Oxidation of Corticosteroids to Steroidal Carboxylic Acids by an Enzyme Preparation from Hamster Liver†

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ABSTRACT: Enzyme activity which catalyzes the oxidation of 11-deoxycorticosterone to 21-oic acids accompanies the "detritiating" enzyme (isomerase) of hamster liver recently isolated by Martin, K. O., et al. ((1977) *Biochemistry 16* (preceding paper in this issue)). The metabolites isolated were 20α- and 20β-hydroxy-3-oxo-pregn-4-en-21-oic acid and 3,20-dioxo-pregn-4-en-21-oic acid. When 21-hydroxy[4-^{14}C, 21-^{3}H]pregn-4-en-3,20-dione was the substrate, about half of the tritium was retained in position 20 of the hydroxy acids. The system which catalyzes the conversion of the ketol side chain of corticosteroids to acid metabolites appears to be a cluster of closely related enzymes. As a result of these studies, we believe that the hamster liver enzyme preparation provides a useful model system for studies on the biosynthesis of acid metabolites of the corticosteroids in man.

The demonstration by Schneider (1965) that 11-deoxycorticosterone is converted to 21-oic acid metabolites by guinea pig liver slices provided the first concrete evidence that the corticosteroid side chain could undergo net oxidation. Subsequent work amply supported Schneider's original observation (Bradlow et al., 1973a,b; Martin and Monder, 1976). Although a number of pathways leading to the 21-oic acids have been proposed (Schneider, 1965; Bradlow et al., 1973a,b; Martin and Monder, 1976), the sequence of events which leads to the final product has not been established. The discovery that 21-oic acids are important end products of corticosteroid metabolism in humans convinced us that it would be profitable to carefully examine the oxidative transformations of the corticosteroid side chain. We synthesized 21-^{3}H-labeled corticosteroids in order to measure the oxidation of the steroid side chain by following the loss of tritium during the reaction (Willingham and Monder, 1973, 1974). An enzyme that catalyzed the transfer of tritium from steroid to water was purified from hamster liver, but it proved to be an isomerase which effected the loss of tritium with retention of the ketol side chain (Martin et al., 1977). An accompanying enzyme activity which catalyzed the net oxidation of corticosteroids to steroidal 21-oic acids was also present. It was unstable. Our attempts to separate and stabilize the enzyme (or enzymes) have thus far been unsuccessful. We describe in this paper the isolation and identification of the acidic end products.

Materials and Methods

Male golden hamsters weighing about 100 g were purchased from Chick Line Co., Newfield, N.J., and maintained on a standard chow diet. 11-Deoxy-[4-^{14}C]corticosterone was purchased from New England Nuclear Corp. Unlabeled corticosteroids were obtained from Steraloids, Inc., Wilton, N.H. When necessary, the steroids were purified by thin-layer chromatography on silica gel supports with appropriate developing solvents. All other chemicals and solvents were reagent grade.

Instrumentation. High and low resolution mass spectra of steroids were obtained using a direct inlet system on the JEOL GCMS 1 mass spectrometer. The instrument was operated at an electron energy of 75 eV, an accelerator voltage of 7 kV, and a trap current of 200 μA. The direct and ion source temperatures were 200 and 260 °C, respectively.

Infrared spectra were obtained on a Perkin-Elmer Model 211 instrument. The samples were incorporated into potassium bromide pellets. Ultraviolet absorption spectra were obtained in ethanol on a Cary Model 15 spectrophotometer. Melting points of steroids were measured on a Fisher-Johns micro apparatus and are uncorrected.

Radioactivity of ^{3}H and ^{14}C was measured on a Packard 3380 scintillation spectrometer. Correction for quenching was made by the external ratios method and all the reported values were corrected to 100% efficiency. Radioactivity on thin-layer chromatograms was located with a Packard Model 7201 radiochromatogram scanner.

Synthesis of 21-^{3}H-Labeled Corticosteroids. Corticosteroids labeled with tritium at C-21 were synthesized from the respective 21-dehydrocorticosteroids with sodium borotritiide by the method of Willingham and Monder (1973). The 21-dehydrocorticosteroids were prepared by oxidation of corresponding corticosteroids with cupric acetate (Monder and Furfine, 1969; Lewbart and Mattox, 1963). Specific activity of tritiated corticosteroids ranged from 36 to 50 Ci/mol. Steroid substrates and metabolites were purified by preparative chromatography on 0.5-mm thick plates of silica gel (GF_{254}, E. Merck, Darmstadt). Analytical separations of steroids were performed on 0.3-mm thick plates in the solvent systems listed in Table I. Table I also summarizes the R_f values in these systems of the steroids referred to in this paper.

Chemical Synthesis of 20-Hydroxy-3-oxo-pregn-4-en-21-oic Acid. The 21-methyl ester of 20-hydroxy-3-oxo-pregn-4-en-21-oic acid was prepared by rearrangement of 3,20-dioxo-pregn-4-en-21-al catalyzed by cupric ion (Lewbart and Mattox, 1963). Steroid methyl ester was purified on silica gel plates using solvent system B. The area containing steroid ester was scraped off the plate and the steroid was eluted from the silica gel with acetone. To 20 mg of steroid methyl ester dissolved in 0.5 mL of methanol were added 1 mL of water and

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TABLE I: Chromatographic Mobilities of Steroids.[a]

Solvent	Steroid									
	1	2	3	4	5	6	7	8	9	10
AH	0.66	0.41	0.40	0.43	0.68	0.56	0.67	0.76	0.76	0.75
B	0.32	0	0	0.02	0.34	0.11	0.26	0.26	0.52	0.54
C	0.43	0.34	0.34	0.35	0.35	0.54	0.42	0.42	0.55	0.56
E	0.37	0.03	0.04	0.04	0.25	0.27	0.39	0.39	0.60	0.60
FH	0.61	0.59	0.59	0.47	0.65	0.66	0.65	0.65	0.68	0.67

[a] Mobilities, expressed as R_f values, were determined on 0.25-mm thick plates of silica gel (EM Cat 5775) containing fluorescent indicator. Developing solvents were (AH) chloroform–methanol–formic acid, 90:10–0.5; (B) chloroform–methanol, 98:2; (C) upper phase of toluene–acetic acid–water, 5:5:1; (E) dichloromethane–acetone, 9:2; (FH) ethyl acetate–formic acid, 99:1. Steroids were: (1) 11-deoxycorticosterone; (2) 20β-hydroxy-3-keto-pregn-4-en-21-oic acid; (3) 20β-hydroxy-3-keto-pregn-4-en-21-oic acid; (4) 3,20-diketo-pregn-4-en-21-oic acid; (5) 3,20-diketo-pregn-4-en-21-al; (6) 3-keto-androst-4-en-17-carboxylic acid; (7) 20α-hydroxy-3-keto-pregn-4-en-21-oic acid 21-methyl ester; (8) 20β-hydroxy-3-keto-pregn-4-en-21-oic acid 21-methyl ester; (9) 3,20-diketo-pregn-4-en-21-oic acid 21-methyl ester; (10) 3-keto-androst-4-en-17-carboxylic acid methyl ester.

1 mL of 1.5 N NaOH. The reaction mixture was shaken for 30 min at room temperature. The milky suspension became a clear pale yellow solution. This was acidified with 3 N HCl and extracted three times with 10 mL of ethyl acetate. The organic phase was reextracted with 4 × 1 mL of 2% $NaHCO_3$. The aqueous solution was acidified with 3 N HCl. White needles of free acid formed slowly. The collected free acid was dissolved in methanol and further purified by chromatography using solvent system C and D. Melting point of the purified acid was 158–160 °C; λ_{max} (ethanol) 238 nm (ϵ = 13 300); IR (KBr) 2.95 (bonded OH), 3.4 ($-CH_2-$), 4, shoulder (COOH), 5.8 (C=O), 6.1 (C=C), 7.2 (COOH), and 9.2 (>COH). Anal. Calcd for $C_{21}H_{30}O_4 \cdot H_2O$: C, 69.2; H, 8.8. Found: C, 69.4; H, 8.9.

The 21-methyl ester of 20-hydroxy-3-oxo-pregn-4-en-21-oic acid was synthesized by esterification of 20-hydroxy-3-oxo-pregn-4-en-21-oic acid with excess diazomethane. Melting point was 204–207 °C with decomposition; IR (KBr) 2.95 (bonded OH), 3.4 ($-CH_2$), 6.0, 6.3 (C=C-C=O), 7.1 (OH), and 9.5 (>COH); MS (high resolution) *m/e* 360.2279 (expected molecular ion, 360.2258) for $C_{22}H_{32}O_4$; *m/e* 124.0905 (expected base peak 124.0922) for $C_8H_{12}O$.

Enzyme was prepared as described by Martin et al. (1977). The preparation was used immediately or within 4 days, for the enzymes responsible for acid formation were unstable and became inactive in a few days. The stability of the acid forming activity varied from preparation to preparation. In some cases, it was active as long as 2 weeks. It has not yet been possible to separate the oxidizing activity from the isomerase without immediately inactivating the former.

Results

Isolation and Identification of Metabolites. A large scale incubation was set up in order to identify the metabolites derived from 11-deoxycorticosterone. The system, containing 100 mg of 11-deoxy[4-^{14}C]corticosterone in 5 mL of methanol, and 70 mg of enzyme preparation in 200 mL of 0.025 M Tris-HCl buffer, pH 8.0, was incubated at 37 °C for 20 h. The incubation mixture was adjusted to pH 1–2 with 2 N HCl, and the steroids were extracted with ethyl acetate. The organic extract was evaporated at 30 °C with a stream of nitrogen, and the resulting residue, dissolved in methanol, was streaked onto silica gel plates, 0.5-mm thick, containing fluorescent indicator. After the plates were developed with chloroform–methanol (98:2), two bands were observed under ultraviolet light: a mobile one, corresponded to the substrate itself; the other remained at the origin. Two radioactive peaks, detected on the plate with a radiochromatogram scanner, coincided with the

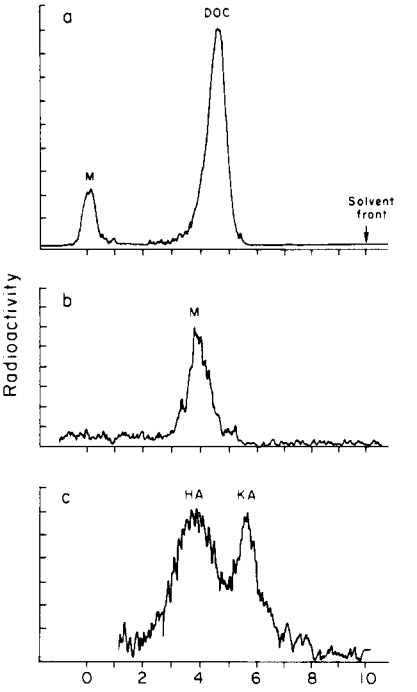


FIGURE 1: Thin-layer chromatography of products resulting from chromatography of [4-^{14}C]-11-deoxycorticosterone with 21-detritiating enzyme. Chromatograms were developed on silica gel plates, 0.5-mm thick, containing a fluorescent indicator. (a) Unfractionated steroid after 20 h of incubation. Plates developed with chloroform–ethanol (98:2); M = polar metabolites; DOC = 11-deoxycorticosterone. (b) Peak M of a after development in the upper phase of toluene–acetic acid–water (50:50:10). (c) Peak M of b after conversion to methyl ester. Developing solvent: chloroform–methanol (98:2).

UV opaque components (Figure 1a). The portion containing the nonmobile, polar metabolite was scraped off the plate and eluted with methanol. The methanol extract was further purified by thin-layer chromatography using solvent systems AH and C. A component with R_f values in these systems of 0.40 and 0.35, respectively, was obtained (Figure 1b) but was not completely symmetrical. It was esterified by reaction with diazomethane. Chromatography of the methyl ester derivatives on thin-layer plates with chloroform–methanol (98:2 v/v) resolved the acid fraction into two components, as shown in Figure 1c. The more abundant component, designated component HA, migrated with 20-hydroxy-3-oxo-pregn-4-en-21-oic acid 21-methyl ester on thin-layer plates in solvent systems AH, B, E, and FH. The free acid coincided with 20-hydroxy-3-oxo-pregn-4-en-21-oic acid in systems C and FH.

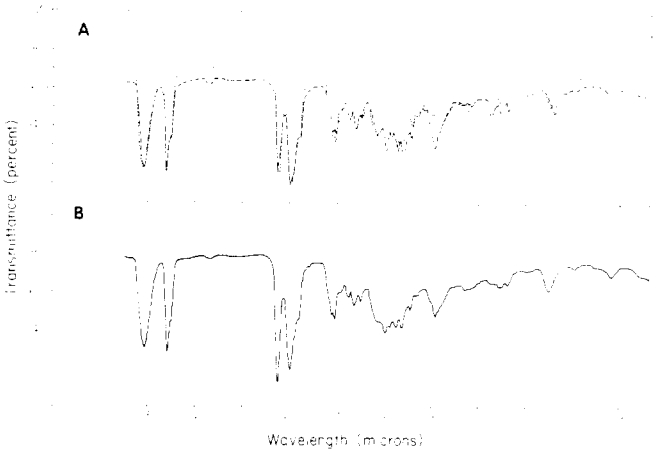


FIGURE 2: Infrared spectra of steroids acids. (A) 20α-Hydroxy-3-keto-pregn-4-en-21-oic acid 21-methyl ester (- - -); 20β-hydroxy-3-keto-pregn-4-en-21-oic acid 21-methyl ester (— —). (B) 21-Methyl ester of acid metabolite (—).

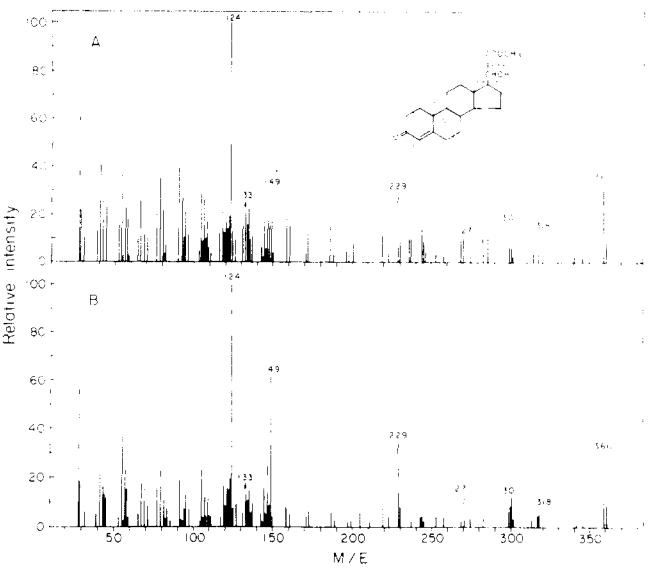


FIGURE 3: Mass fragmentation patterns of steroid acids. (A) 20-Hydroxy-3-keto-pregn-4-en-21-oic acid 21-methyl ester; (B) 21-methyl ester of acid metabolite.

Treatment of the 21-ester of the unknown with pyridine–acetic anhydride led to the formation of the 20-acetate whose chromatographic mobility was identical with that of the authentic steroid derivative in solvent systems AH, B, C, E, and FH.

The infrared and ultraviolet spectra of the acid metabolite and its 21-methyl ester were the same as those of authentic 20-hydroxy acid and its methyl ester. A comparison of the infrared spectra of the 21-methyl esters of the enzymatically and chemically synthesized products is shown in Figure 2. Profiles of the chemically synthesized 20α- and 20β-hydroxy steroid esters, though generally similar, showed different characteristics in the "fingerprint" region. The infrared pattern of the isolated metabolite suggested a mixture of α and β epimers.

Further support for the structure of the metabolite was obtained by mass spectrometry. The fragmentation pattern derived from the spectrum of the 21-methyl ester is reproduced in Figure 3 and compared with the spectrum of the authentic steroid. With high resolution analysis, the molecular ion of the unknown was found to have a mass of 360.2279 (expected: 360.2258) for the molecular formula $C_{22}H_{32}O_4$. The mass of the base peak, C_8H_2O, was 124.0905 (expected, 124.0922). Most of the intense ions were characteristic of fragments derived from Δ^4-3-keto steroidal nucleus with a 17β-side chain (Biemann and Spiteller, 1962; Budzibiewicz, 1972). The base peak at *m/e* 124 and a second intense peak at *m/e* 149 resulted from ring B cleavage. Other important ions were 318 ($M^+ - CH_2CO$), 301 ($M^+ - COOCH_3$), 271 ($M^+ - CHOHCOOCH_3$), 229 (*m/e* 271 $- CH_2CO$).

Retention of Tritium in Steroid Acid. Since the products of enzymic oxidation were carboxylic acids, retention of nonexchangeable tritium at position 21 when [21-^{3}H]DOC was the substrate was impossible. Tritium was, nevertheless, present in the steroid acid after purification. An estimate was made of the mole proportion of total tritium initially present in the substrate that was left in the steroid acid. A mixture was prepared to contain 11-deoxy[21-^{3}H]corticosterone (specific activity 45 Ci/mol) and 11-deoxy[4-^{14}C]corticosterone (specific activity 54 Ci/mol) ($^3H/^{14}C$ = 5.77), 0.4 mg of enzyme, and 0.025 M Tris-HCl buffer, pH 8.0, in a final volume of 2 mL. After 2 h at 37 °C, the mixture was lyophilized and the resulting residue redissolved in 5 mL of water. The aqueous solution was adjusted to pH 1 with HCl and extracted with ethyl acetate. Metabolites and substrate were separated by thin-layer chromatography.

In the experiment illustrated in Table II, the condensate obtained by lyophilization contained 67% of the total ^{3}H and no ^{14}C. The residue contained 38% of ^{3}H activity and 90% of ^{14}C activity. About 10% of the ^{14}C was lost in the aqueous phase during solvent extraction. After separation of the components by thin-layer chromatography, $^3H/^{14}C$ of the 20-hydroxy acid was 2.35 and of the substrate, 2.44. Since the side chain of the metabolite can retain no more than 50% of the tritium of the substrate, it was calculated that tritium retained was 82% of theoretical. The substrate lost about 58% of its tritium during the reaction via enzyme mediated enolization. No tritium was lost from the [4-^{14}C, 21-^{3}H]cortisol in the absence of enzyme. The loss was not due to nonspecific protein–substrate interaction, since the $^3H/^{14}C$ ratio remained constant at 5.8 when enzyme was replaced by bovine serum albumin. In control incubations, steroid acid was never detected.

In order to determine the location of the residual tritium in the acid product, the side chain was removed. The acid was resistant to cleavage with periodic acid or bismuthate, but lead tetraacetate in glacial acetic acid was effective in removing the side chain. A minor radioactive component which migrated with androstenedione in chloroform–acetone (9:1) on silica gel thin-layer plates was eluted and cocrystallized with authentic androstenedione. The recrystallized androstenedione contained only ^{14}C and no significant residual tritium ($^3H/^{14}C$ = 0.02).

The major degradation product resulting from oxidation of 20-hydroxy steroid with lead tetraacetate was 3-oxo-androst-4-ene-17-carboxylic acid. The $^3H/^{14}C$ ratio of this etienic acid was 0.025 (theory = 0.00). It was therefore concluded that at least 99% of the tritium of the hydroxy acid ($^3H/^{14}C$ = 2.35) was in the side chain.

Stereochemistry of the Side Chain. In order to establish the configuration at carbon 20, the enzymically synthesized steroid acid was converted to 20,21-dihydroxy-pregn-4-en-3-one. To 10 mg of lithium aluminum hydride in 10 mL of dry tetrahydrofuran was added 1.5 mg of 20-hydroxy-3-oxo-pregn-4-en-21-oic acid. The mixture was refluxed for 80 min. One milliliter of ethyl acetate, 1 mL of saturated aqueous sodium sulfate, and 50 mg of magnesium sulfate were added with stirring; the suspension was filtered and the filtrate dried. The

TABLE II: Distribution of Radioactivity after Incubation of [4-^{14}C, 21-^{3}H]-11-Deoxycorticosterone with Hamster Liver Enzyme.[a]

Treatment	Fraction	^{3}H (dpm)	^{14}C (dpm)	^{3}H/^{14}C	% of substrate ^{3}H	% of substrate ^{14}C
1. Incubation, no enzyme	11-Deoxycorticosterone	736 000	128 000	5.77	100	100
2. Incubation with complete system and lyophilization	Condensate	490 000	0		67	0
	Residue	287 000	114 000	2.52	38	90
3. Chromatographic fractionation of residue	11-Deoxycorticosterone	189 800	78 000	2.44	26	61
	20-Hydroxy-3-oxo-4-pregnen-2-oic acid	9000	40 000	2.35	13	31
4. Oxidation of 20-hydroxy-21-oic acid with lead chromatographic purification and cocrystallization with indicated steroid	4-Androstene-3,17-dione	7	360	0.02		
	3-Oxo-androst-4-ene-17-carboxylic acid	14	563	0.025		

[a] The incubation mixture contained [21-^{3}H]DOC (45 Ci/mol) and [4-^{14}C]DOC (54 Ci/mol) (^{3}H/^{14}C = 5.77), 400 μg of enzyme protein, in 2 mL of 0.025 M Tris, pH 8.0. After 2 h at 37 °C, the incubation mixture was frozen and lyophilized. The "condensate" refers to the water in the receiving trap. The "residue" refers to the final residual activity remaining in the distilling flask. Metabolites were extracted from the residue with ethyl acetate and separated as described under Figure 1.

reduced material was oxidized by stirring for 21 h with manganese dioxide in dry chloroform, filtered, concentrated, and chromatographed with ethyl acetate as the developing solvent on silica gel plates impregnated with boric acid against authentic 20β,21-dihydroxypregn-4-en-3-one and 20α,21-dihydroxy-pregn-4-en-3-one derived from reduction of 3,20-dioxo-pregn-4-en-21-oic acid with sodium borohydride. The product from the enzymically synthesized acid moved as two components with 20α- and 20β,21-diol (R_f: for 20β = 0.19; for 20α = 0.24).

Reverse Isotope Dilution. In order to confirm the identity of the hydroxy acids, the unresolved radioactive acids from the incubation mixture were converted to the 21-methyl esters with diazomethane and cocrystallized with authentic 3-oxo-20α-hydroxypregn-4-en-21-oic acid from mixtures of dichloromethane and hexane. Initial specific activity was 675 dpm/mg. Successive crystallizations yielded 386, 361, 326, and 327 dpm/mg. Cocrystallization with 20β-hydroxy ester also occurred to constant specific activity. From an initial value of 35 dpm/mg, successive crystallizations gave 25 and 24 dpm/mg. These results confirmed that both 20α and 20β isomeric forms of 3-oxo-20-hydroxy-pregn-4-en-21-oic acid was produced from 11-deoxycorticosterone.

3,20-Dioxo-pregn-4-en-21-oic Acid. The less polar of the two acid esters shown on the chromatographic profile of Figure 1c (peak KA) had a mobility on thin-layer plates that corresponded to that of 3,20-dioxo-pregn-4-en-21-oic acid 21-methyl ester in solvent systems AH, B, E, and FH. The free acid moved with the authentic oxo acid in systems C and D. When incubated with pyridine–acetic anhydride (1:1) overnight, the chromatographic mobility of the C_{21} acid did not change, indicating that no hydroxyl groups were on the side chain.

This product was devoid of tritium, indicating that oxidation of DOC to a keto acid occurred without incorporation of the label into the steroid ring. To confirm the identity of the steroid, the product was cocrystallized with authentic 3,20-dioxo-pregn-4-en-21-oic acid 21-methyl ester. The specific activity of the crystals, expressed as dpm per mg of steroid, remained constant (273, 277, 260 dpm/mg) as did the specific activity of the mother liquor. The identification of the metabolite as 3,20-dioxo-pregn-4-en-21-oic acid was therefore considered proven.

Studies on [4-^{14}C]-3,20-Dioxo-pregn-4-en-21-al (21-DehydroDOC) as a Possible Substrate. Experiments were performed to determine if 21-dehydroDOC was converted to keto acids by direct oxidation of the 21-aldehyde group as described by Monder and Wang (1973a,b) or to hydroxy acids by internal dismutation, similar to the rearrangement catalyzed by glyoxalase. Enzyme and 21-dehydro[4-^{14}C]DOC were incubated for 2 h at 37 °C in 0.025 M Tris, pH 8.0. Controls with no enzyme and with enzyme inactivated by boiling were also run. The 21-dehydroDOC underwent some decomposition during the incubation. The chromatographic profiles developed in chloroform–methanol (9:1) of the resulting steroids were the same with the active enzyme or controls. No acid steroids were formed under these conditions. The pattern remained unchanged when NAD^+ was included in the incubation mixture.

Incubation of 20-Hydroxy-3-oxo-pregn-4-en-21-oic Acid. The [4-^{14}C]-20-hydroxy acid was incubated with enzyme under the same conditions used for [4-^{14}C]DOC oxidation to acid. No keto acid was formed by the enzyme. The hydroxy acid was recovered unchanged.

Discussion

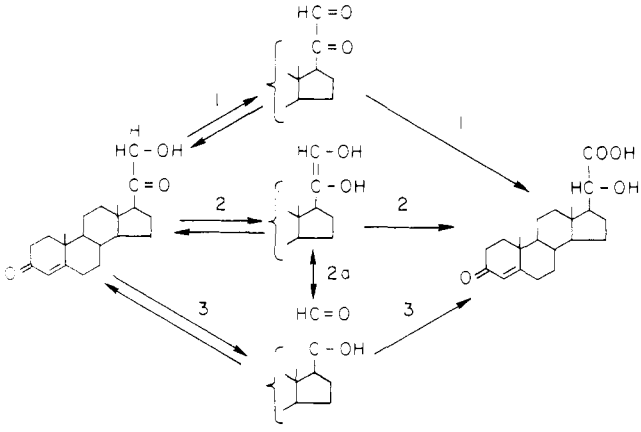
In this paper we have demonstrated that an enzyme preparation from hamster liver which catalyzes the reversible enolization of the ketol side chain of DOC can also catalyze the conversion of DOC to C_{21} carboxylic acids. We have described a similar metabolic conversion of corticosteroids to steroid acids in human subjects (Bradlow et al., 1973a,b) and by an enzyme preparation from human liver (Martin and Monder, 1976). Incubation of 21-^{3}H-labeled DOC with the enzyme preparation led to the synthesis of 3,20-dioxo-pregn-4-en-21-oic acid which, as expected, contained no tritium. The 20-hydroxy acids retained tritium at position 20. We have shown earlier that a major part of the tritium lost from [21-^{3}H]DOC was incorporated into water (Martin et al., 1977). The extent of this transfer was far in excess of that which would

have occurred because of the oxidation of steroid to acid. We propose, therefore, that the loss of tritium is a measure of the formation of a reversible enzyme-bound intermediate which may revert to the ketol or continue to the acid.

In order to postulate a mechanism for the overall reaction, we must account for both migration of part of the tritium from carbon 21 to carbon 20 during the formation of the steroidal hydroxy acid, and the incorporation of the remainder of the tritium into water. Three alternative pathways are shown in Scheme I. Pathway 1 was considered because enzyme-catalyzed oxidation of oxoaldehydes to hydroxy acids is well known (Knox, 1960). The mechanism of the glyoxalase reaction requires an internal dismutation in which the aldehyde proton migrates via a hydride shift to the α carbon (Rose, 1957). No direct demonstration of enzymic oxidation of corticosteroids to 21-dehydrocorticosteroids (which contain the glyoxal side chain) has been achieved. Indirect evidence suggests formation of little or no steroidal glyoxals during corticosteroid metabolism in humans (Monder et al., 1975). The enzymic reduction of 21-dehydrosteroids is well established (Monder and White, 1963) and therefore the requirement that the ketol be regenerated is met. However, our observations are inconsistent with a glyoxalase-catalyzed reaction. Incubation of 21-dehydro-DOC with the enzyme did not yield a hydroxy acid. On the contrary, the 21-dehydrosteroid was a noncompetitive inhibitor of the detritiation reaction and of acid formation. In man, administered 21-dehydrocortisol was poorly converted to a steroidal hydroxy acid (Monder et al., 1975). Consequently, pathway 1 in Scheme I does not appear to be a likely one. Pathway 2 provides a rationale for the loss of tritium from position 21 of the substrate. This route is in accord with the proposals of Eger et al. (1972) who have suggested that, in the relatively nonpolar environment of the specific steroid binding site of a protein, the formation of corticosteroid 17β-enediols may be facilitated by the presence of metal ions such as cobaltous ion. The ability of ferrous and cobaltous ions to enhance detritiation by the isomerase-oxidase complex (Martin et al., 1977) is consistent with this proposal. Similar arguments apply to pathway 3, in which a 20-ol-21-al intermediate is proposed. As yet there is insufficient information to distinguish between pathway 2 and 3. Since the enediol and hydroxyaldehyde are tautomers by step 2a, both pathways may be operating concurrently.

It is evident that any pathway from ketol to hydroxy acid requires the formation of intermediates. The large molecular mass of the enzyme (Martin et al., 1977) is consistent with a complex overall reaction. We have recently succeeded in synthesizing the postulated hydroxy aldehyde intermediate (Oh and Monder, 1976) and are now in a position to test its possible role in the synthesis of steroid acids directly.

SCHEME I: Postulated Pathways of Steroid Acid Synthesis.



How the enzymic synthesis of the keto acid occurs cannot yet be explained. In these studies, it represented 16 to 25% of the total acid formed. It was not made from the steroidal ketoaldehyde, 21-dehydro-11-deoxycorticosterone, nor was it formed from the hydroxy acid. We have not been able to formulate a reasonable alternative mechanism that could account for its presence.

The formation of both 20α and 20β isomers of the hydroxy acid can be explained in one of two ways. There may be two enzymes of restricted stereospecificity which act on the common substrate 11-deoxycorticosterone to generate the two enantiomeric hydroxy aldehydes. An alternative, equally reasonable explanation is that the enzyme is an isomerase-epimerase complex. In either case, the significant intermediates would be the 20α- and 20β-hydroxy-21-aldehyde, which would then be oxidized to the corresponding hydroxy acids.

In humans, corticosteroids are oxidized to 20-hydroxy-21-oic acids. Both 20α and 20β isomers have been isolated from urine of patients given [4-^{14}C, 21-^{3}H]cortisol (Bradlow et al., 1973a). The enzyme described in this paper catalyzes a conversion of the corticosteroid ketol side chain to 20α- and 20β-hydroxy acids. It may therefore be considered to be a model system for studies on the synthesis of the two isomeric forms of acid isolated from human urine.

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