respect to NAD^+ or $NADP^+$ than for non-steroidal α-ketoaldehydes. Steroids therefore appear to be more efficient substrates than other ketoaldehydes, a circumstance which is fortunate in view of the low solubility of the steroids in water.

The effects of sequential addition of substrates and p-HMB shown in Table 2, lead to the conclusion that 21-dehydrocorticosterone, but not NAD^+, protects the enzyme from inactivation. These results suggest that the steroid reacts reversibly with a sulfhydryl group on the enzyme to form a thiohemiacetal, which is then dehydrogenated by NAD^+. A similar mechanism has been proposed for the role of cyanide ion in the chemical oxidation of 21-dehydrosteroids to steroidal keto acids[12].

Recently Gerhards *et al.*[7] reported that among the metabolites of fluocortolone (6α-fluoro-11β,21-dihydroxy-16α-methyl-1,4-pregnadiene-3,20-dione) isolated from human urine were conjugates of a carboxylic acid identified as 6α-fluoro-11β-hydroxy-3,20-dioxo-16α methyl-1,4-pregnadiene-21-oic acid. It is reasonable to propose that α-ketoaldehyde dehydrogenase participated in the sequence of oxidations which led to the formation of the steroid acid described by these authors. The factors that determine whether the end products of the oxidative metabolism of corticosteroids are 21-oic or 20-oic acids remain undetermined.

The available data suggest that discreet enzymes catalyze the conversion of corticosteroids to steroid acids. When the properties of these enzymes have been adequately characterized, it should be possible to study the factors that result in the oxidation of the corticosteroid side-chain and the physiological consequences of these transformations.

ACKNOWLEDGEMENTS

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